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AEROBIC AND ANAEROBIC STRATEGIES FOR HYDROGEN-MEDIATED ELECTROMICROBIAL PROTEIN PRODUCTION

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TIIVISTELMÄ

Veeti Laine: Aerobiset ja anaerobiset vedynhapettajat mikrobielektrosynteesiin perustuvassa proteiinintuotannossa
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Proteiinintuotanto mikrobielektrosynteesillä ja uusiutuvalla energialla on potentiaalinen ratkaisu tulevaisuuden ruoantuotannon haasteisiin. Tämän tutkielman tavoitteena on tutkia proteiinintuotantoprosessia, jossa vedynhapettajabakteerit sitovat hiilidioksidia ja ammoniakkia proteiiniksi käyttäen sähkökemiallisesti tuotettua vetykaasua energianlähteenään. Kirjallisuudessa on hyödynnetty sekä anaerobisia että aerobisia vedynhapettajia proteiinintuotantoon. Molemmat kykenevät litoautotrofiseen elämäntapaan, mutta niiden aineenvaihdunnan lopputuotteet eroavat oleellisesti. Keskeisenä tutkimuskysymyksenä tutkielmassa on aerobisten ja anaerobisten vedynhapettajien erot ja miten ne vaikuttavat tuotantoprosessiin.

Vetyä voidaan tuottaa erillisessä elektrolyysikennossa tai samassa bioreaktorissa kuin bakteereja viljellään. Valinta yhden tai kahden reaktorin välillä on oleellinen vetyvälitteisessä mikrobielektrosynteesissä ja se vaikuttaa merkittävästi prosessin suunnitteluun. Kummallakin vaihtoehdolla on hyvät ja huonot puolensa jotka kytkeytyvät vedyn kemialliseen luonteeseen ja elektrolyysireaktioon, jolla sitä tuotetaan. Ongelmallisia yhden reaktorin mikrobielektrosynteesissä ovat kilpailevissa reaktioissa muodostuvat haitalliset happiradikaalit sekä anaerobisessa prosessissa elektrolyysin toinen tuote eli happi, jotka estävät mikrobien kasvua. Kahden reaktorin systeemissä huomioitavaa on vetykaasun reaktiivisuus hapen kanssa sekä kaasujen heikko liukoisuus kasvatusliuokseen.

Aerobisten vedynhapettajien viljelyprosessi on verrattain yksinkertainen, ja sen lopputuotteena on proteiinitiheä biomassa. Anaerobisten vedynhapettajien metabolia tuottaa pääosin asetaattia, joka voidaan hyödyntää edelleen energianlähteenä heterotrofisille mikrobeille, esimerkiksi mikrosienille. Tuotantoprosessien vertailu keskittyy tarvittavaan pH:n säätelyyn, viljelmien koostumukseen ja mahdollisiin hiilen ja typen lähteisiin. Eräs anaerobisten vedynhapettajien potentiaalinen etu on, että ne voisivat hyödyntää jätevirtojen ammoniakkia tai niitä voitaisiin muokata sitomaan typpeä entsymaattisesti. Lisäksi vertaillaan tuotantomenetelmien tehokkuutta ja taloudellisuutta.

Viimeisessä osiossa sivutaan mikrobielektrosynteesillä tuotetun proteiinin potentiaalia toimia ratkaisuna nykyisiin ja tuleviin ruoantuotannon ongelmiin. Solumaatalouden merkittävimpiä etuja perinteiseen maatalouteen verrattuna on vähentynyt ammoniakin tarve, pienempi hiilijalanjälki sekä tehokkaampi maankäyttö. Mikrobielektrosynteesiin perustuva proteiinintuotanto ei vaadi viljelykelpoista maata ja on ilmastosta riippumatonta.

Avainsanat: mikrobielektrosynteesi, proteiinintuotanto, vedynhapettajat

ABSTRACT

Veeti Laine: Aerobic and anaerobic strategies for hydrogen-mediated electromicrobial protein production
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Electromicrobial protein production using renewable energy is a potential solution to the challenges of future food production. The aim of this thesis is to investigate a protein production process in which hydrogenotrophic bacteria fix carbon dioxide and ammonia into protein using electrochemically produced hydrogen gas as their energy source. Both anaerobic and aerobic hydrogenotrophs are utilized for protein production in literature. Both can grow lithoautotrophically, but their metabolic end products differ fundamentally. A central question for this thesis is the difference between the production strategies required for each type.

Hydrogen can be produced in a separate electrolysis cell or in the same bioreactor as the bacteria are cultivated. The choice between single-reactor or two-reactor design is relevant in hydrogen-mediated microbial electrosynthesis as it influences process design greatly. Both options have their advantages and disadvantages related to the chemical nature of hydrogen and the electrolytic reaction it is synthesized through. Challenges in single-reactor microbial electrosynthesis are posed by cytotoxic radical oxygen species formed in side reactions, as well as by oxygen gas for the anaerobic process. Of concern in a two-reactor system are hydrogen's reactivity with oxygen and the low solubility of the gaseous substrates.

The cultivation of aerobic hydrogenotrophs is relatively simple, as it yields protein rich biomass as its end product. Anaerobic hydrogenotrophs, however, produce primarily acetate, which requires additional heterotrophic microbes such as fungi and yeasts that can utilize it as an electron donor. The comparison of these processes will include required pH control, impact on culture composition, and possible carbon and nitrogen sources. A potential benefit of anaerobic hydrogenotrophs is that they could possibly utilize waste stream ammonia or they could be engineered to fix nitrogen enzymatically. The efficiency and economical viability of these production strategies are also investigated.

In the final section, the relevance of electromicrobial protein production is considered from the wider perspective of global food production and as a solution to its current and future challenges. The most important advantages of cellular agriculture compared to traditional agriculture is the lesser requirement for ammonia, the reduced carbon footprint, and the improved land use efficiency. Electromicrobial protein production does not require arable land and is climate independent.

Keywords: microbial electrosynthesis, electromicrobial protein production, hydrogenotrophs

CONTENTS

1 INTRODUCTION

Anthropogenic climate change and faltering food security are some of the greatest challenges humanity is facing in the 21st century. As population growth and worsening yields increase demand for agricultural land (Nyyssölä et al., 2021), deforestation and increased carbon emissions of agriculture are expected to further expedite the ecological catastrophe. The concepts of cellular agriculture and power-to-protein have been proposed as a solution to feed a growing population with high-quality protein while massively reducing the impact food production has on the environment (Abel et al., 2022; Molitor et al., 2019).

Cellular agriculture is an alternative to the unsustainable agricultural system. It involves the production of cellular foods, particularly animal cells or microorganisms, in bioreactors and fermenters, removing the requirement for arable land (Nyyssölä et al., 2022). However, thus far most commercial single-cell protein (SCP) products are based on heterotrophic bioprocesses that ultimately rely on organic carbon, sourced from agricultural crops (Nyyssölä et al., 2022).

Electromicrobial protein production, powered by renewable energy and lithoautotrophic growth, has the potential to circumvent this issue. There has been particular interest in the use of hydrogenotrophic bacteria as a protein production platform due to hydrogenotrophs being highly efficient at conserving energy as biomass and converting ammonia into protein, resulting in considerably lower carbon emissions compared to traditional agriculture or heterotrophic SCP (Abel et al., 2022). Both aerobic and anaerobic hydrogenotrophs have been investigated for use in electromicrobial protein production.

