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ELECTRO-FERMENTATION: SUSTAINABLE BIOPRODUCTIONS STEERED BY ELECTRICITY

Bernardino Virdis[a], Robert Hoelzle[b], Angela Marchetti[c], Santiago T. Boto[d,e], Miriam A. Rosenbaum[d,e], Ramiro Blasco-Gómez[f], Sebastià Puig[f], Stefano Freguia[g], Marianna Villano[c,*]

[a] Australian Centre for Water and Environmental Biotechnology (ACWEB, formerly AWMC), The University of Queensland, Brisbane, QLD 4072, Australia

[b] School of Earth and Environmental Sciences, The University of Queensland, Brisbane, QLD 4072, Australia

[c] Department of Chemistry, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy

[d] Bio Pilot Plant, Leibniz Institute for Natural Product Research and Infection Biology – Hans-Knöll-Institute (HKI), 07745 Jena, Germany

[e] Faculty of Biological Sciences, Friedrich Schiller University (FSU), 07743 Jena, Germany

[f] LEQUIA, Institute of the Environment, University of Girona, Maria Aurèlia Capmany 69, 17003 Girona, Spain

[g] Department of Chemical Engineering, The University of Melbourne, Parkville, VIC 3010, Australia

*Corresponding author: marianna.villano@uniroma1.it

ABSTRACT

The market of biobased products obtainable via fermentation processes is steadily increasing over the past few years, driven by the need to create a decarbonized economy. To date, industrial fermentation (IF) employs either pure or mixed microbial cultures (MMC) whereby the type of the microbial catalysts and the used feedstock affect metabolic pathways and, in turn, the type of product(s) generated. In many cases, especially when dealing with MMC, the economic viability of IF is hindered by factors such as the low attained product titer and selectivity, which ultimately challenge the downstream recovery and purification steps. In this context, electro-fermentation (EF) represents an innovative approach, based on the use of a polarized electrode interface to trigger changes in the rate, yield, titer or product distribution deriving from traditional fermentation processes. In principle, the electrode in EF can act as an electron acceptor (i.e., anodic electro-fermentation, AEF) or donor (i.e., cathodic electro-fermentation, CEF), or simply as a mean to control the oxidation-reduction potential of the fermentation broth. However, the molecular and biochemical basis underlying the EF process are still largely unknown. This review paper provides a comprehensive overview of recent literature studies including both AEF and CEF examples with either pure or mixed microbial cultures. A critical analysis of biochemical, microbiological, and engineering aspects which presently hamper the transition of the EF technology from the laboratory to the market is also presented.

Keywords: electrode potential; microbial electrochemical technology; anodic electro-fermentation; cathodic electro-fermentation; redox mediator; pure culture; microbial mixed cultures; bioelectrochemical system

1. INTRODUCTION

Electro-fermentation (EF) is an innovative field of biotechnology that involves the use of electrochemical means to steer and control fermentative processes (Chandrasekhar et al., 2021; Chu et al., 2020; Moscoviz et al., 2016; Schievano et al., 2016; Vassilev et al., 2021). It merges principles of traditional industrial fermentation (IF) with the ones of applied electrochemistry. As such, knowledge of both fields is an essential prerequisite to understand the fundamental aspects and know-how of EF.

To start with, while IF originated as a process to improve food transformation, production, and conservation, nowadays it represents a broader biotechnological platform whereby organic materials are transformed by microorganisms into a variety of products (Bader et al., 2010; Wainaina et al., 2019). These include organic acids, amino acids, alcohols, solvents, vitamins and other secondary metabolites, enzymes, as well as biofuels and biopolymers, all of which are valuable products that find applications in a variety of industrial sectors, including chemical food, pharmaceutical, and medical applications, as well as textile, leather processing and plastic manufacturing (Jiang et al., 2017; Lee et al., 2011, 2014).

However, IF is typically based on the use of axenic microbial cultures or dedicated biological agents (e.g., *Saccharomyces cerevisiae* for bioethanol production) and this often represents a significant economic constrain. Indeed, even though pure culture-based processes typically allow high selectivity and productivity, the maintenance of sterile conditions also implies the utilization of specific and purified substrates (e.g., carbohydrates, corn starch, sugarcane or chemicals), whose availability can require dedicated land use and agro-industrial transformations, strongly impacting the overall IF cost (Rago et al., 2019; Straathof et al., 2019). Over the last years, these issues have re-directed many research efforts towards the employment of mixed microbial cultures that, besides requiring simplified process operation and favouring the economic viability

and sustainability of the technology, also benefit from the possibility of using cheap or no cost and non-sterile substrates as feedstock. In this context, the opportunity to extract added-value substances even from waste organic matter gives to IF processes a significant role in the development and implementation of the biorefinery concept as well as in the diffusion of the more recent model of the circular bioeconomy (Alibardi et al., 2020; Leong et al., 2021). This model is being rapidly established due to the multiple drivers involved, which include the pressing issues toward the creation of a fossil fuel independent economy to reduce climate change.

While IF is well placed in this regard, it typically suffers of low product selectivity, especially when using mixed cultures. In this context, EF technology has emerged as an innovative tool to boost the production of desired compounds and thereby improving penetration of IF into chemical markets. In the following Section 1.1, a broader overview on market-relevant IF processes and evolving trends will be firstly provided, before outlining the principles of EF in Section 1.2.

1.1 INDUSTRIAL FERMENTATION MARKET AND EMERGING APPLICATIONS

Over the past two decades, the global market of fermentation products (grouped in **Table 1** based on category) has increased steadily, valued at $127 billion in 2013, and projected to exceed $200 billion by 2023 (De Jong et al., 2020; Deloitte, 2014; Wood et al., 2021). While this rise is certainly linked to the rapid growth of the bioethanol industry and the increasing demand for bioethanol blends in the transportation sector - presently contributing to over 80% of the overall market share – other relevant factors play important roles. Undoubtedly, these include the ever-growing interest in fermentation products driven by the quest for sustainability. An example is given by bio-based organic acids, which have broad and relevant industrial applications as polymer building blocks and commodity chemicals (Agler et al., 2011). Among them, the 3-hydroxy-propionic acid and itaconic acid represent emerging fermentation-derived compounds, that can be used as

precursors for the synthesis of acrylic acid and methyl methacrylate (used in Plexiglas production), respectively; and have been identified as top value chemicals from biomass (Becker et al., 2015). Importantly, organic acids are also direct precursors for the biosynthesis of polyhydroxyalkanoates (PHA), which are fully biodegradable polymers with a broad portfolio of industrial applications, including the packaging and medical sectors (Medeiros Garcia Alcântara et al., 2020; Raza et al., 2018; Valentino et al., 2017). Amino acids and biogas (comprising gaseous hydrocarbons) represent other key segments of the global fermentation market. In particular, the fermentative production of amino acids from inexpensive carbon and nitrogen sources, typically using *Corynebacterium glutamicum* as a biocatalyst, presently sustains a market with strong prospects of growth, also in consideration of the increasing demand for these molecules from the food, pharmaceutical, and nutraceutical industries (Sanchez et al., 2018). In this context, important research efforts are presently undertaken to improve production yields (e.g., through manipulating intracellular availability of redox carriers) and broaden the range of attainable products. In this respect, great interest has been directed toward methionine (i.e., a poultry feed additive) which is presently produced exclusively by chemical synthesis, for which, however, a fermentative production route is feasible though not yet competitive (Mitsuhashi, 2014).

Biogas production via anaerobic digestion (AD) can be also regarded as another relevant and growing branch of the global fermentation market. AD is a complex anaerobic fermentation process whereby waste organic substrates are converted into a methane-rich gas and digestate as products (Appels et al., 2011; Fu et al., 2021). While initially regarded primarily as a waste treatment technology, in recent years AD is rapidly becoming a central hub of the future circular bioeconomy. Indeed, innovative applications of AD move beyond the production of biomethane and focus on the production of cleaner gas blends such as hythane and/or valuable liquid chemical feedstocks such as medium-chain fatty acids (Da Ros et al., 2020; Menon and Lyng, 2021).

An overview of the global market of fermentation products (grouped by categories), including examples of bio-based compounds holding emerging application interest, is summarized in **Table 1**.

Table 1. Overview of the global fermentation market and emerging applications.

PRODUCT CATEGORY	KEY COMMERCIAL MOLECULES	PREVALENT INDUSTRIAL PRODUCTION ROUTE (beyond fermentation)	GLOBAL MARKET SIZE IN VALUE (BILLION USD) (De Jong et al., 2020)	EXAMPLES OF FERMENTATION-BASED MOLECULES/PROCESSES OF EMERGING INTEREST
Alcohols and Ketones	• Ethanol • n-Butanol • 2,3-Butanediol • Acetone	• Ethene • Propylene • Butene • Propylene + benzene	110.0	*1,4-Butanediol, obtained from direct fermentation of sugars, has a very high demand for a wide range of applications (textiles, electronics, automotive, consumer goods)*
Amino acids	• L-glutamic acid • L-lysine • L-phenylalanine • L-tryptophan	Almost exclusively produced by fermentation	11.0	*Methionine, an essential amino acid usually supplied as feed additive is, so far, mainly produced by chemical synthesis*
Organic acids	• Citric acid • Lactic acid • Succinic acid	• Fermentation only • Acetaldehyde • Maleic acid	3.5	*- 3-hydroxypropionic acid, has a great market potential as chemical precursor for acrylic acid* *- Itaconic acid, obtained from the fermentation of sugars, is increasingly considered for the production of biobased polymers*
Polymers	• Xanthan • Starch • Triacylglycerols	• Fermentation only • Extraction from plants • Extraction from plants	0.6	*Polyhydroxyalkanoates (PHA), microbial storage polymers obtained from volatile fatty acids, hold great promise for several applications*
Biogas & gaseous biofuels	• Methane • Hydrogen	• Natural gas • Steam methane reforming	0.2	*- Hythane, a gaseous blend of methane (80-95%) and hydrogen (20-5%) obtained from the fermentation of organic wastes has improved combustion yields relative to methane* *- Short-chain alkanes, obtained from CO_2, are sustainable drop-in fuels and chemicals*