The production strategies largely differ between the two types, and this thesis aims to define and compare these processes by reviewing implementations found in recent literature. The first section will explain the basics of hydrogenotrophic metabolism. The second section serves as an overview of the general principles of hydrogen-mediated protein production. In the third section, different aspects of the aerobic and anaerobic protein production strategies will be compared, focusing on process design, energetic efficiencies, and economical viability. The final section discusses the potential of electromicrobial protein production for reducing the environmental impacts of the food industry and for ensuring food security in an uncertain future.

2 HYDROGENOTROPHIC BACTERIA

Hydrogenotrophs, or hydrogen-oxidizing bacteria, are a wide group of bacterial species characterized by their ability to oxidize H_2 to power their metabolism. Hydrogenotrophs can be aerobic or anaerobic. The former are also referred to as knallgas bacteria. Anaerobic hydrogenotrophs are acetogenic, producing scarce edible biomass, but the acetate they produce may be fed to heterotrophic microorganisms to yield protein-rich biomass (Molitor et al., 2019). The ability to grow lithoautotrophically on H_2 and CO_2 confers hydrogenotrophs unique advantages both in nature and as a bioproduction platform. In the protein production processes discussed later, the hydrogenotrophs are cultivated in bioreactors on electrochemically and biologically generated H_2.

2.1 Metabolism

Knallgas bacteria are aerobic lithoautotrophs that can utilize H_2 as their electron donor, and O_2 as the terminal electron acceptor (Koller, 2016). Some species are able to use other, possibly organic substrates as the source of electrons (and carbon) in addition to H_2 (Pander et al., 2020). Heterotrophic growth in presence of organic carbon is therefore possible, but only autotrophic growth is of interest here.

Lithoautotrophic growth of *C. necator*, a model organism for knallgas bacteria metabolism, is fueled by H_2 oxidation. Electrons derived from H_2 are shuttled through *C. necator*'s respiratory chain towards O_2, yielding ATP in the process. Oxidative phosphorylation in knallgas bacteria is catalyzed by enzymes similar to those of the eukaryotic mitochondrial complex (Koller, 2016). In addition to conserving energy in the form of ATP, H_2 oxidation reduces NADH and NADPH to be used as reductive power during carbon fixation under autotrophic growth (Brigham, 2019). Furthermore, a regulatory system is required to recognize the presence of H_2 and activate hydrogen-specific pathways in response (Koller, 2016). Oxidative phosphorylation, NADH and NADPH reduction, and H_2 sensing are mediated by the membrane-bound (MBH), soluble (SH), and regulatory (RH) hydrogenases, respectively (Koller, 2016).

Like knallgas bacteria, anaerobic hydrogenotrophs can utilize a variety of electron donors, some even the cathode directly (Nevin et al., 2011), but H_2 is the electron donor of interest here. Unlike in knallgas bacteria, the terminal electron acceptor is not O_2, but instead the electrons are used to fix CO_2.

2.1.1 Carbon fixation

Fixation of inorganic carbon, namely CO_2, into organic carbon skeletons is essential for growth under autotrophic conditions. Several pathways exist for incorporating CO_2 into carbon skeletons; the most important of these to *C. necator* and many other knallgas bacteria is the Calvin-Bensom-Bassham (CBB) cycle. Other pathways possibly utilized by other knallgas bacteria include the Wood-Ljungdahl pathway (WLP) and the reverse citric acid cycle (Salimijazi et al., 2020). WLP is the main carbon fixation pathway used by anaerobic hydrogenotrophs.

In knallgas bacteria, the CBB cycle functions largely the same as it does in photoautotrophs, with the key difference of the metabolic energy originating from H_2 rather than light (Koller, 2016). The expression of central CBB enzymes under autotrophic conditions is likewise induced by the presence of H_2 instead of light (Koller, 2016).

The key enzyme of the cycle, ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo), binds CO_2 with ribulose 1,5-bisphosphate to produce two 3-phosphoglycerate molecules, which are then further metabolized to yield glyceraldehyde 3-phosphate. The reduction of 3-phosphoglycerate into the more energy-rich compound glyceraldehyde 3-phosphate, and further into ribulose 1,5-bisphosphate, requires ATP from oxidative phosphorylation, and NADPH produced by the cytoplasmic SH complex. For every three CO_2 molecules incorporated, one glyceraldehyde 3-phosphate is output. The three high-energy ribulose 1,5-bisphosphates required to bind the CO_2 molecules are ultimately regenerated with ATP energy, completing the cycle. (Park et al., 2011)

Different species of anaerobic hydrogenotrophs have slightly differing strategies for carbon fixation, with most opting for a variation of WLP. *Clostridium ljungdahlii*, used for protein production by Molitor et al. (2019), serves as a model organism for one carbon fixation strategy (Schuchmann et al, 2014). In *C. ljungdahlii*, as in all acetogens, WLP is used to fix CO_2 into acetate. Carbon fixation through WLP requires the reducing equivalent ferrodoxin, which is reduced through H_2 oxidation (Schuchmann et al, 2014). The total reaction for converting 4 H_2 and 2 CO_2 into acetate and ATP is:

$$2CO_2 + 4H_2 + nADP + nP_i \rightarrow CH_3COOH + nATP + (2+n)H_2O$$

3 HYDROGEN-MEDIATED ELECTROMICROBIAL PROTEIN PRODUCTION

Electromicrobial production (EMP) refers to biotechnological processes which employ microorganisms to carry out microbial electrosynthesis (MES), the conversion of CO_2 into multicarbon compounds using electricity as the source of reductive power (Abel et al., 2022; Nevin et al., 2010). MES is traditionally exploited to produce extracellular products such as acetate, methane, or higher-value products such as biofuels (Nevin et al., 2010). In electromicrobial protein production (EMPP), the growth of the microbial biomass is often the desired outcome. The biomass is then processed into the edible single cell protein (SCP) product. In some applications, intermediate products such as acetate and methane may be yielded by MES, to be used for the cultivation of a heterotrophic species which is then processed into the SCP (Molitor et al., 2019).

At the center of the EMP process is the bioreactor in which lithoautotrophic microorganisms are cultivated. The growth of microorganisms in bioreactors requires a nutrient-rich growth medium that includes ammonia, as well as an electron donor and a terminal electron acceptor. For lithoautotrophic microorganisms such as hydrogenotrophs, a source of inorganic carbon (CO_2) is also required. CO_2 can act as an electron acceptor for anaerobes, and as a carbon source for autotrophic growth. Lithotrophs utilize inorganic electron donors such as H_2 that can be synthesized electrochemically. The synthesis of these simple redox mediators can be achieved by two different system designs: a single-reactor system where the redox mediator is synthesized *in situ*, and a two-reactor system which introduces an additional electrosynthesis reactor in addition to the bioreactor.

In a single-reactor system, electrodes are introduced to the bioreactor, and electrons are transferred from the cathode to the microorganisms through both biological and electrochemical activity (Salimijazi et al., 2020). Electroautotrophic microorganisms can import electrons from the cathode using extracellular electron transfer (EET) (Tremblay et al., 2017). EET can be direct or indirect. Direct electron transfer (DET) is made possible by the formation of a conductive biofilm from which the electroautotrophs intake electrons via type IV pilii (Tremblay et al., 2017). Indirect EET involves the use of complex (e.g. flavins) or simple redox mediators to shuttle electrons from the cathode to the microorganism (Salimijazi et al., 2020). The main EET routes are summarized in figure 1.

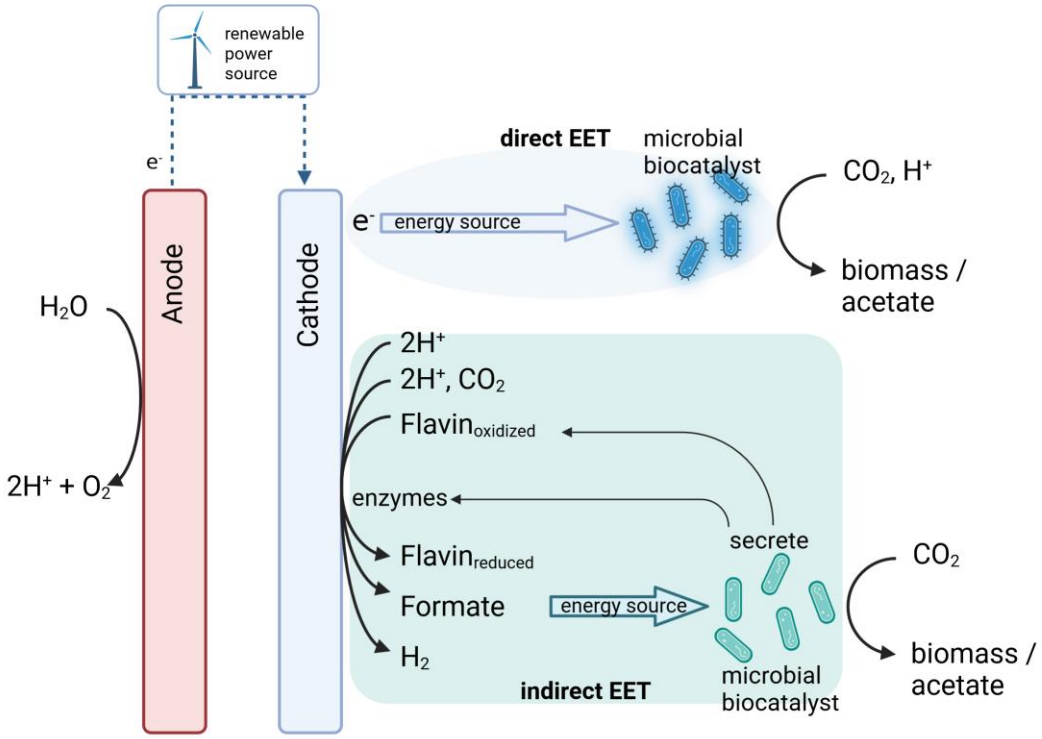

Figure 1: Direct and indirect extracellular electron transfer routes utilized by electroautotrophs. Modified from Tremblay et al. (2017). Created with BioRender.com

Electroautotrophs using indirect routes of EET often secrete enzymes that catalyze the formation of the simple redox mediators at the cathode, mainly H_2 or formate, which the electroautotrophs then feed on (Salimijazi et al., 2020). If the microorganism cultivated for biomass is not itself electroautotrophic, additional, electroautotrophic species may be introduced to the growth medium to biologically facilitate the formation of a redox mediator (Pous et al., 2022).

However, electroautotrophic activity is not strictly necessary, as the integrated electrodes allow for non-microbial electrosynthesis of H_2 or formate. These electrochemical reactions are catalyzed by inorganic catalysts.

In a two-reactor design, there is no biological aspect to redox mediator production, as it is produced in a separate electrosynthesis reactor. This simplifies the bioreactor design and allows for continuous supply of the electron donor to the bioreactor (Tremblay et al., 2017; Ruuskanen et al., 2021).

3.1 Hydrogen as a redox mediator

H_2 is considered a key redox mediator in single-reactor EMP due to the slow rates of other EET routes, such as DET (Perona-Vico et al., 2020). Hydrogenotrophy allows the employed

microorganisms to feed on a simple redox mediator that can be produced electrochemically from water via electrolysis.

Electrolysis of water occurs in two steps. First, water is split into oxygen, electrons, and protons at the anode. This is an oxygen evolution reaction (OER). In the second step, the hydrogen evolution reaction (HER), the protons are reduced to H_2 at the cathode. To drive electron flow from anode to cathode through an electric circuit, a relatively high external voltage must be applied as oxygen has a large reduction potential that needs to be overcome.

In a two-reactor system, H_2 is produced in an electrolyser separate from the bioreactor, and then mixed into the bioreactor's growth medium (Pous et al., 2022). The main advantages of using a commercial electrolyser include high efficiency and a stable supply of the redox mediator to the bioreactor (Tremblay et al., 2017), as well as the simplicity of such a system making the purification of the product easier (Ruuskanen et al., 2021).

An issue with using H_2 in two-reactor systems is the risk of the H_2 and O_2 explosively mixing, which is avoided in single-reactor systems (Ruuskanen et al., 2021). However, this risk could be mitigated by adjusting the H_2:O_2 ratio of the gas mixture so that it is higher than 10:1, which is a "safe ratio" (Abel et al., 2022). To prevent oxygen's low partial pressure from hindering its mass transfer and therefore the biomass growth rate, increasing the partial pressures of both while retaining the safe ratio is required (Abel et al., 2022).

Mass transfer of the reactant gases appears to largely influence the efficiency and productivity of H_2-mediated EMP (Abel et al., 2022; Ruuskanen et al., 2021). In two-reactor setups, the less soluble gaseous substrates H_2, O_2, and CO_2 require sparging to mix the gases into the growth medium (Pous et al., 2022). In low-pressure systems where diffusion is not enough to circulate the gaseous substrates, additional agitation is needed for improved mass transfer, and substantial energy costs are associated with agitation systems (Salimijazi et al., 2020). Issues related to H_2's low solubility and mass transfer rate might be alleviated by *in situ* electrolysis in single-reactor systems (Li et al., 2012; Pous et al., 2022). *In situ* electrolysis refers to H_2 production inside the system that requires it.

A major downside of the single-reactor system is that *in situ* electrolysis also causes oxidative stress that limits cell growth. Oxidative stress results from the formation of cytotoxic reactive oxygen species (ROS) at the cathode and the anode (Torella et al., 2015), as well as other toxic compounds such as reactive nitrogen species at the anode (Li et al., 2012). ROS formation occurs at the high anode overpotentials required for OER and the low cathode potentials required for HER (under -600

mV (Nevin et al., 2010)), with the cathodic ROS presenting a larger threat to the health of the microbial culture (Torella et al., 2015).