1.2 FUNDAMENTAL ASPECTS OF ELECTRO-FERMENTATION PROCESSES

From a strictly metabolic standpoint, fermentations are microbially catalysed catabolic reactions in which a substrate is broken down in the absence of an external electron acceptor (such as oxygen, nitrate, or sulphate), generating energy by substrate-level phosphorylation (El-Mansi et al., 2006; Hoelzle et al., 2014; Madigan et al., 2018). Fermentations are internally balanced redox bioprocesses whereby a fermentable substrate becomes both oxidized and reduced. A typical example is glucose fermentation into ethanol (i.e., the reduced product) and carbon dioxide (i.e., the oxidized product). Fermentation processes can also lead to a single product, as in the case of lactic acid production from glucose via homolactic fermentation. Depending on the type of involved microorganisms, glucose fermentation may also lead to the production of a mixture of ethanol, lactic acid and carbon dioxide (i.e., heterolactic fermentation) (Madigan et al., 2018). Notably, in some fermentation processes (e.g., based on mixed- or co-cultures), the redox balance is ensured by the production of molecular hydrogen, which may ultimately become an essential electron donor for other reactions possibly taking place in the biological environment. Typically, redox reactions occurring during fermentation processes involve the cycling of electron carriers, such as nicotinamide adenine dinucleotide (NAD^+), which is reduced into NADH upon substrate oxidation. To regenerate the electron carrier (i.e., NAD^+) for continued reuse in the fermentation pathway, electrons stored in NADH are transferred to an oxidized molecule (e.g., acetaldehyde in the case of alcoholic fermentation), thereby generating a reduced product (e.g., ethanol). Both the reduced and oxidized end-products (e.g., ethanol and carbon dioxide) are excreted from the microbial cell into the environment, as shown in **Figure 1A**. In principle, the relative amounts of obtainable products can be changed by altering the electron flow in the biochemical pathway by means, for example, of control of the hydrogen partial pressure or of the pH in the reaction environment (Cheah et al., 2019; Zhou et al., 2017). Besides these conventional strategies, an

imbalance of the cellular redox state can be also achieved by modifying the intracellular NAD^+/NADH ratio with a polarized electrode. This is a central role of the EF process, which can in principle operate as a tool to enhance the generation of either an oxidized or a reduced product. Indeed, in the anodic electro-fermentation (AEF) the electrode functions as an electron acceptor, possibly sinking a fraction of the electrons deriving from substrate oxidation, which causes a decrease in the intracellular NADH content making electrons unavailable for the generation of a reduced compound (**Figure 1B**). AEF can potentially be exploited when the reduced compound is an undesired product, and it has been proposed as a strategic approach to replace oxygen required in traditional aerobic respiration processes with the anode. The opportunity to make aerobic processes anaerobic brings the considerable advantages of energy and cost savings (Vassilev et al., 2021).

On the contrary, in the cathodic electro-fermentation (CEF) processes the electrode functions as an electron donor and a surplus of electrons, besides those deriving from substrate oxidation, is made available to the cells. This triggers an increase in the intracellular NADH content which may ultimately translate into an increased formation of the reduced end-product, relative to the oxidized end-product (**Figure 1C**). However, it is still unclear whether extracellular electron transfer is involved in EF processes or if the polarized electrode simply acts as a mean to control the oxidation-reduction potential (ORP) of the fermentation broth at its interface with the electrode and, in turn, the intracellular redox state which alters the formation and distribution of reduced and oxidized products (**Figure 1D**). However, even though the occurrence of an electron flow cannot be excluded (e.g., involving redox active species in the fermentation broth), this often cannot explain the change in products distribution (Moscoviz et al., 2016). In addition, when redox mediators are used in high concentrations in a well-mixed reactor, the radius of influence of the electrode may be extended to the whole reactor. Also, synthrophic and cooperative interactions,

based on interspecies electron transfer among different microorganisms, can be triggered by the presence of a polarized electrode (Chu et al., 2020).

Finally, it has been recently reported that changes in products formation during EF can also be induced by changes in the composition of the bacterial community (Toledo-Alarcón et al., 2021).

These aspects, including some relevant examples of both AEF and CEF applications with either pure or mixed microbial cultures, as well as EF reactors and equipment will be deeply discussed in the following sections.

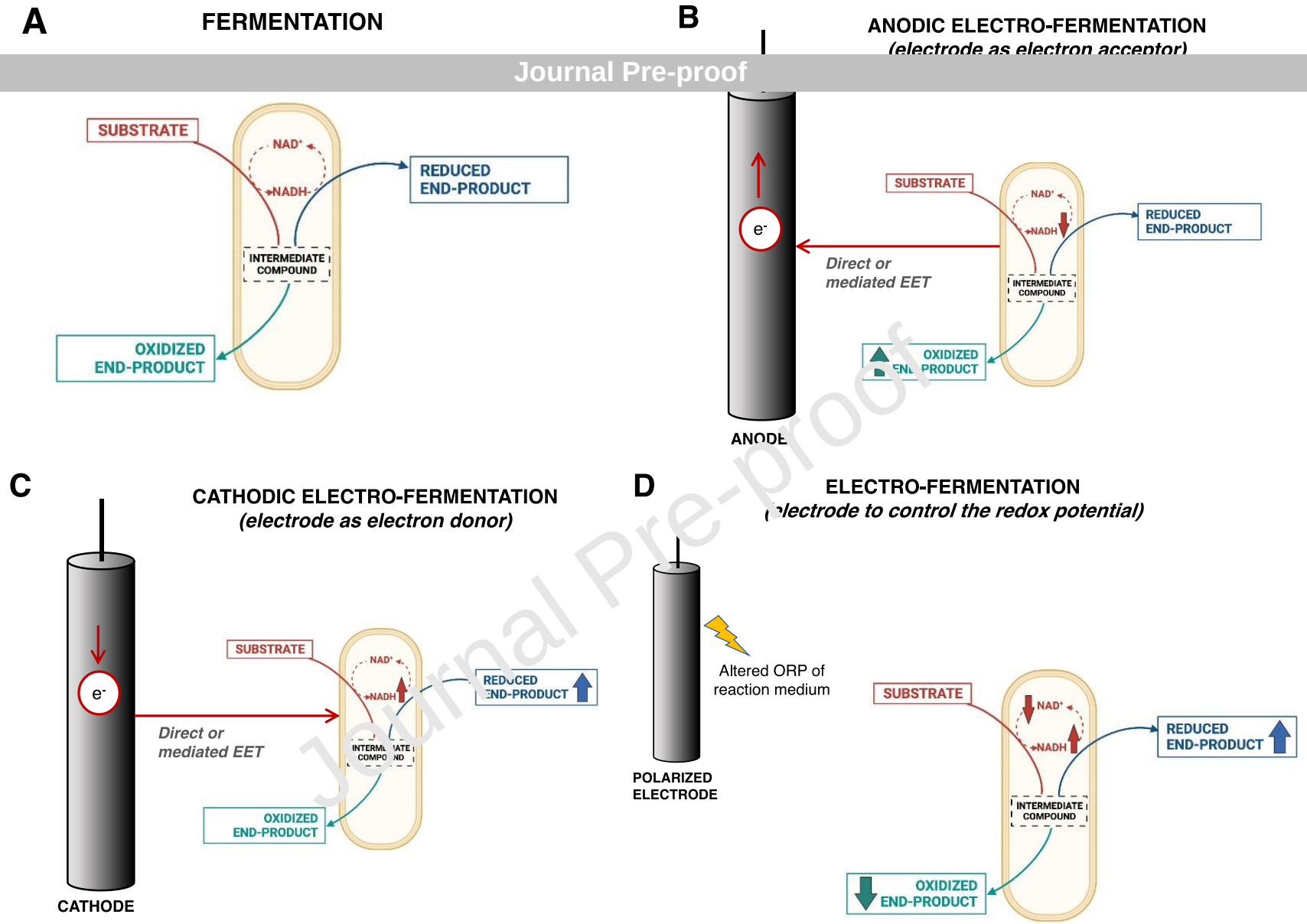

Figure 1. Substrate conversion into products via anaerobic fermentation (A), and via electro-fermentation with the electrode serving as electron acceptor (B), as electron donor (C), or as a mean to control the redox potential of the reaction environment (D). In Figure 1B and 1C the red arrows placed next to NADH highlight the fact that the exchange of electrons with the polarized electrode directly affects the NADH content and, consequently, products distribution.

2. CATHODIC *VS.* ANODIC ELECTRO-FERMENTATIONS

As highlighted previously, depending on the microorganisms and target production pathway, EF can either couple bioproductions to anodic oxidations or cathodic reductions. As a third option, it is also possible to steer and influence microbial fermentations by passively adjusting the redox conditions in the fermentation broth without a direct involvement of the electrochemical system in the microbial production pathways. In this section, we will give an overview of recent developments of these different electro-fermentations, while more detailed examples of EF processes starting from specific substrates is given in the following section.

a) Electrodes as electron acceptors

While the majority of research into microbial electrophysiology and electroactive model organisms has been conducted at the anode, fairly little research has been devoted to anodic bioproductions. Instead, the major application of anodic microbial processes is still centred around electric current generation in microbial fuel cells, reviewed elsewhere (Logan et al., 2019). Here, the harvest of electrons from an oxidation of a complex or defined substrate is the primary goal without the generation of an added value product. Organisms that are efficient in anodic electron discharge, like *Shewanella oneidensis* or *Geobacter sulfurreducens*, are not known to natively make any biotechnologically relevant products. Microorganisms with well-known biotechnological potential, like *Pseudomonas* sp., are not that efficient in anodic electron discharge. Therefore, strong efforts are underway to heterologously utilize and re-engineer the efficient cytochrome-based electron discharge pathways from *S. oneidensis* in the biotech host *E. coli* (Goldbeck et al., 2013; Jensen et al., 2010; Sturm-Richter et al., 2015; Teravest et al., 2014), but the timeline and final degree of success of these efforts are still unknown. Similarly, efforts are ongoing to improve mediator-

based electron discharge in *Pseudomonas* sp. (Askitosari et al., 2019; Schmitz et al., 2015) and couple it to cellular energy generation to be able to drive anaerobic *Pseudomonas* bioproductions. Nevertheless, among all these fundamental efforts some research studies already showed a benefit of coupling a microbial production to anodic electron discharge and provide good examples for AEF. One of the first proof-of-principle studies used the native electron transfer pathway of *S. oneidensis* and combined it with a heterologous fermentation of glycerol to ethanol (Flynn et al., 2010). Later a similar process was obtained in *E. coli* for an unbalanced fermentation of glycerol to acetate via electron discharge with heterologous *S. oneidensis* cytochromes and a synthetic redox mediator (Sturm-Richter et al., 2015). Even more recently, fermentation of glycerol to 3-hydroxypropionate was boosted in *Klebsiella pneumoniae* by regenerating NAD^+ through anodic electron discharge (Kim et al., 2017). This study was followed up by a deeper analysis of the metabolic shift during more oxidative anodic electron discharge conditions compared to conventional fermentation (Kim et al., 2019). Gene expression and metabolic flux analysis revealed a shift towards more oxidative metabolism driven by NAD^+ recycling, whereby the route of electron discharge, in this case, is not clear yet.