Some solutions to protecting the cells from ROS toxicity have been proposed. Anodic ROS could be physically stalled from reaching a cathode culture by a porous ceramic cup around the anode, giving time for the ROS to be neutralized in the medium (Li et al., 2012). In a similar manner, the cathodic ROS could be depleted by a biofilm that confluently covers the cathode, absorbing the ROS before they can harm the vulnerable bacterial community; some bacterial species have been found to gain a fitness benefit under oxidative stress from their ability to form such a biofilm (Reiner et al., 2020). The addition of biofilms to cathodes to facilitate redox mediator formation seemingly also improves growth conditions (Pous et al., 2022) which could indicate that the biofilms physically shield the microorganisms. Some success has also been found in adding catalase to the solution to neutralize hydrogen peroxide (Nyyssölä et al., 2021; Torella et al., 2015).

Efficient electrocatalysts that can better select for HER over ROS formation, as well as lower the anode potential required for OER, have been developed. A cobalt phosphate (CoP_i) anode allows OER to be performed at significantly lower cell voltages (>1.3 V lower) and in neutral pH (Torella et al., 2015), which would normally cause slow reaction kinetics (Ruuskanen et al., 2021). This catalyst can perform electrolysis in natural waters as well as biological growth mediums (Torella et al., 2015). A NiMoZn cathode has also been shown to avoid ROS formation (Torella et al., 2015). Continued development of better inorganic catalysts through which the electrode potentials can be optimized is likely to increase the efficiency of the system while reducing oxidative stress.

Single-reactor EMP has typically required the use of a potentiostat to avoid fluctuations in cathode potential which could be damaging to the cells (Giddings et al., 2015; Nevin et al., 2010). Potentiostat control presents barriers to scale-up such as increased complexity and cost, and the potentiostat does not function effectively in larger-scale reactors (Giddings et al., 2015). However, there have been successful experiments where some bacterial species such as *S. ovata* have been cultivated on direct current, without potentiostat control (Giddings et al., 2015).

3.2 Alternatives to hydrogen

H_2 as a redox mediator could prove impractical due to the safety and solubility issues, oxidative stress, and the large energy inputs and low cathode potentials (under -600 mV) required to drive electrolysis of water (Nevin et al., 2010). Alternative electron transfer pathways will be briefly discussed next.

Some studies have used formate instead of H_2 as a redox mediator, though not necessarily for protein production (Li et al., 2012). Many hydrogenotrophs can also metabolize formate, which can be produced electrochemically as well as biologically similarly to H_2 (Torella et al., 2015). Electrosynthesis of formate from CO_2 and H_2O can achieve relatively high efficiency, and formate is highly soluble, as opposed to H_2 (Li et al., 2012). However, the electrosynthesis is still less efficient and therefore more energy-costly than that of H_2 (Leger et al., 2021). Formate production is possible at higher cathode potentials than does HER, avoiding some of the oxidative stress (Li et al., 2012). Formate also does not pose the risk of explosively reacting with oxygen.

Direct electron transfer (DET) from the cathode has been shown to be possible by species from the phylum *Clostridium*, including species *Sporomusa ovata* (which served as initial proof of concept by Nevin et al. (2010)), *S. sphaeroides*, *S. silvacetica*, *C. ljungdahlii* and *C. aceticum*, as well as *M. thermoacetica* (Nevin et al., 2011). However, these were unable to reach rates of current consumption demonstrated by *S. ovata* (Nevin et al., 2011). Later studies have shown DET-capable microorganisms, in particular certain acetogens, to produce organic compounds with a high energy efficiency of >80% (Igarashi & Kato, 2017). DET-capable acetogens can produce acetate at cathode potentials above -500 mV, higher than those required for HER, thus reducing oxidative stress.

Certain electroautotrophs can shuttle electrons from the cathode by secreting complex biological redox mediators, such as flavins, which are reduced at the cathode and subsequently oxidized by the microorganism to yield energy for metabolism (Salimijazi et al., 2020). There has also been some research into using artifical redox mediators that act in a similar manner to biological redox mediators. The main issue with them is cytotoxicity, though advancements have been made into developing more biocompatible mediators (Igarashi & Kato, 2017). The main advantage of using such redox mediators is avoiding the use of expensive inorganic catalysts required for H_2 or formate electrosynthesis (Igarashi & Kato, 2017).

4 ANAEROBIC AND AEROBIC STRATEGIES FOR PROTEIN PRODUCTION

H_2 appears a promising redox mediator for use in EMPP despite of the previously discussed issues. Hydrogen-mediated EMPP has been widely studied and can achieve high efficiencies compared to other redox mediators (Abel et al., 2022). Both anaerobic and aerobic hydrogenotrophs have been utilized for protein production, and the processes designed around them will now be compared in more detail.

In the aerobic process, bacterial biomass cultivated in the bioreactor becomes the SCP product through filtering and drying. In the the anaerobic process, however, anaerobic metabolism results in 70-80% of carbon fixed being secreted as acetate (Pander et al., 2020), which means the acetogens can not directly be used as SCP. A second stage is thus required in which the acetate is fed to heterotrophic microorganisms such as yeast or fungi to cultivate protein-rich biomass (Molitor et al., 2019).

In either process, one or two reactors are required to produce H_2 and cultivate the hydrogenotrophs. In the proof-of-concept study of the anaerobic strategy by Molitor et al. (2019), the researches used a separate electrolyser unit to supply the acetogens with H_2. Two-reactor systems for the aerobic and anaerobic processes are illustrated in figure 2.