A different approach utilizes synthetic redox mediators to couple the discharge of surplus reducing equivalents to an anode. The effect of different external redox mediators on the fermentation profile of *Clostridium cellulolyticum* revealed that the type greatly influences the total amount and spectrum of fermentation products (Sund et al., 2007). Similarly, the activity of different redox mediators for anaerobic electron discharge with *Pseudomonas putida* was investigated (Lai et al., 2016). Here, the well-known redox mediator ferricyanide showed the best potential to couple microbial electron discharge to the production of 2-ketogluconate, which was then further improved by genetic engineering of the production pathway (Yu et al., 2018). A recent study also

showed anaerobic L-lysine production in recombinant *Corynebacterium glutamicum* (Vassilev et al., 2018a).

b) Electrodes as electron donors

Unlike research conducted on the anode, research at the cathode has been focused mainly on bioproduct formation and much less on the specific microbial processes of electron uptake. In a broader perspective, cathodic reactions have proven to be a feasible way of reducing inorganic carbon sources like carbon dioxide with electrons derived from the cathode in order to generate methane or multi-carbon organic molecules, processes specifically referred to as electromethanogenesis (Cheng et al., 2009; Villano et al., 2010) or microbial electrosynthesis (MES) (Nevin et al., 2010), respectively. Hereby, the bacteria serve as biocatalysts facilitating the transfer of electrons towards the production of cellular reducing equivalents. Since the first reports of cathodic electro-autotrophic growth of *Geobacter metallireducens* coupled to nitrate reduction (Gregory et al., 2004) or the production of acetate and 2-oxobutyrate by *Sporomusa ovata* (Nevin et al., 2010), several bacterial groups have been found to be able to perform cathodic reactions. A central group of interest are acetogens, belonging to the genera *Sporomusa, Clostridium* or *Moorella,* which are capable of reducing carbon dioxide using cathode-derived electrons to produce short-chain organic acids and in some cases their corresponding alcohols *via* the Wood-Ljungdahl carbon fixation pathway (Aryal et al., 2017; Chen et al., 2018; Nevin et al., 2011). Initially, it has been suggested that electrons were consumed via direct electron transfer by acetogens (Nevin et al., 2011). However, there is still no scientific confirmation of a direct electron consumption in these organisms, even though foundational cytochrome- based mechanistic models for electron transport into cells have recently been proposed (Lovley, 2022). Instead, it has been proposed that electron transfer could be mediated by electrochemically-generated and

microbially-boosted hydrogen or formate as electron mediators (Deutzmann et al., 2015; Jourdin et al., 2015; Philips, 2020). Other technical innovative approaches such as the use of thermophilic mixed cultures, the external addition of ethanol as a supplementary organic electron donor, the fine-tuning of reaction conditions, and the employment of alternative reactor configurations have also expanded the opportunities for acetogens in microbial electrosynthesis (Jiang et al., 2020a, 2018; Rovira-Alsina et al., 2020; Vassilev et al., 2019, 2018b). As a process strategy, electrolytic hydrogen formation and microbial fermentation can also be uncoupled, as has been shown for *Clostridium autoethanogenum* in a co-culture with *Clostridium kluyveri,* which produced butanol and hexanol using hydrogen generated in a separate electrolyser cell (Haas et al., 2018). This "electrolyser-assisted fermentation" was recently suggested as the only economically viable strategy for scaling up microbial electrosynthesis (Wood et al., 2021). Beyond that, cathodic electron transfer can be enhanced and directed towards a certain product formation via a metabolic shift induced by the introduction of synthetic mediators such as Neutral Red and Brilliant Blue as demonstrated with *Clostridium pasteurianum* (Utesch et al., 2019).

Cathodic electroactivity has also been described in some iron-oxidizing, ammonia-oxidizing and sulfate-reducing bacteria, but these groups are considerably less known because the research focus has been around acetogens and methanogens. Some sulfate-reducing strains such as *Desulfosporosinus orientis* are capable of electro-autotrophically forming acetate and butyrate (Agostino et al., 2020). In a recent investigation, a PHB-producing knallgas bacterium was isolated from a sulfur-based extremophilic community, expanding the range of possible cathodic products (Reiner et al., 2020). Synthetic biology developments have allowed the implementation of bioelectrosynthetic processes in *E. coli*, supplemented with synthetic cathodic electron mediators, through the increase of the metabolic FAD pool to produce succinate (Z. Wu et al., 2019) or for the bioconversion of chiral alcohols (Mayr et al., 2020).

The developing concept of CEF closely relies on the possibility to obtain products from the reduction of substrates different from carbon dioxide. Yet, the involved mechanisms whereby cathodes are used as direct or indirect electron donors for the generation of target products, remain scarcely investigated. Overall, the lack of a scientifically proven understanding of electron uptake pathways has greatly limited the commercialization of CEF. For example, a hydrogen-driven process would require very different design and operation compared to a process driven by direct microbial electron uptake (Wood et al., 2021). Therefore, a more thorough physiological clarification of electron consumption is a prerequisite for taking CEF to the next level.

c) Electrodes as means to control the redox potential of fermentation broth

A technically much simpler strategy for EF compared to the specific reactions of microorganisms with an anode or cathode as presented above is to broadly utilize electrochemistry to manipulate the redox environment (ORP) of microbial cells, which affects the metabolic activity and, in turn, the redistribution of fermentation products. Several studies have shown that redox potential control can greatly influence the metabolic spectrum of microbial fermentations especially under anaerobic or microaerobic conditions. In the past, this has been mainly achieved by adding redox-active chemicals to the fermentation broth such as sorbitol, dithiothreitol, sodium sulfide or oxygen/air (Chen et al., 2012; Kastner et al., 2003; Li et al., 2010). Overall, the degree of reduction of extracellular chemicals is also reflected by intracellular redox balances such as the NAD^+/NADH level (Berríos-Rivera et al., 2002; De Graef et al., 1999). More directly, redox-active molecules have also been shown to act as transcription factors to control specific gene expression (Pei et al., 2011; Wietzke and Bahl, 2012). Only in more recent years, the use of electrochemical bioreactors became a relevant strategy to control the redox potential of the fermentation medium (Li et al., 2010; Riondet et al., 2000). Especially in anaerobic solventogenic fermentations with Clostridia

(Arbter et al., 2021; Engel et al., 2019; Liu et al., 2017, 2013; Mahamkali et al., 2020), the redox potential is an important control parameter, since it correlates directly with intracellular $NAD(P)^+$/NAD(P)H levels, which in turn influence the production of solvents like butanol, acetone or ethanol. A fine-tuned analysis of the influence of different redox potentials on the metabolic spectrum could enable a simple strategy for changing the balance of microbial products. Comparative analyses at different redox potentials and the effect on fermentation products have been performed among others for *Saccharomyces cerevisiae* ethanol formation (Liu et al., 2016), for *Rhodosporidium toruloides* lipid production (Arbter et al., 2019), and for *Clostridium thermosuccinogenes* succinate formation (Sridhar and Eiteman, 2001, 1999). More detailed, systematic work investigating the specific physiological effects for individual microorganisms is still scarce but has recently been performed for *Escherichia coli* by Arunasri et al. 2020. The work compared the organic acid production profile with the level of related gene expression when the electrochemical potential of the working electrode was varied from -0.6 to +1.0 V vs. Standard Hydrogen Electrode (SHE) and found a switch from a hydrogen-dominated to a lactate-dominated metabolism. The use of synthetic mediators can also boost the effect of the applied potentials for certain reactions. Neutral red and AQDS are able to increase butyrate production and selectivity in fermentations from glucose (Paiano et al., 2019), while butanol production can be promoted using Neutral red with *Clostridium beijerinckii* (Zhang et al., 2021).

Mainly originating from environmental engineering and target applications such as wastewater treatment, bioelectrochemical-based potential control was quickly also proposed for steering anaerobic fermentations of undefined mixed microbial cultures. Here, the means of process control are in general limited and steering the activity of the community to, as an example, a more reduced product spectrum (as reported in Figure 1D), like fuel gases (hydrogen and methane) or alcohols from volatile fatty acids or sugars has proven very successful (Gavilanes et al., 2019;

Sasaki et al., 2018; Shanthi Sravan et al., 2018; Villano et al., 2017). However, the possibility to enhance the production of oxidized compounds via ORP-driven EF as explained above cannot be ruled out.

3. PURE- AND MIXED MICROBIAL CULTURE-BASED ELECTRO-FERMENTATION STUDIES

Fermentation, as previously specified, is broadly defined as carbon catabolism in the absence of an external terminal electron acceptor (i.e., O_2, NO_3^-, or SO_4^{2-}), whereby cells instead utilize oxidative substrate-level phosphorylation coupled with substrate reduction to balance electron load and generate ATP (El-Mansi et al., 2006; Hoelzle et al., 2014; Madigan et al., 2018). The cellular mechanisms, which regulate electron transport and ATP-generating processes, including electron bifurcation (Buckel and Thauer, 2013; Peters et al., 2016), proton motive force ATP generation (Konings et al., 1995; Maris et al., 2004; Mitchell, 1978; Rodriguez et al., 2006), and redox pair homeostasis (Green and Paget, 2004; Liu et al., 2013; Moscoviz et al., 2016), drive intracellular electron balancing by generating the large of organic acids, alcohols, and other compounds associated with fermentation processes. EF makes use of theses mechanisms by altering the cellular electron load, forcing cells to adjust substrate reduction stoichiometry to maintain the electron balance.

The range of biochemicals that can be produced from fermentation processes is determined by the complex metabolic pathways that have evolved in microbes to balance electron loads (**Figure 2**). However, any given microbe only possesses a subset of these pathways as determined by its genetic makeup, and so the range and yield of these products, as well as the substrates used to generate them, are determined by the specific microbial community present in the process. Pure (Arbter et al., 2019; Emde and Schink, 1990; Utesch et al., 2019) and defined co-culture (Awate et al., 2017; Speers et al., 2014) processes utilize one to two specific microbial strains. The substrate

utilization and product spectrum potentials of these systems are highly limited by the specific metabolisms of those strains, though lack of competition enables very high product yield and specificity. Mixed and open-culture processes (Dennis et al., 2013; Jiang et al., 2018; Paiano et al., 2019; Raes et al., 2020), by contrast, utilize diverse consortia of microbes which convert substrates to products via total metabolism of the culture. Their metabolic potential is limited only by the genetic range of the whole community, thus enabling a both broader substrate utilization and product spectrum. Process controls, such as applied potential (Jiang et al., 2018; Paiano et al., 2019), pH (Atasoy et al., 2019; Lu et al., 2011; Mohd-Zaki et al., 2016; Temudo et al., 2007), substrate load (Eng et al., 1986; Hoelzle et al., 2021; Romli et al., 1995), and substrate type (Raes et al., 2020) can be used to tune mixed-culture metabolic outputs toward desired products. The range of microbial species inherently competes for resources in mixed culture systems, however, resulting in lower product yield and specificity compared to defined culture processes.