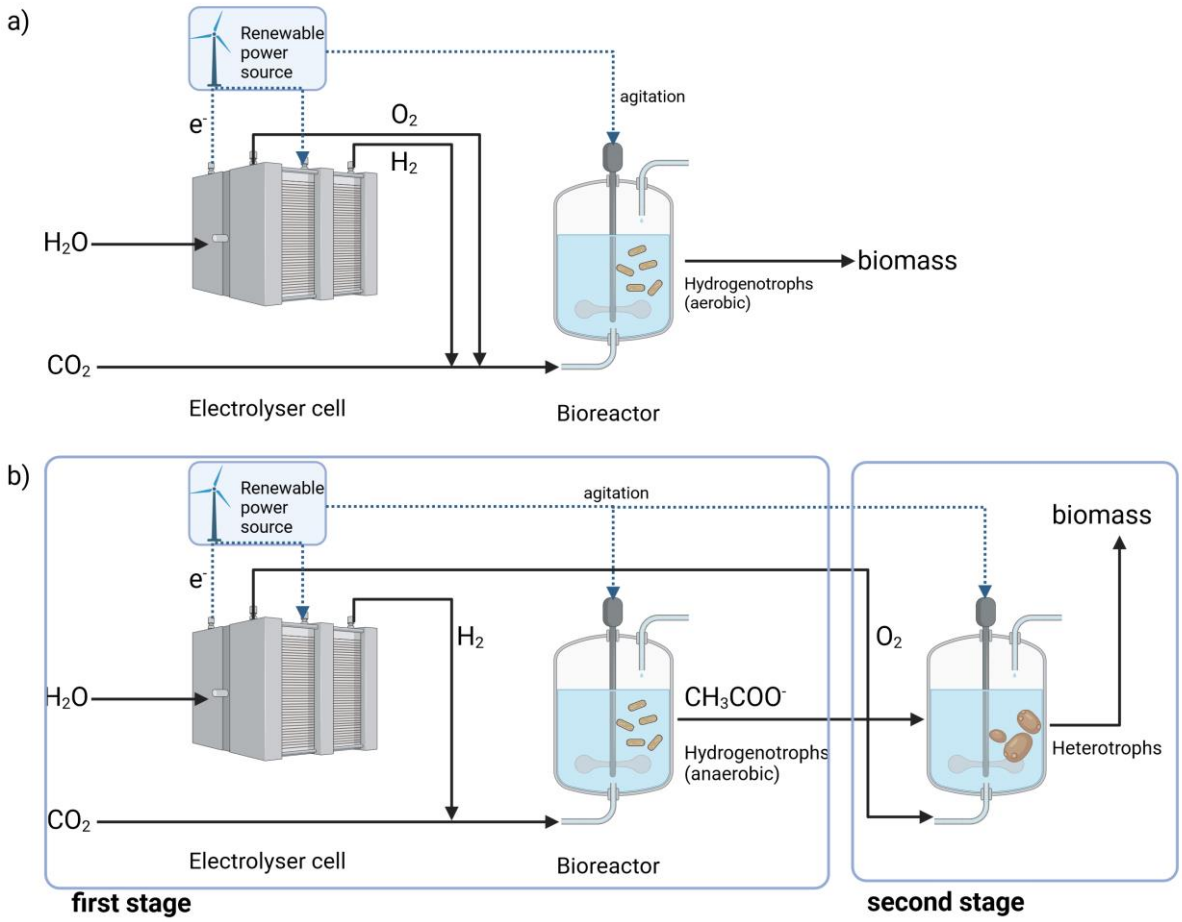

Figure 2: Two-reactor systems for cultivation of a) aerobic and b) anaerobic hydrogenotrophs. In the second stage of the anaerobic process, another bioreactor is added to cultivate aerobic heterotrophic microorganisms on the acetate produced in the first stage. Modified from Mishra et al. (2020). Created with BioRender.com.

Alternatively, H_2 could be produced through *in situ* electrolysis in the bioreactor. However, *in situ* electrolysis has been found rather limited in efficiency (Ruuskanen et al., 2021). Biological H_2 production can help remedy this. Ideally, a biofilm of electroautotrophs growing on the cathode (a biocathode) can secrete dehydrogenase enzymes that facilitate H_2 formation (Pous et al., 2022). Species from the genera *Rhodobacter*, *Rhodopseudomonas*, *Rhodocyclus*, *Desulfovibrio*, and *Sporomusa* have been found to contribute to H_2 evolution at the biocathode, most promising of which are *D. desulfuricans* and *D. paquesii* (Perona-Vico et al., 2020). The role of hydrogen-producing bacteria in coculture with hydrogenotrophs is illustrated in figure 3.

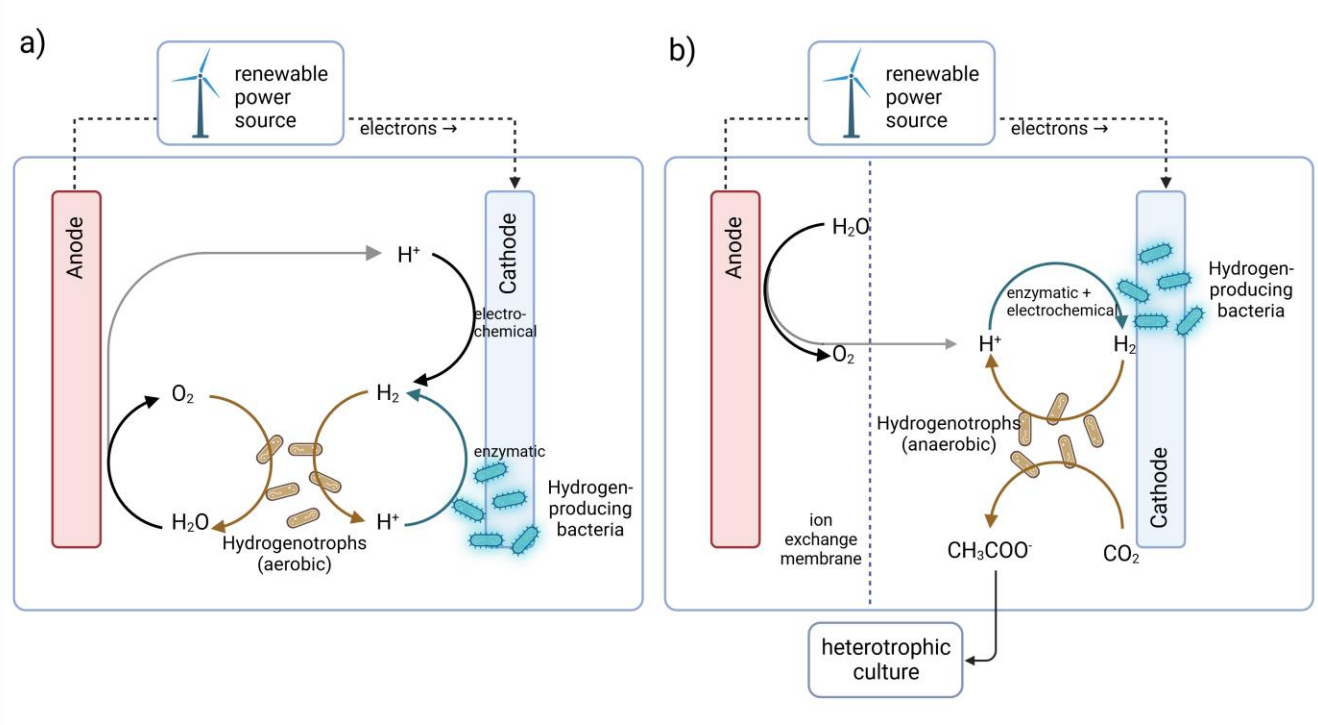

Figure 3: Single-reactor system for a) aerobic and b) anaerobic hydrogenotrophs, with added hydrogen-producing bacteria to facilitate H_2 production. Modified from Pous et al. (2022). Created with BioRender.com.

With strictly anaerobic hydrogenotrophs, the main issue presented by *in situ* electrolysis is the presence of oxygen in the culture medium, which could require the separation of the anode and cathode by an ion exchange membrane (Giddings et al., 2015). An ion-exchange membrane (IEM) is a semi-permeable membrane used to prevent exposure of anaerobic microorganisms to oxygen, as well as preventing the oxygen from consuming electrons at the cathode (Giddings et al., 2015). However, Giddings et al. (2015) discovered deliberate positioning of electrodes to avoid oxygen exposure allowed the anaerobic *S. ovata* to thrive even without an IEM. This greatly simplifies reactor design and removes barriers for scale-up associated with the use of an IEM.

4.1 pH control

pH control is often necessary in EMP due to biochemical reactions producing or consuming protons. A stable pH optimal to cell growth is maintained usually by adding NaOH or HCl. Their production is energy-expensive, but for cultivation of knallgas bacteria represents only a small portion of the total energy costs (Abel et al., 2022).

For the anaerobic process, pH control is a significant energy sink, since acetate production lowers the pH of the hydrogenotrophic culture, and the consumption of acetate raises the pH of the heterotrophic culture (Abel et al., 2022). Large amounts of NaOH and HCl are needed for maintaining stable pH in either culture, thus lowering the overall efficiency of the process (Abel et al., 2022).