In addition to the trade-offs in metabolic potential between defined and mixed-culture systems, process costs must also be considered. Defined-culture systems often require expensive methods of process sterilization in order to maintain the desired community (Angenent et al., 2004; Hoelzle et al., 2014; Spirito et al., 2014). They may also require defined "clean" substrates, specific nutrient additives, and rigorous process controls, which all further increase the cost of operation. By contrast, community heterogeneity is generally desired in mixed culture systems, and so simple systems without sterilization are often sufficient. "Dirty" substrates, such as municipal waste streams, agricultural byproducts, and other organic wastes, can also be used as feedstock with minimal pre-treatment (Awate et al., 2017; Shanthi Sravan et al., 2018). Due to these metabolic and equipment trade-offs, defined-culture systems are typically used for production of high-value products which justify the higher costs of construction and operation (Damasceno et al., 2004; Singh et al., 2013; Xie, 2017), whereas mixed-culture systems are used for production of lower-

value products or where varied substrate utilization or product selection is desirable (Brar et al., 2014; Sreela-or et al., 2011).

Fermenting organisms use different metabolic strategies to generate energy depending on the substrate type. Carbohydrates, which are processed through central carbon metabolism pathways, tend to be widely accessible substrates for diverse ranges of microbes (Madigan et al., 2018). 6-carbon carbohydrates are catabolized through either Embden-Meyerhof-Parnas (EMP) glycolysis or the Entner-Doudoroff (ED) pathway, though EMP glycolysis is more typical for fermenting organisms (Peekhaus and Conway, 1998). 5-carbon carbohydrates, by contrast, are catabolized through the pentose-phosphate (PP) pathway (Kruger and von Schaewen, 2003). Glycerol fermentation branches from carbohydrate fermentation, and is utilized by some organisms to generate highly reduced products. In particular, species of *Klebsiella*, *Clostridia*, *Citrobacter*, *Lactobacillus*, and *Enterobacter* are able to generate energy by consuming glycerol through the later steps of either EMP glycolysis or the ED pathway while balancing the electron load through direct reduction of some of the glycerol to 1,3-propanediol (Saxena et al., 2009). This process only occurs in the absence of carbohydrates, which act as an inhibitor to 1,3-propanediol production (Cameron et al., 1998). Finally, the products of carbohydrate and glycerol fermentation, which are generally short chain fatty acids (organic acids with ≤4 carbons, also called SCFAs) and alcohols, can be catabolized through secondary fermentation pathways into acetate, ethanol, and medium (C5-C12) and long (C13+) chain fatty acids (MCFAs and LCFAs, respectively) (Batstone et al., 2002; Spirito et al., 2014). Based on these considerations, examples of EF studies grouped on substrate typology with either pure or mixed microbial cultures are hereafter discussed.

3.1 CARBOHYDRATE FERMENTATION

Plant-based carbohydrates account for the largest proportion of biomass and organic carbon on earth, primarily in the forms of cellulose and hemicellulose (Abdel-Hamid et al., 2013; Bar-On et al., 2018; Boerjan et al., 2003). Starchy and hemicellulose-rich plants are often farmed for biofuel and biochemical production, and carbohydrates also account for a large proportion of wastewater organic content (Huang et al., 2010). As such, carbohydrates are a heavily researched substrate for the development of a bioeconomy (Agler et al., 2011; De Groof et al., 2019; Hoelzle et al., 2014).

In fermentation processes, carbohydrates are metabolized through central carbon metabolism, thus serving as both the primary source of cellular energy as well as the primary carbon source for biomass (Hoelzle et al., 2014; Rodriguez et al., 2006). EMP glycolysis and the ED and PP pathways are among the most highly conserved forms of carbon metabolism (Flamholz et al., 2013; Madigan et al., 2018)**,** and much of this carbon is further oxidized to acetate and butyrate to generate further ATP (Decker et al., 1970; Jungermann et al., 1973; Schröder et al., 1994)**.** These oxidative, energy-generating pathways result in excess electrons which are used to reduce a portion of the carbon to a variety of additional products, thus maintaining an overall redox balance. Using EF, it is possible to influence the redox balance of the fermentation products by altering the intracellular redox balance via interaction with electron-mediating cofactors (Hoelzle et al., 2014). As already discussed, anodic EF will typically shift metabolism towards more oxidized products, while cathodic EF will generally shift metabolism towards more reduced products.

The highest value products are those used in the production of bioplastics, pharmaceuticals, or food additives. These include 2-ketogluconic acid (2KGA), polyhydroxybutyrate (PHB), acetoin, L-lysine, propionate, lactate, and butanol. 2KGA, PHB, and acetoin are each relatively oxidized products (**Figure 2**), and therefore tend to benefit from anodic pressure. Lai and colleagues and Yu and colleagues (Lai et al., 2016; Yu et al., 2018) each achieved up to 6.4-fold increased 2KGA production via an anodic ferrocyanide (E' = +0.420 V_{SHE}) mediation system with pure cultures of

Pseudomonas putida. Similarly, another study (Nishio et al., 2014) applied anodic PMF (i.e., Poly2-methacryloyloxyethyl phosphorylcholine-co-vinylferrocene) mediation (E' = +0.500 V_{SHE}) to a *Ralstonia eutropha* pure culture to increase PHB production by 60% *vs.* OCV (open circuit voltage). In another pure culture study with engineered *E. coli*, Förster and colleagues (Förster et al., 2017) improved acetoin production by 8.2-fold using the mediator methylene blue (E' = -0.076 V_{SHE}).

By contrast, the more reduced compounds (e.g., propionate and butanol) or even those with an identical degree of reduction (e.g., lactate) increase in production in cathodic systems. Emde and Schink (Emde and Schink, 1990) and Schuppert and colleagues (Schuppert et al., 1992) each improved propionate production from a pure culture of *Propionibacterium* (*freudenreichii* and *acidipropionici*, respectively) with two separate mediators, AQDS (E' = -0.224 V_{SHE}) and CoS (E' = -0.390 V_{SHE}). Sasaki and colleagues (Sasaki et al., 2014) similarly used AQDS to achieve a 1.47-fold increase in lactate production from *Corynebacterium glutamicum*, while Kracke and colleagues (Kracke et al., 2016) achieved a 35-fold increase with *Clostridium autoethanogenum* and a cobalt mediator ([Co(*trans*-diammac)]$^{3+}$, E' = -0.555 V_{SHE}). Finally, another study (Kim and Kim, 1988) demonstrated 1.3-fold improved butanol yield from *Clostridium acetobutylicum* using methyl viologen (E' = -0.450 V_{SHE}).

L-lysine presents an interesting case, as it is itself a relatively reduced product, but its metabolism first requires oxidation of pyruvate to oxaloacetate. In two pure culture *Corynebacterium glutamicum* studies, Xafenias and colleagues (Xafenias et al., 2017) and Vassilev and colleagues (Vassilev et al., 2018a) each achieved 2.1-fold increase in L-lysine production with potentials of +0.450 to +0.697 V_{SHE}, reflecting the oxidative intermediate step. However, the greatest production increase (4.8-fold) was achieved cathodically by Xafenias and colleagues (Xafenias et al., 2017) with the electron mediator AQ2S (E' = -0.45 V_{SHE}).

Mixed-culture systems, while easier to set up and operate than pure culture systems, tend to produce predominantly lower value acetate and butyrate. The high functional diversity of mixed cultures generally leads to consumption of the higher value products into secondary fermentation (see below). However, the correlation between applied potential and product redox distribution is still evident, with increased acetate and butyrate production observed within the potential range of -0.79 to +0.01 V_{SHE} (Jiang et al., 2018; Paiano et al., 2019; Shanthi Sravan et al., 2018; Villano et al., 2017), Table 2.

3.2 GLYCEROL FERMENTATION

Glycerol finds several uses in a variety of industries, including cosmetics, food, pharmaceuticals, automotive. Being also a by-product of the biofuel industry has made it a cheap source of reduced organic carbon, and it has pushed researchers into developing biotechnologies for its conversion into valuable products. Several bacterial species can ferment glycerol to produce valuable products including organic acids and solvents (Temudo et al., 2008). Among these, 1,3-propanediol (1,3-PDO) has attracted attention due to its comparatively higher market value and its use in bioplastic production. Glycerol conversion to 1,3-PDO occurs through two sequential reactions: glycerol → 3-hydroxypropionaldehyde (3-HPA) by means of glycerol dehydratase with coenzyme B_{12} as a cofactor, and 3-HPA → 1,3-PDO by 1,3-propanediol oxidoreductase (Kim et al., 2020, 2017). The latter reaction requires reducing equivalents to deliver the two additional moles of electrons necessary to balance glycerol conversion to 1,3-PDO (Kim et al., 2020). These are typically sourced from NADH produced through the nonreductive metabolism of glycerol, which products include acetate, ethanol, succinate, lactate, propionate (Temudo et al., 2008). Since 1,3-PDO production is a sink for excess reducing equivalents, production yields are highly affected by the internal carbon and electron fluxes. Under the rationale that the provision of additional

reducing power from electrodes could improve 1,3-PDO yields and rates, several studies have focused on CEF to improve the reductive metabolism of glycerol, employing both mixed and pure cultures (**Table 2**). Using mixed cultures, Zhou and co-workers reported a CEF system whereby the application of -0.90 V_{SHE} resulted in a 2-fold increase in 1,3-PDO production yield relative to open circuit fermentation control (Zhou et al., 2013). Similar results were reported by the same group for EF under galvanostatic operational mode (applied currents: 1-10 mA). In this case, the continuous supply of electrons drove the microbial competition between glycerol reduction and microbially mediated hydrogen evolution (Zhou et al., 2015). Using batch EF with electrode potentials in the range of -0.80 V_{SHE} and -1.1 V_{SHE}, Xafenias and co-workers reached a 6-fold increase in production rates and a 2.3-fold increase in product concentration (up to 42 $g_{1,3\text{-}PDO}\ L^{-1}$) relative to the open circuit controls (Xafenias et al., 2015). To remove the role of hydrogen as a possible indirect electron donor during glycerol metabolism, Moscoviz and colleagues (Moscoviz et al., 2018) applied a cathode potential of -0.656 V_{SHE} which could not support abiotic hydrogen evolution. A metabolic shift towards higher 1,3-PDO production yields was observed (10% higher relatively to open circuit controls). Interestingly, the total electron input represented only less than 1% of the total electrons provided by glycerol, which indicates that the electric current is not sufficient to explain redirection of metabolic pathways. This confirmed observations by Choi *et al*. who, by exposing a pure culture of *C. pasteurianum* to an even higher cathodic potential of +0.045 V_{SHE}, noted that a small electron uptake significantly promotes NADH-consuming pathways over stoichiometric conversions of cathode-derived electrons (Choi et al., 2014). This suggested that unbalanced $NADH/NAD^+$ ratios have a greater effect on diverting metabolic pathway through cellular regulation than through direct electron supply (Choi et al., 2014; Moscoviz et al., 2016).

Among species natively capable of 1,3-PDO production from glycerol, which include members of the *Klebsiella*, *Clostridia*, *Citrobacter* and *Lactobacillus* genera, *Klebsiella* is of note because one of

its members, *K. pneumoniae*, natively possesses active enzymes essential to glycerol reductive pathways (Kim et al., 2020; Kumar and Park, 2018). Strain *K. pneumoniae* L17, was shown as able of extracellular electron transfer during glucose metabolism in the presence of redox mediators in microbial fuel cell (Kim et al., 2016), which made it a good candidate to study metabolic regulation in EF of glycerol. Under CEF conditions, Kim *et al*. observed a 1.5-fold increase in 1,3-PDO concentration at a polarization of -0.69 V_{SHE} in the presence of neutral red, with transcriptional analysis indicating over expression of glycerol dehydratase and 1,3-PDO oxidoreductase driving the shift in carbon flux toward reductive pathways (Kim et al., 2020). AEF of glycerol by *K. pneumoniae* has mostly targeted the production of 3-hydroxypropionic acid (3-HP), a chemical with important applications in industrial chemical synthesis (Kumar et al., 2013). 3-HP production in *K. pneumoniae* shares the first step of the 1,3-PDO production pathway, conversion of glycerol → 3-HPA by glycerol dehydratase, followed by 3-HPA → 3-HP conversion by aldehyde dehydrogenase, requiring NAD^+ to balance excess redox equivalents (as opposed to the reductive metabolism to 1,3-PDO). Regeneration of NAD^+ through aerobic respiration is however not desirable as the synthetic pathway for coenzyme B12 in *K. pneumoniae* is significantly suppressed in the presence of oxygen (Kim et al., 2017). As such, AEF has been proposed to achieve NAD^+ regeneration for 3-HP production. Kim *et al.* cultured recombinant *K. pneumoniae* overexpressing aldehyde dehydrogenase under anodic polarization of *ca*. +0.710 V_{SHE}, in the presence HNQ as redox mediator to regenerate NAD^+ (**Table 2**), resulting in a 1.7-fold increase in 3-HP production compared to an unpolarized control (Kim et al., 2017). Another notable study compared mRNA expression levels for relevant glycerol conversion enzymes under poised and non-poised conditions, observing a positive correlation between activation of NAD^+-dependent pathways under poised electrode conditions (Kim et al., 2019).

Other examples of products deriving from oxidative glycerol metabolism explored through EF include ethanol and acetate. In Sturm-Richter *et al*., researchers achieved a 183% increase of the anodic electron transfer by reprogramming *E. coli* for glycerol fermentation via heterologous expression of *c*-type cytochromes CymA, MtrA, and STC from exoelectrogen *S. oneidensis,* resulting in glycerol conversion into more oxidized products acetate and ethanol in the presence of suitable redox mediators (Sturm-Richter et al., 2015). Flynn *et al*. engineered *S. oneidensis* to stoichiometrically convert glycerol to ethanol, achieving a 33% increase in ethanol production compared to the wild type, demonstrating that linking fermentative glycerol metabolism with current production is potentially an effective strategy to increase product selectivity by eliminate metabolic redox constrains (Flynn et al., 2010). Another possible strategy to improve product selectivity was suggested by Speers *et al.*, who combined the synergistic metabolisms of exoelectrogen *Geobacter sulfurreducens* with the glycerol fermenter *Clostridium cellobioparum*. While the latter performed glycerol fermentation to ethanol at high yield, the former converted the fermentation byproducts into electricity, thereby increasing product purity (Speers et al., 2014).

3.3 SECONDARY FERMENTATION

Products of primary fermentation, SCFAs such as acetate (C2), butyrate (C4), and propionate (C3), as well as ethanol, can be further converted into MCFAs such as valerate (C5), caproate (C6), caprylate (C8), as well as other products through secondary fermentation (**Figure 2**). Production of MCFAs occurs through the reverse-beta-oxidation (RBO) chain-elongation pathway, which works by cyclically adding an acetyl-CoA molecule (typically derived from the oxidation of ethanol or lactate) to a carboxylate, thereby adding 2 carbon units at the time, e.g., to produce *n*-butyrate from acetate, *n*-caproate from *n*-butyrate, or *n*-valerate from propionate, *n*-heptanoate from *n*-

valerate, etc. (Angenent et al., 2016; Chu et al., 2021). RBO occurs at close to thermodynamic equilibrium, as such, it becomes feasible only under favourable environmental conditions, which include the presence of energy-rich compounds as a source of reducing equivalents and acetyl-CoA (Angenent et al., 2016; González-Cabaleiro et al., 2013; Spirito et al., 2014). Much of the fundamental understanding of the RBO pathway has been obtained through pure culture studies. Besides the non-acetogenic model organism *Clostridium kluyveri* (Seedorf et al., 2008), organisms belonging to the genera *Megasphaera* (Choi et al., 2013) and *Eubacterium* (Genthner et al., 1981) have been often reported as capable of chain elongation. While pure cultures allow full control of process parameters to fit specific organisms, reactor microbiomes (i.e., open culture of microbial consortia), which typically produce and consume SCFAs in anaerobic digestion, have been employed for such conversions in recent years (**Table 2**). In fact, not only can open cultures utilize more complex substrates, they also easily adapt to nonsterile conditions typical to waste processing (Angenent et al., 2016; Q. Wu et al., 2019).

Ethanol and lactate are ideal electron donors for chain elongation since they are cheaper than other compounds (such as hydrogen and sugars) and practical to be added in the reaction medium. However, the EF approach could also provide an efficient and practical strategy for the chain elongation process, since reducing equivalents can be supplied - directly or indirectly - by a polarized electrode. Under the rationale that H_2 could also act as the source of reducing equivalents, several researchers have attempted using the electro-fermentative RBO to produce longer-chain chemicals. Steinbusch and co-workers reported on the conversion of acetate into ethanol (as well as butyrate) in an electrochemical system amended with MV as the redox mediator and with the electrode poised at -0.55 V_{SHE} (Steinbusch et al., 2010). Interestingly, the high yield of ethanol was obtained thanks to the inhibitory effect of MV on side reactions such as methanogenesis and *n*-butyrate production, confirming previous observations (Peguin et al., 1994;

Wolin et al., 1964). Alcohol production was also observed in the absence of mediators by Sharma *et al*. (Sharma et al., 2013) and by Gavilanes *et al*. (Gavilanes et al., 2019) in the presence of acetate and butyrate, and at a poised potential of -0.651 V_{SHE} or at an applied cell voltage of 1.5 V. The possibility to use electrodes to drive alcohol formation from SCFA offers opportunities to use EF to promote RBO even when ethanol is not available. In a follow-up study, Steinbusch *et al*. reported on caproate and caprylate production from acetate using either H_2 or ethanol as the electron donors (Steinbusch et al., 2011). This was in line with observations by Van Eerten-Jansen *et al.* who also reported caproate and caprylate production from acetate in the absence of redox mediators with the electrode poised at -0.90 V_{SHE} (Van Eerten-Jansen et al., 2013); and Raes *et al*. who reported production of mostly butyrate from acetate in the absence of ethanol, though, as correctly pointed out by the researchers, the absence of ethanol detection during the fermentation could be due to the complete conversion into RBO products rather than direct acetate conversion (Raes et al., 2017). In general, the rate of RBO observed when hydrogen is the (sole) electron donor is considerably lower than when ethanol is provided in addition to the SCFA, probably because of the typically slow rate of acetate hydrogenation observed in reactor microbiomes (Ganigué et al., 2016; Steinbusch et al., 2008). More recently, Jiang *et al*. observed improved specificity towards caproate production in the presence of acetate, ethanol and electrochemical control (Jiang et al., 2020b), while Raes *et al*. further investigated the role of different supplied substrates in the specificity of product formation (Raes et al., 2020). These works suggested that, similar to what was observed for glycerol EF, electrochemical control offers the opportunity to steer fermentation pathways towards the formation of specific products through intracellular redox regulation rather than through stoichiometric electron utilization, although further research is warranted to elucidate the mechanisms.

Overall, the main results obtained in EF experiments by using the electrode as either anode or cathode are reported in **Table 2** (grouped on the typology of compound used as substrate). Examples of redox mediators typically employed in (electro)-fermentation studies are also reported in **Table 3**.