To circumvent this issue, an alternative implementation of the two-stage anaerobic process was proposed by Abel et al. (2022) in which the acetogens and heterotrophs are instead cocultured in a single bioreactor. The main issue with this is the heterotrophs' requirement for oxygen conflicting with the oxygen sensitivity of the acetogens. However, a potential solution to anaerobes' oxygen sensitivity has been investigated: using strains which have been evolved to tolerate small amounts of oxygen, which has been proven to be possible with *S. ovata* (Abel et al., 2022).

4.2 Culture composition

The composition of an EMP culture determines how easily the process can be controlled, and its food safety. A pure culture with highly defined composition may be required for high-value products or edible biomass meant for human consumption, but it might also require extensive sterility precautions such as media autoclaving to protect it from contamination (Matassa et al., 2016).

Mixed cultures or even open cultures without a strictly defined composition that do not require sterilization of medium have been used in literature and may be a desirable alternative particularly for the anaerobic process (Igarashi & Kato, 2017). Mixed cultures often yield primarily acetate (Giddings et al., 2015), which is a desired intermediate in the anaerobic process. However, a pure culture of acetogens was still used in the study by Molitor et al. (2019). Mixed cultures may be beneficial for the cultivation of both knallgas bacteria and acetogens as they allow the introduction of additional microbial species that biologically facilitate H_2 production (Pous et al., 2022).

Open cultures are the least defined and may therefore pose a risk of pathogenic contamination (Mishra et al., 2020). In an open culture study by Matassa et al. (2016), a highly stable composition could be achieved under continuous culture conditions due to *Sulfuricurvum spp*. dominating over predatory microorganisms (over 90 days). A stable culture composition has also been achieved in studies using diverse acetogenic communities such as *Acetobacterium spp.* (Igarashi & Kato, 2017). The risk of pathogenic contamination might also not be as relevant in acetogenic cultures, as the acetogens are not directly used as SCP.

Even non-pathogenic microorganisms may be harmful when consumed due to the endotoxins contained in the cell walls of Gram-negative bacteria (Ruuskanen et al., 2021), and therefore the employed microorganisms need to be especially carefully chosen when the biomass is to be used as food.

4.3 Carbon and nitrogen sources

CO_2 to be fixed by the microorganisms could potentially be sourced from industrial offgas (Mishra et al., 2020) or syngas. However, in most scenarios, direct air capture (DAC) of CO_2 would be used instead (Abel et al., 2022).

An organic nitrogen source is necessary for the synthesis of amino acids. Options for supplying the culture with nitrogen include catalytic reduction at the cathode by combining atmospheric N_2 with H_2 (Mishra et al., 2020), recovery of organic nitrogen species such as urea or ammonia from wastewaters (Molitor et al., 2019), and nitrogen fixation by the microorganisms themselves (Wise et al., 2022). Nitrogen fixation would require engineering of the microorganisms to incorporate the nitrogenase genes. Currently, all known nitrogenases are oxygen sensitive, rendering them unusable by aerobic bacteria (Wise et al., 2022) but not by anaerobic hydrogenotrophs.

Recovery of nitrogen from wastewater could be a sustainable alternative to nitrogen fixation. In accordance with the circular economy principles, dilute organic nitrogen and CO_2 from waste effluents can be recovered and recycled into edible protein (Matassa et al., 2016). In an open continuous culture, the knallgas bacterium *Sulfuricurvum spp*. was able to assimilate organic nitrogen from wastewater (Matassa et al., 2016). The anaerobic process proposed by Molitor et al. (2019) could be the next evolution of microbial wastewater recovery because it circumvents some of the purity concerns associated with an open culture grown on wastewater.

4.4 Sustainability

Comparing the energetic efficiencies of different EMPP setups is useful for investigating the systems with the most potential for large-scale implementation. The energy costs also determine the global warming potential and economic viability of these systems. The energetic efficiency of converting electricity to the chemical energy of protein is the product of the efficiencies of each subprocess, including the electrosynthesis of the redox mediator, NH_3 production, direct air capture of CO_2, microbial growth, and the processing of biomass into the final product (Leger et al., 2021).

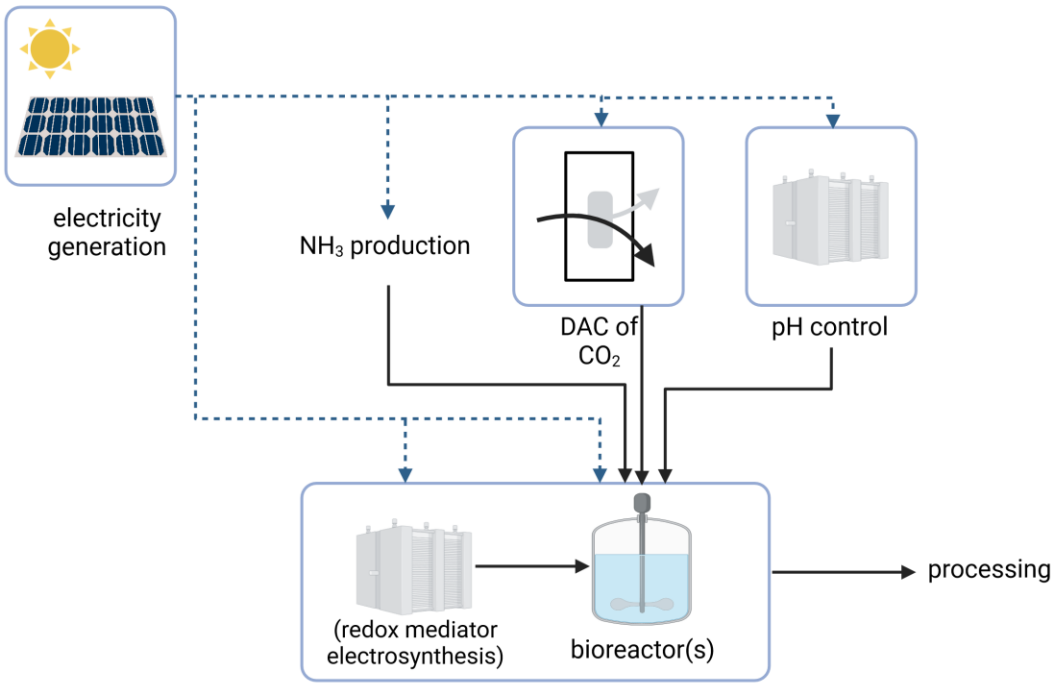

Figure 4: Subprocesses constituting the total energetic efficiency of an electromicrobial protein production system. Dashed arrows denote electricity and solid arrows material flow. Modified from Abel et al. (2022). Created with BioRender.com.

With a growing world population, the solar-to-protein efficiency of food production becomes increasingly relevant (Wise et al., 2022), since the solar-to-protein and solar-to-biomass efficiencies are useful for assessing the land use efficiency of different food production systems. In addition to the previously mentioned factors, the energetic efficiency of solar-to-protein also includes the efficiency of photovoltaic electricity generation, which is assumed as the renewable energy source (Leger et al., 2021).

According to a life-cycle assessment (LCA) of three EMP systems by Abel et al. (2022), electromicrobial protein production has lesser global warming potential (GWP) than traditional agricultural systems if 90% renewable energy sources are available, due to the considerable emissions associated with ammonia production in traditional agriculture. The global warming potential (GWP) is defined as kg of CO_2 emitted per kg of biomass produced, and it largely depends on the energetic efficiency of the process.

The energetic efficiency of H_2 electrosynthesis is 70% (±5%) (Leger et al., 2021). It is the same for the aerobic and anaerobic processes, as is the efficiency of direct air capture of CO_2. The GWP of the anaerobic process is greater than that of the aerobic process due to the massive energy costs associated with pH control, although this could be mitigated by combining the two stages (Abel et al.,

2022). The anaerobic process also requires slightly more NH_3 and CO_2, as acetogen biomass is cultivated in addition to the edible biomass (Abel et al., 2022).

The LCA only considered the GWP for production of biomass, but the final SCP product emits more CO_2 per kg since the non-protein content is removed in the final processing step. Because high nucleotide contents are harmful to human health (but not animal health), a filtration step is required before drying to remove fatty acids, carbohydrates, and nucleotides (Leger et al., 2021). For the knallgas process, the solar-to-protein efficiency was thus calculated to be 0.4% for SCP and 0.7% for animal feed (Leger et al., 2021), a considerable difference in efficiency.

Of the three EMP systems (and one heterotrophic system fed on glucose) compared in the LCA, knallgas bacteria would appear to be the ideal protein production platform for minimizing environmental impact (Abel et al., 2022).

4.5 Economical viability

In order for an electromicrobial protein production strategy to see any real-world application, it needs to be commercially competitive with existing protein products. Two important factors contributing to the economic viability of an SCP or feed product are the scalability of the process, and the costs of producing protein with the system.

According to Salimijazi et al. (2020), the cell density in the reactor could present a limiting factor for efficiency in systems that rely on agitation to ensure sufficient mass transfer. With greater densities and smaller volumes, agitation rapidly becomes less effective. However, a high cell density and small land footprint is desired, because the system should be small enough to be housed under the PV panels supplying it to not negatively affect the land use efficiency. This conflict of energetic and land use efficiency appears to be solved by an increase in total electrical power supplied to the system: if sufficiently high amounts of power are input (above 10^6 W), a high energetic efficiency can be maintained even with cell densities of 10^{12} m^{-3} required to keep the system footprint under that of the PV system. This analysis would suggest that a larger scale is required for systems reliant on agitation to benefit from more efficient mixing, and small-scale applications might not be as viable.

Productivity determines whether a process can be scaled to produce relevant amounts of protein or not. Productivity can be defined as the growth rate of the culture: how much biomass/protein is produced per hour in a liter of culture medium. Industrially relevant protein production rates are approximately 1 g L^{-1} h^{-1} (Molitor et al., 2019). The anaerobic process implemented by Molitor et al. (2019) reached a yield of 1/14 g L^{-1} h^{-1} and the aerobic process by Matassa et al. (2016) reached a

yield of 1/3 g L^{-1} h^{-1}. The models crafted by Abel et al. (2022) predicted potential productivities of up to 0.65 g L^{-1} h^{-1} (biomass) for knallgas bacteria and much lower productivities of 0.20 g L^{-1} h^{-1} for anaerobic hydrogenotrophs.

In addition to productivity, the cost and complexity of an EMP reactor can be a barrier to scale-up. Materials like ion exchange membranes potentially needed for anaerobic systems are rather expensive, but alternative solutions have been investigated (Giddings et al., 2015).

Energetic efficiency largely determines the cost of the actual product, and its commercial competitiveness. For knallgas bacteria, Leger et al. (2021) estimated average costs of animal feed to be $2.6/kg, and a competitive price of $4-5/kg for a SCP product; this was the lowest cost predicted of the EMPP systems compared.

5 POTENTIAL OF POWER-TO-PROTEIN

Power-to-protein is part of the power-to-X concept, where renewable energy is coupled to the production of chemicals, food, and other commodities traditionally derived from agricultural or fossil resources (Molitor et al., 2019). Once a suitable degree of renewable energy in the power grid has been achieved, electricity can be converted to biomass, biofuels, and other high-value products through electromicrobial production processes. In accordance with the circular economy model, renewable energy can also be used to recover and upcycle many resources which are wasted globally, such as ammonia (Matassa et al., 2016; Molitor et al., 2019). A life cycle assessment suggests that power-to-protein can be an ecologically sustainable alternative (Abel et al., 2022), helping solve or at least alleviate many of the environmental issues caused by traditional agriculture (Molitor et al., 2019).

A further advantage of power-to-X is that it can be used to store electrical energy as chemical energy. A major challenge of renewables is that their energy production is inconsistent and excess energy is difficult to store (Nevin et al., 2011). Electromicrobial production of biofuels during peaks in renewable energy production circumvents this by serving as energy storage which is easy to distribute within the currently existing fuel grid (Nevin et al., 2011; Torella et al., 2015). Similarly, excess renewable energy can be converted into biomass to be used as a food source. Therefore, not only does the increased renewable energy production support the implementation of a power-to-X economy, electromicrobial production capabilities also help solve some of the challenges of renewable energy, creating a positive feedback loop that will aid both nascent technologies in reaching their full potential.

5.1 Ammonia use

The most glaring of the issues with traditional agriculture is the wasteful use of ammonia. Protein production in bioreactors would allow full utilization of the ammonia due to the bioreactor being a closed system, and prevent ammonia runoff into waters (Ruuskanen et al., 2021; Wise et al., 2022) which is a considerable waste of energy and a cause of eutrophication (Pous et al., 2022). To produce the same amount of protein, traditional agriculture requires much more fertilizer, whose production via the Haber-Bosch process has a large carbon footprint (Abel et al., 2022).

The lesser ammonia requirement and more efficient ammonia production methods made possible by EMPP reduces the carbon footprint of electromicrobially produced SCP significantly (Abel et al.,

2022). Although not considered in the LCA by Abel et. al, the possibility of ammonia recovery from wastewater would also largely reduce the need for ammonia production (Matassa et al., 2016).

5.2 Land use

Climate change, in addition to population growth, will increase need for arable land (Nyyssölä et al., 2021). A transition towards cellular agriculture would help avoid the projected conversion of natural ecosystems into fields (Ruuskanen et al., 2021). A power-to-protein economy is also climate-independent, avoiding the risks climate change poses to food security (Leger et al., 2021) Electromicrobial protein production requires no arable land, though the greatly increased solar-to-protein efficiency compared to systems that rely on agriculture (Leger et al., 2021) can lead to significantly reduced land use if agricultural land is repurposed for solar energy generation. Figure 5 highlights the difference in protein yields between plant-based, heterotrophic cellular, and electromicrobial protein production.