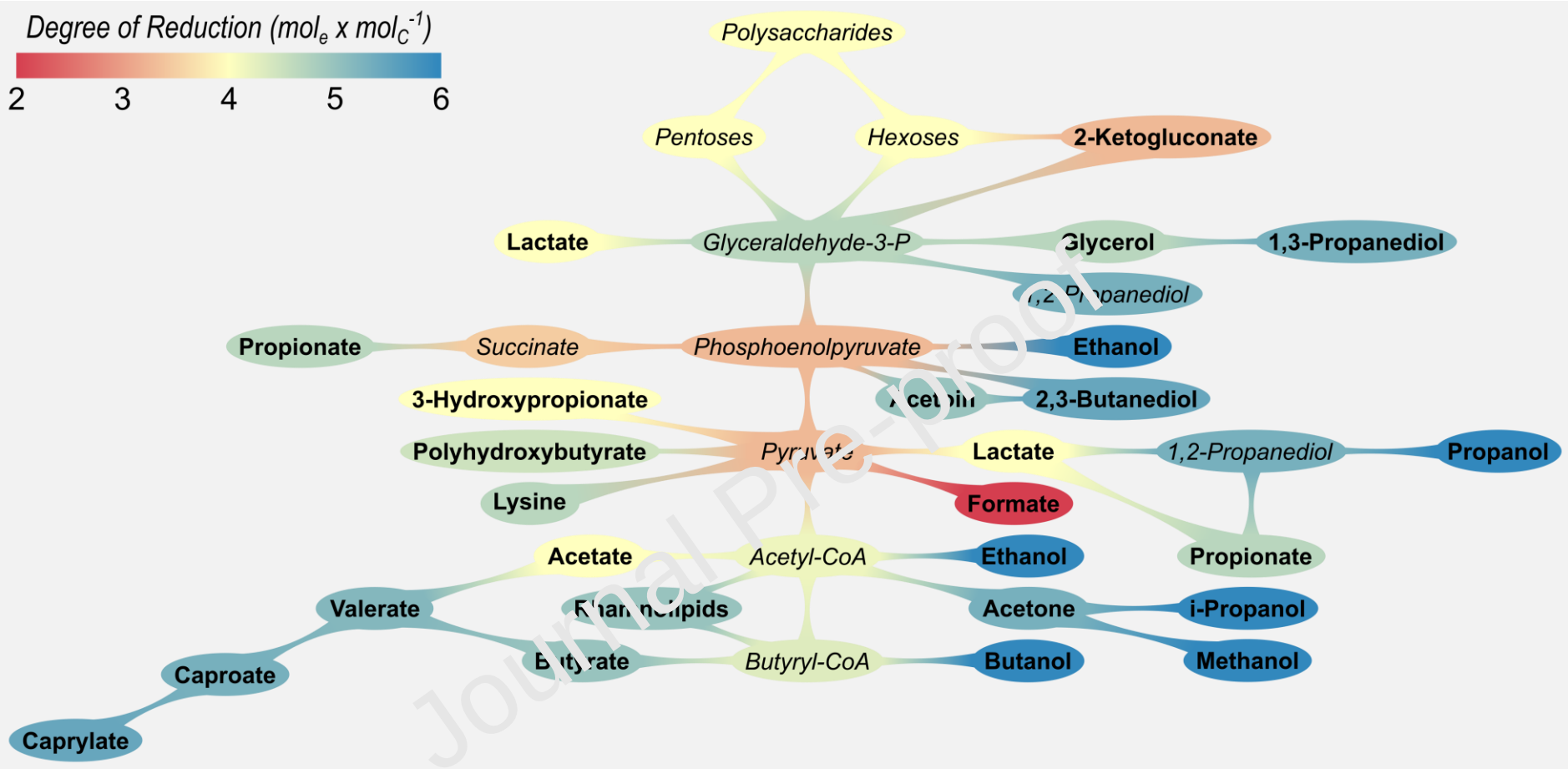

Figure 2. Metabolic pathways for producing common carbon metabolism products, and their degree of reduction. Pathway intermediates have been simplified to only the key branching metabolites. Individual organisms typically possess only a subset of these pathways, though mixed cultures typically possess high metabolic diversity. Chemical species are coloured according to their degree of reduction (in units of mol_e / mol_C), as determined according to (Kracke and Krömer, 2014). In the figure, red indicates oxidative pathways, and blue indicates reductive pathways. Species indicated in bold are those listed in Table 2.

Table 2. Electro-fermentation studies grouped on substrate typology.

Substrate	Microbial catalyst	Type of EF	Applied electrochemical conditions*	External mediator	Main EF Product(s)	Notes on improvement	Reference
Carbohydrates fermentation							
Glucose	Engineered *Corynebacterium glutamicum lysC*	Anodic	+0.697 V_{SHE}	FC	l-lysine	2.1-fold increase (mol/mol) compared to OCV	(Vassilev et al., 2018a)
Glucose	*Pseudomonas putida* F1	Anodic	+0.697 V_{SHE}	FC $[Co(bpy)_3](ClO_4)_3$ TC	2-ketogluconic acid (2KGA)	Increased productivity correlated with the potential of the mediator used	(Lai et al., 2016)
Glucose	Engineered *Pseudomonas putida* KT2440	Anodic	+0.697 V_{SHE} (0.5 V Ag/AgCl, sat KCl)	FC	2-ketogluconic acid (2KGA)	644% increase compared to wild-type	(Yu et al., 2018)
Glucose	Engineered *Pseudomonas putida* KT2440 *phz*	Anodic	+0.697 V_{SHE} (0.2 V Ag/AgCl, sat KCl)	no, self-secreted phenazines	Rhamnolipids	First proof of oxygen-limited synthesis	(Askitosari et al., 2020)
Fructose	*Ralstonia eutropha*	Anodic	+0.600 V_{SHE}	PMF	Polyhydroxybutyrate (PHB)	60% increase in PHB production rate vs OCV	(Nishio et al., 2014)
Cellobiose	*Cellulomonas uda* + Engineered *G. sulfurreducens*	Anodic	+0.450 V_{SHE} (+0.24 V Ag/AgCl 3M KCl)	no	Ethanol	3.7-fold increase (g/g)	(Awate et al., 2017)
Glucose	Engineered *Escherichia coli*	Anodic	+0.200 V_{SHE}	MB	Acetoin	8.2-fold increase in acetoin concentration vs OCV	(Förster et al., 2017)
Glucose	Mixed microbial culture	Cathodic / Anodic	-0.656 V_{SHE} ÷ 1.144 V_{SHE} (-0.900 V ÷ +0.900 V SCE)	no	Butyrate, ethanol	Shift from lactate to butyrate and EtOH production vs OCV. Strong link to microbial community	(Toledo-Alarcón et al., 2019)
Glucose	Mixed microbial culture	Cathodic / Anodic	+0.010 V_{SHE}, -0.390 V_{SHE}, -0.790 V_{SHE}	no	Acetate	Increased by 55% when applied potential shifted	(Jiang et al., 2018)

			(-0.2 V, -0.6, -1.0 V Ag/AgCl 3M KCl)			from +0.010 V_{SHE} to -0.790 V_{SHE}	
					Propionate	No significant change associated to applied potential	
					Butyrate	At +0.010 V_{SHE}, +21% increase relatively to OCV	
Glucose	*Corynebacterium glutamicum* ZW04	Cathodic / Anodic	-1.25 V_{SHE}, +0.45 V_{SHE}	AQ2S	l-lysine	4.8-fold increase on titers (g/g) vs OCV	(Xafenias et al., 2017)
				no		2.1-fold increase on titers (g/g) vs OCV	
Glucose	*Saccharomyces cerevisiae*	Electrostatic fermentation	3÷15 V (voltage across anode and cathode)	n/	Ethanol	2.6-fold increase in the specific ethanol production rate vs OCV	(Mathew et al., 2015)
Sucrose	*Clostridium tyrobutyricum* BAS 7	Cathodic	-0.190 V_{SHE} (-0.40 V Ag/AgCl, 3M KCl)	0.1 mM NR	Butyrate	1.8-fold increase in concentration 1.3-fold increase in yield vs control without mediator	(Choi et al., 2012)
Glucose	*Propionibacterium freudenreichii*	Cathodic	-0.224 V_{SHE}	0.5 mM AQDS	Propionate	1.1-fold increase in concentration vs OCV	(Emde and Schink, 1990)
			-0.390 V_{SHE}	0.4 mM CoS		1.5-fold increase in concentration vs OCV	
Glucose	*Clostridium acetobutylicum*	Cathodic	-0.403 V_{SHE}	no	Acetone	2.04-fold yield increase vs OCV	(Engel et al., 2019)
					Butanol	1.31-fold yield increase vs OCV	
					Ethanol	1.67-fold yield increase vs OCV	
Glucose	*Clostridium pasteurianum* DSM 525	Cathodic	+0.045 V_{SHE}	no	Butanol	2.5-fold increase in concentration vs OCV	(Choi et al., 2014)
Composite food waste	Mixed microbial culture	Cathodic	-0.395 V_{SHE} (-0.60 V Ag/AgCl,	no	Acetate, propionate, butyrate	1.7-fold increase in VFA production	(Shanthi Sravan et

			3.5 M KCl)			(g/g)	al., 2018)
Glucose	*Corynebacterium glutamicum*	Cathodic	-0.400 V_{SHE}	0.2 mM AQDS	Lactate	1.47-fold yield increase	(Sasaki et al., 2014)
				no		1.27-fold yield increase	
Glucose	*Escherichia coli*	Cathodic	*ca.* -0.440 V_{SHE} (-0.650 V Ag/AgCl, KCl not specified)	0.2 mM NR	Ethanol, acetate, lactate	1.2-, 1.2-, and 1.2-fold increase (conc) vs OCV	(Harrington et al., 2015)
Glycerol	*Klebsiella pneumoniae*				Ethanol, propanediol, lactate	2.3-, 1.1-, and 2.1-fold increase (conc) vs OCV	
Glucose, acetate, ethanol	Mixed microbial culture	Cathodic	-0.700 V_{SHE}	no	*i*-Butyrate	20-fold increase of yield (mol/mol) vs OCV	(Villano et al., 2017)
Glucose	*Clostridium acetobutylicum*	Cathodic	-2.50 V_{SHE}	2 mM MV	Butanol	1.3-fold increase (conc.) vs OCV	(Kim and Kim, 1988)
Glucose	Mixed microbial culture	Cathodic	-0.700 V_{SHE}	no	(*i*+*n*)-Butyrate	1.7 ÷ 2.0 fold increase of yield (mol/mol) vs OCV	(Paiano et al., 2019)
			-0.700 V_{SHE}	NR	*n*-Butyrate	1.5 ÷ 3.0 fold increase of yield (mol/mol) vs OCV	
			-0.300 V_{SHE}	AQDS	*n*-Butyrate	1.6 ÷ 7.0 fold increase of yield (mol/mol) vs OCV	
Fructose	*Clostridium autoethanogenum*	Cathodic	-0.603 V_{SHE}	[Co(*trans*-diammac)]$^{3+}$	Lactate, 2,3-butanediol	35-fold and 3-fold increase (conc and yields) vs OCV	(Kracke et al., 2016)
Lactose	*Propionibacterium acidi-propionici*	Cathodic	-0.470 V_{SHE}	0.8 mM CoS	Propionate	1.4-fold increase in concentration vs OCV	(Schuppert et al., 1992)
Glycerol fermentation							
Glycerol	Engineered *Klebsiella pneumoniae* L17	Anodic	*ca*. +0.710 V_{SHE}	0.1 mM HNQ	3-Hydroxypropionic acid	1.7-fold increase in concentration vs OCV	(Kim et al., 2017)
Glycerol	*Klebsiella pneumoniae* L17	Anodic	Operated with a 1 kΩ in MFC	0.1 mM HNQ	Acetate	1.5-fold (concentration)	(Kim et al., 2019)

			mode		3-Hydroxypropionic acid	1.4-fold (concentration)	
					1.3-Propanediol	1.2-fold (concentration)	
Glycerol	*Clostridium cellobioparum* + *Geobacter sulfurreducens* (synthrophic growth)	Anodic	+0.450 V_{SHE} (+0.240 V Ag/AgCl, 3M KCl)	no	Ethanol	1.3-fold increase (conc.) in cocolture *vs* monocultures	(Speers et al., 2014)
Glycerol	Engineered *Shewanella oneidensis*	Anodic	+0.400 V_{SHE}	no	Ethanol	33% increase in ethanol production vs wild type	(Flynn et al., 2010)
Glycerol	Engineered *Escherichia coli*	Anodic	+0.200 V_{SHE}	50 mM MSO 0.010 mM MB	Ethanol, acetate	183% increase in extracellular electron transfer	(Sturm-Richter et al., 2015)
Glycerol	*Clostridium pasteurianum* DSM 525	Cathodic	+0.045 V_{SHE}	no	1,3-Propanediol	*ca*. 1.6-fold increase in concentration vs OCV	(Choi et al., 2014)
Glycerol	Mixed culture (pre-colonised with *G. sulfurreducens*)	Cathodic	-0.6[illegible]6 V_{SHE} (-0.900 V SCE)	no	1,3-Propanediol	*ca*. 10% increase (conc and yields) vs OCV	(Moscoviz et al., 2018)
Glycerol	Engineered *Klebsiella pneumoniae* L17	Cathodic	-0.690 V_{SHE}	NR	1,3-Propanediol	1.5-fold increase (conc.) vs OCV	(Kim et al., 2020)
Glycerol	Mixed microbial culture	Cathodic	-0.800 ÷ -1.10 V_{SHE}	no	1,3-Propanediol	6-fold increase (production rate) vs OCV 2.3-fold increase (conc) vs OCV	(Xafenias et al., 2015)
Glycerol	Mixed microbial culture	Cathodic	-0.900 V_{SHE}	no	1,3-Propanediol	2-fold increase (yield) vs OCV	(Zhou et al., 2013)
Glycerol	*Clostridium pasteurianum*	Cathodic	Applied current: 400 mA	NR	Butanol	33% increase (yield) vs OCV	(Utesch et al., 2019)
				BB	1,3-Propanediol	21% increase (yield) vs OCV	
Glycerol	Mixed microbial culture	Cathodic	Applied current: -3.2 A m^{-2}	no	1,3-Propanediol, carboxylic acids, alcohols	*Product profile associated to microbial composition*	(Dennis et al., 2013)

Glycerol	Mixed microbial culture	Cathodic	Applied current: -1 A m^{-2}, -10 A m^{-2}	no	1,3-Propanediol	2.1-fold increase (conc) when current increased from 1 A m^{-2} to 10 A m^{-2}	(Zhou et al., 2015)
Secondary fermentation							
Lactate	Engineered *Shewanella oneidensis*	Anodic	0 V_{SHE}	no	Acetoin	78% of the theoretical maximum acetoin achievable	(Bursac et al., 2017)
Acetate	Mixed microbial culture	Cathodic	-0.550 V_{SHE}	MV	Ethanol	n.a.	(Steinbusch et al., 2010)
Acetate + butyrate	Mixed microbial culture	Cathodic	-0.651 V_{SHE}	no	Ethanol, methanol, propanol, butanol, acetone	n.a.	(Sharma et al., 2013)
Acetate + butyrate	Mixed microbial culture	Cathodic	Applied voltage: 1.5 V	no	Methanol, ethanol, propanol	n.a.	(Gavilanes et al., 2019)
Acetate	Mixed microbial culture	Cathodic	-0.900 V_{SHE}	no	Butyrate, caproate, caprylate	n.a.	(Van Eerten-Jansen et al., 2013)
Acetate	Mixed microbial culture	Cathodic	Applied current: 3.1 and 9.3 A m^2	no	Butyrate	4.5 higher butyrate concentration and volumetric productivity achieved at higher applied current	(Raes et al., 2017)
Acetate + Ethanol	Mixed microbial culture	Cathodic	-0.590 V_{SHE}, -0.890 V_{SHE}	no	Caproate	28% higher specificity towards caproate than in the OCP control	(Jiang et al., 2020b)
Acetate	Mixed microbial culture	Cathodic	-0.507 ÷ -0.549 V_{SHE}	no	*n*-Butyrate, *n*-caproate	94.1% C4, 4.6% C6	(Raes et al., 2020)
Acetate + propionate					*n*-Butyrate, *n*-valerate	23.7% C4, 73.8% C5	
Acetate + butyrate					*n*-Butyrate, *n*-valerate, *n*-caproate	14.5% C4, 5.8% C5, 83.4% C6	
Acetate+proprionate+butyrate					*n*-Valerate, *n*-caproate	95,4% C5, 4.6% C6	

*Values in brackets are those reported in the original manuscript.

Table 3. Examples of synthetic and natural soluble redox mediators mainly employed in (electro)-fermentation studies.

Compound	Abbreviation	$E^{0'}$ (V *vs* SHE)	Reference
Synthetic mediators			
Poly(2-methacryloyloxyethyl phosphorylcholine-*co*-vinylferrocene)	PMF	+0.500 V	(Nishio et al., 2013)
Ferricyanide	FC	+0.425 V	(O'Reilly, 1973)
$[Co(bpy)_3](ClO_4)_2$	-	+0.310 V	(Lai et al., 2016)
Thionine Chloride	TC	+0.208 V	(Lai et al., 2016)
Dimethyl sulfoxide	DMSO	+0.160 V	(Wood, 1981)
Methylene Blue	MB	-0.011 V	(Fultz and Durst, 1982)
Anthraquinone 2,6-disulfonate	AQDS	-0.184 V	(Emde and Schink, 1990)
Anthraquinone-2-sulfonate	AQ2S	-0.230 V	(Fultz and Durst, 1982)
Neutral Red	NR	-0.325 V	(Fultz and Durst, 1982)
Cobalt Sepulchrate	CoS	-0.350 V	(Emde and Schink, 1990)
Methyl Viologen	MV	-0.440 V	(Fultz and Durst, 1982)
Brilliant Blue (measured for brilliant blue FCF at pH 6.5)	BB	-0.530 V	(Chen et al., 2008)
$[Co(trans\text{-diammac})]^{3+}$	-	-0.555 V	(Kracke et al., 2016)
2-hydroxy-1,4-naphthoquinone	HNQ	-0.600 V	(Kim et al., 2020)
Natural mediators produced by biocatalyst			
Pyocyanin	PYO	-0.034 V	(Askitosari et al., 2020)
Phenazine-1-carboxylic acid	PCA	-0.116 V	(Askitosari et al., 2020)

Riboflavin	RF	-0.210 V	(Wu et al., 2014)

4. REACTOR DESIGN AND ISSUES FOR UPSCALING

The most widely adopted design for bench-scale EF systems is the typical “H-type” geometry (Hongo and Iwahara, 1979), consisting of two glass bottles, separated by an ion exchange membrane, each forming an electrochemical half-cell hosting the anode and cathode, respectively. Alternatively, single chamber reactors have also been employed because of their relative ease of construction and operation (Paiano et al., 2021), with the main drawback being the possible production at the counter electrode of enzymes- or cell-toxic compounds that can inhibit the process (Chen et al., 2020). The characteristics of the EF process – for example, whether based on planktonic or biofilm biocatalysts - highly determines the reactor engineering. In biofilm based systems, effort should aim at maximising biocompatibility, biofilm adhesion, and interfacial contact between electrodes and microbial cells (Cercado et al., 2013; Roy et al., 2016; Sleutels et al., 2009). On the other hand, when the EF process relies on microbial cells in suspension, which may use redox mediators (**Table 3**), the design should guarantee complete mixing conditions and an appropriate biomass retention. Interestingly, to solve some of the challenges associated with standardization and performance comparison, researchers have introduced conversion kits to allow the upgrade of commercial bioreactors, such as those widely used in traditional fermentation research, into bio-electro-reactors (Rosa et al., 2019, 2017). The researchers designed lids for conventional bioreactors which were fitted with ports allowing for the housing of electrodes. These ports also enable the introduction of multiple electrodes to maximise their radius of influence. Following this approach, the anodic glucose fermentation catalysed by an engineered *Corynebacterium glutamicum* for the production of lysine and organic acids was successfully upscaled from 0.35 L to 2.40 L with no significant loss in process performance (Krieg et al., 2018b).

The ultimate goal of reactor and process engineering remains the improvements of reaction yields and rates to bring electro-fermentation to higher technology readiness levels (TRLs) (Jadhav et al., 2022) (**Figure 3**). Electro-biotechnology research should take advantage of ongoing advancements in the field of industrial bioprocessing (Chen et al., 2020; Roy et al., 2016). For example, (*i*) the combination of bioelectrocatalysis and synthetic biology (including novel target pathways and electro-stimulation of microbial metabolism), (*ii*) design and application of new reaction media that favour the bioprocess itself and downstream steps, and (*iii*) engineering applications for large scale implementations.

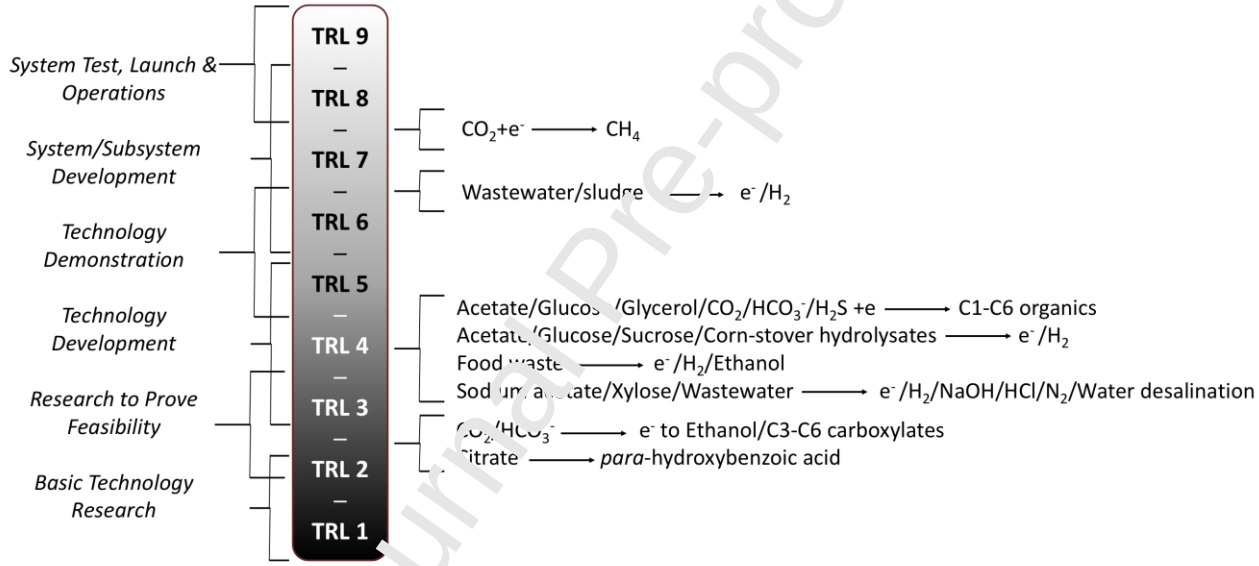

Figure 3. Current TRL state of different examples of electro-fermentation applications.