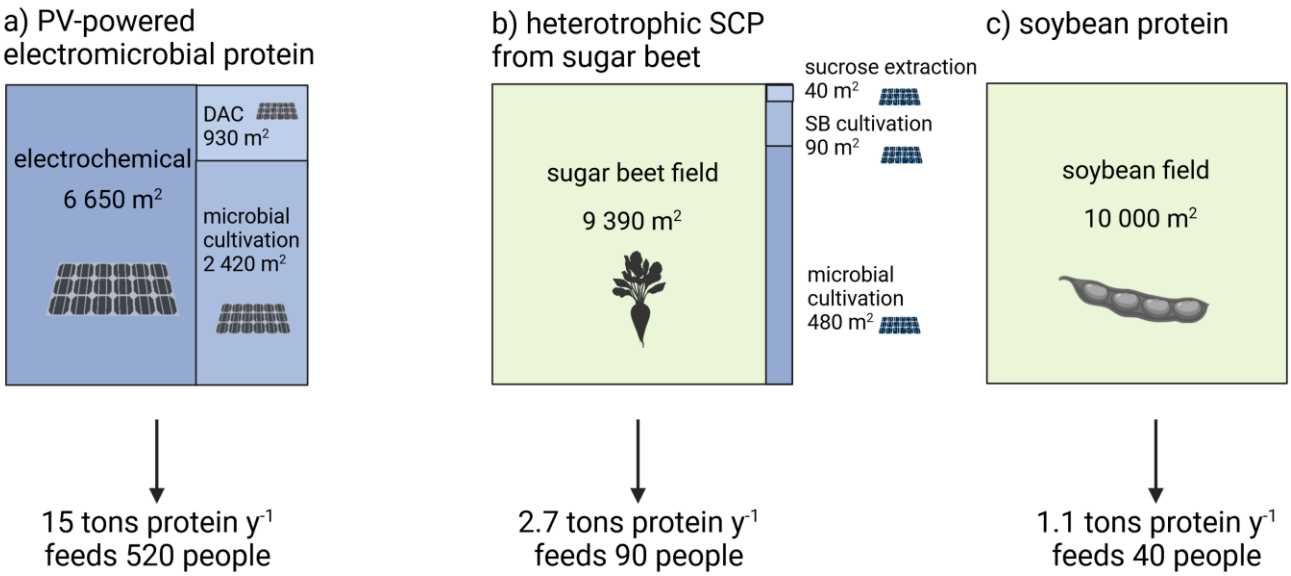

Figure 5: Protein yields for a land area of 10 000 m² for three different protein production strategies. Modified from Leger et al. (2021). Created with BioRender.com.

Caloric yield and protein yield are metrics used to measure the amount of biomass or protein that can be produced a year per m² of land (Leger et al., 2021). They are useful for comparing how much protein a unit of land can yield when allocated to different protein production strategies. Caloric yield is calculated by multiplying the solar-to-protein efficiency of the system by irradiance, which is the amount of solar energy available to a m² of land in a year (Leger et al., 2021). When protein yield is calculated, the non-protein content of the food product is omitted.

Irradiance depends on latitude: in the study by Leger et al. (2021), the caloric and protein yields of different SCP production strategies were calculated for a spectrum of latitudes. The yields were also corrected with a correction function to account for the negative effect of high irradiance levels on photovoltaic efficiency, though the crop yields were not corrected for the effect of varying levels of sunlight.

Compared to the “baseline” of soy protein, which is the highest-protein crop used in agriculture, all SCP production strategies showed a significant increase (sometimes over tenfold) in protein yields, including one heterotrophic SCP fed on plant-derived sucrose (Leger et al., 2021). However, since the latter is still based on an agricultural product, its protein yield is significantly lower than the EMPP strategies. PV-powered EMPP has protein yields of nearly 15 times that of a soybean field (Leger et al., 2021). In the lifecycle assessment conducted by Abel et al. (2022), it was estimated that the land occupation for an aerobic hydrogenotrophic protein production system is 95% lower than for a sucrose-fed heterotrophic system, and almost the same for the anaerobic system (not considered by Leger et al.).

The vastly increased land use efficiency arises from the lithoautotrophs’ superior solar-to-protein efficiencies compared to photoautotrophs (Abel et al., 2022), in addition to photovoltaics being very efficient (100 times more) at harvesting solar energy in comparison to natural photosystems (Nevin et al., 2011). Other forms of renewable energy may be even more efficient in terms of land use; solar was used as the comparison point here since both traditional and cellular agriculture can harness solar energy for biomass production.

5.3 Improving the energetic efficiency

The solar-to-protein efficiencies of EMPP strategies can be further improved by advancements in photovoltaics efficiency, which are expected to happen with the continuing development of PV technologies (Leger et al., 2021; Torella et al., 2015). In addition, the energetic efficiency of the EMP systems themselves will improve with optimization of the growth medium, the reactor design, and the catalysts (Torella et al., 2015).

Wise et al. (2022) predicted the thermodynamic upper limits for electricity-to-protein and solar-to-protein efficiencies. The highest efficiencies can be achieved in H_2-mediated EMPP using the Wood-Ljungdahl pathway or the reverse citric acid cycle. The hypothetical lowest energy cost for protein using WLP would be 64 kJ g^{-1}. However, the CBB cycle does not fall much behind. Knallgas bacteria using the CBB cycle have potential to achieve energy costs of as low as 67.9 kJ g^{-1}. The solar-to-

protein efficiency in this scenario would be approximately 6%, which is a significant increase from the 0.4% efficiency of solar-to-protein calculated by (Leger et al., 2021). However, achieving this in a real-world setting would require that the photovoltaic cell be perfectly efficient, and that the nitrogen is fixed by the microorganisms themselves, which is currently not achievable with aerobic microorganisms as the required nitrogenase enzymes are oxygen sensitive (Wise et al., 2022).

6 CONCLUSIONS

Bioreactor cultivation of microorganisms can be a potent and efficient method for biomass and SCP production. This thesis has discussed two of the most promising strategies for hydrogen-mediated electromicrobial protein production.

Aerobic and anaerobic hydrogenotrophs have largely differing metabolisms and metabolic end products, warranting different EMPP strategies. Aerobic bacteria using the CBB cycle invest in growth, yielding protein-rich biomass that can be used directly as food. The WLP utilized by the anaerobic hydrogenotrophs yields acetate, requiring an additional heterotrophic production stage to utilize the acetate and convert it into protein-rich biomass. For this process, pH control to balance protons produced and consumed in either stage is a significant energy sink. Because of this, an alternative, combined implementation of the anaerobic process has been considered.

The composition of an EMPP culture determines how easily the process can be controlled, and its food safety. Pure, mixed, and even open cultures that do not require sterilization of medium have been used in literature. Carbon dioxide and organic nitrogen can be obtained from a variety of sources, with some being potentially less environmentally taxing. CO_2 can be sourced from industrial offgas, syngas, or directly from air via DAC. Options for supplying the culture with nitrogen include catalytic reduction at the cathode, recovery of organic nitrogen from wastewaters, and nitrogen fixation by the microorganisms themselves.

The energetic efficiencies of the different strategies are an important factor determining their potential for large-scale implementation and their global warming potential. With the growing world population, the solar-to-protein efficiency and land use efficiency of different food production systems becomes increasingly relevant. According to a life-cycle assessment of three EMPP systems, EMPP has a much lesser environmental impact than traditional agricultural systems if 90% renewable energy sources are available.

Electromicrobial protein production is a field yet in its infancy, and it is difficult to predict when or in what form it will become fully realized and commercially viable. However, with the looming threat of climate change to food security, its future potential appears much greater than its current challenges.

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