To address current issues, a synergy between sibling and complementary research fields such as electro-microbiology, electrochemistry, materials science, and engineering are required (Holtmann and Harnisch, 2018). A rational scale-up approach must be prioritized over empirical methods based on trial-and-error experiments, which have been unsuccessful so far in similar electro-biotechnological processes (Cheng and Logan, 2011; Cusick et al., 2011). Scaled-up electro-fermenters require simple and robust designs that allow changes in the feedstock (gas/liquid streams) and the operating times needed for the growth of electroactive populations.

Additionally, selecting proper electrode materials and fermenter architectures that are effective at higher scales has been pointed out as crucial (Schievano et al., 2016). Larger volumes of electrode chambers might in fact lead to limitations in the homogeneous distribution of substrates. Electrode manufacturing can benefit from using 3D-printing technology that allows the tuning of working electrodes towards the maximization of the ratio of electrode surface versus reactor volume (e.g., >2 square/cubic units) and facilitating biofilm attachment (Kracke et al., 2021; Krige et al., 2021). At larger scales (L or m^3 scale), selecting proper mixing conditions is required to increase the contact time between the biofilm and fresh medium, in addition to avoiding pH gradients (Zeppilli et al., 2021). Information available in the literature about design parameters of bioelectrochemical reactors is scarce and lacks standardization of data representation (Santoro et al., 2021). For example, dimension-less numbers (e.g., Reynolds or Newton numbers) or chemical oxygen demand as the total carbon equivalent for the coulombic efficiency calculation must be given to allow proper benchmarking (Krieg et al., 2018a).

The dichotomy between scaling-up and numbering-up in the field of electro-biotechnology is still on debate. Enzmann and co-workers suggested that once a proof of concept is confirmed in a small reactor, the above-mentioned dimensionless parameters must be assessed in small prototypes for further optimization (Dessì et al., 2021; Enzmann et al., 2019). Advanced modelling of processes such as computational fluid dynamics (CFD) analysis can help optimizing the mixing conditions (Korth and Harnisch, 2017; Sharma et al., 2014; Vilà-Rovira et al., 2015). Then, scaling up towards 10-100 L working volume prototypes should be based on the main parameters responsible for performance, as identified through sensitivity analysis (Enzmann et al., 2019). Finally, several of these reactor units can be connected either in series or in parallel as individual or stacked units, leading to the numbering up of the process (Baeza et al., 2017; Heidrich et al., 2013; Isabel San-Martín et al., 2018; Liang et al., 2018). Some experiences have used scale-up

criterions to transit from lab to pilot scale using similar geometries and operational parameters (Enzmann and Holtmann, 2019). Remarkable deviations were observed in electrode distance, Wagner number and abiotic current density, which led to differences between performance due to high electrode resistances. The use of electrode materials with better electronic properties or stacked configurations offers a solution to alleviate electric resistances created at larger scales. In addition, some electro-fermentation configuration could require sacrificial counter-electrodes that are consumed and need periodic replacement. Similarly, ion exchange membranes need to be exchanged, since they lose selectiveness and a long-term use negatively affects their mechanical, chemical, and electrochemical properties (San-Martín et al., 2019b, 2019a). Modular designs should be preferred when replacement of electrodes and membranes is required, as they avoid disturbing to the biological community. Based on these considerations, a schematic overview of critical steps involved in the upscaling of the EF process is depicted in **Figure 4**.

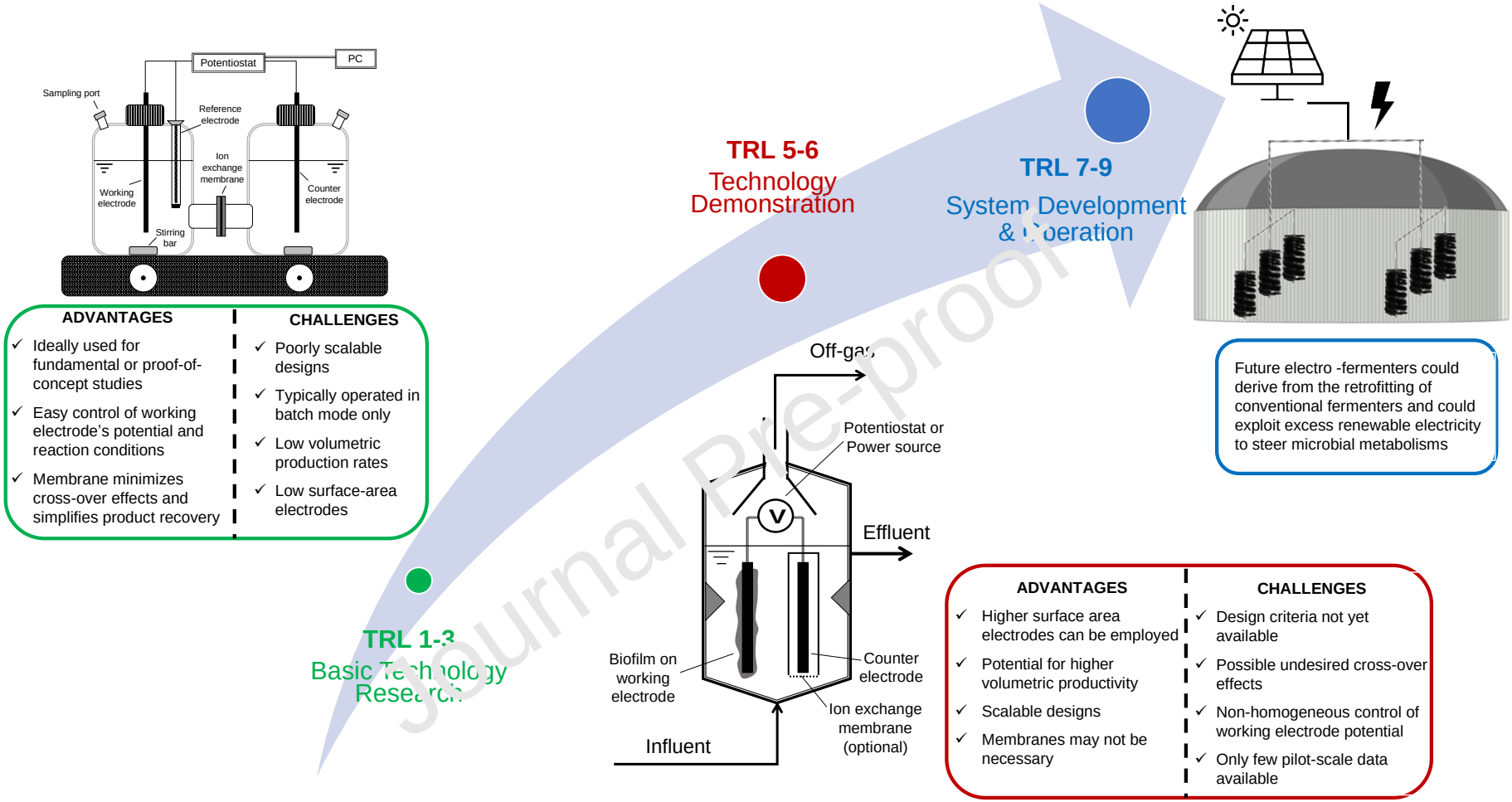

Figure 4. Challenges and perspectives toward the upscaling and deployment of the EF technology.

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This review paper presents a comprehensive analysis of recent literature studies dealing with anaerobic fermentation processes steered by polarized electrodes, an approach commonly referred to as electro-fermentation (EF). Undoubtedly, EF represents an extremely appealing, yet still largely unexploited, biotechnological strategy to gain a greater control over anaerobic fermentation processes, particularly those based on mixed-microbial cultures. Despite the promising results obtained till now, it is apparent that the lack of knowledge on several different scientific and technical aspects still greatly challenge its transition from the laboratory to the market. To start with, the molecular and biochemical basis behind the capability of a polarized electrode to trigger a change in the rate, yield, titer or product distribution during a microbial fermentation process remains largely unknown.

Pure culture experiments partially contributed to the development of mechanistic models which, in some cases, highlighted the pivotal role of the redox state of cells (e.g., $NADH/NAD^+$ ratio) as a major determinant affecting EF performance. However, the validity of such models to other microbial ecosystems or mixed-microbial communities has not yet been confirmed. This typically translated in a poor capacity to model and in turn predict EF performance as well as to define sound design criteria with broad applicability scenarios which could assist the technology upscaling and spur the commercial interest of potential stakeholders. Due to this lack of knowledge, most EF processes are still largely designed and optimized through merely empirical trial-and-error approaches, which typically hold a narrow range of applicability and a very limited validity.

To help address this critical issue, further research efforts are urgently needed which should possibly involve the use of labelled (e.g., ^{13}C) substrates as well as the combined application of a portfolio of chemical, analytical and biomolecular techniques to follow *in vivo* a number of

parameters relevant to the EF process such as the real-time redox state of cells and the intracellular concentration of redox carriers and other key metabolites.

Another important aspect which deserves greater attention is the development of sound design criteria for EF reactors. Indeed, EF has been so far performed using bioelectrochemical reactors traditionally designed and optimized for other purposes and processes such as the microbial fuel cells for energy production or the microbial electrolysis cells for hydrogen or methane production. However, a fundamental difference among the above-mentioned processes is that in EF achieving a high current density is not necessarily the ultimate design criterium to meet process sustainability and economic viability. Indeed, in EF (irrespective of cathodic, anodic or based on an altered ORP of the reaction medium) the electron flow is typically marginal with respect to the carbon flow and productivity of the target metabolite. This finding not only implies that in principle EF is not expected to suffer from the same scalability problems typically affecting all (bio)electrochemical systems but also that traditional anaerobic bioreactors equipped with relatively small surface area electrodes could probably be employed for the purpose; or, in other words the upscaling EF reactors could start from the retrofitting of conventional fermenters. Notwithstanding these aspects, it should be noted that so far, no attempts have been made to identify the role of the electrode material, geometry, topography, morphology, and surface area on the efficacy of EF. Obviously, all these issues come along with the need for a deeper understanding of the underlying microbe-electrode interactions, which are of utmost relevance for the deployment of this exciting technology.

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HIGHLIGHTS

- Polarized electrodes can be used to steer product distribution during fermentations
- Electrodes can serve as electron-donors, acceptors, or as a mean to control ORP
- Biochemical, microbiological, and engineering aspects of EF are reviewed
- Upscaling EF reactors could start from the retrofitting of conventional fermenters