ELECTROMICROBIAL STRATEGIES FOR A SUSTAINABLE GROWTH OF PURPLE PHOTOTROPHIC BACTERIA

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TESIS DOCTORAL

Electromicrobial strategies for a sustainable growth of purple phototrophic bacteria

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“Estamos en este mundo para alumbrar no
para deslumbrar”

F.J. Suárez Torombo

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Summary

The impact of human activity on the environment has led the planet to a climate emergency situation. The concentration of greenhouse gases and the contamination of natural environments is having enormous consequences such as intense droughts, water scarcity, severe fires, rising sea levels, flooding, melting polar ice, catastrophic storms and declining biodiversity.

This situation requires rethinking the model of consumption and production. The food sector is responsible for ca. 25% of current greenhouse gas (GHG) emissions. Consumption habits are changing and, according to the Boston Consulting Group (BCG), that alternative sources of protein will represent 11%-22% of protein consumption in 2035. Therefore, it is crucial to find new sustainable sources of protein for food.

Purple phototrophic bacteria (PPB), the most versatile microorganisms on earth, have been proposed as a possible alternative source of protein. The main limitation is the feedstock used for cultivation, which represents a high percentage of the overall operational expenditure. Finding new affordable feedstocks will make the bioproduct economically viable.

In this thesis, two alternative feedstocks by means of electrodes as protagonists have been studied. The first was electrical current through the use of an electrode for the cultivation of a PPB-dominated microbial consortium (Chapter 2). The second feedstock explored in this thesis was wastewater in combination with electrodes for controlling the metabolism of purple phototrophic bacteria (Chapter 3 and 4).

This thesis report is organized into 5 chapters. In Chapter 1, we present an state of the art of electromicrobiology including the physiology of purple phototrophic bacteria and the interaction with electrodes. Then, the research results are contained in Chapters 2, 3 and 4. Finally, in Chapter 5, the experimental results are discussed and compared with the state of the art.

In the same way that purple phototrophic bacteria use ferrous iron as an electron donor, these microbes can use carbon-based electrodes (Chapter 2). Carbon fixation by extracellular electron uptake allows electricity and carbon dioxide to be used as feedstock to cultivate purple phototrophic bacteria. The electrode, acting as a cathode (-0.6 V *vs*. Ag/AgCl), served to grow a PPB-dominated consortium. Both the electrochemical and the microbial population analysis point to the *Rhodopseudomonas* genus (PPB) as the main actor in the electron uptake, acting as a link between the electrode and the rest of the microbial community.

Wastewater as feedstock is more than just a payable resource as many industries pay for its treatment. Indeed, wastewater treatment is eventually a service. In addition, using a residue such as wastewater to generate a bioproduct is environmentally positive. Although

the wastewater has already been used as feedstock to cultivate purple phototrophic bacteria, the composition of the wastewater determines the metabolic microbial behaviour. The electrode, both as electron acceptor and donor, can help control microbial metabolism, allowing a fine control of purple phototrophic bacteria.

Purple phototrophic bacteria cultured under anodic polarization showed a 2-fold enhancement in the brewery wastewater treatment (vs. non-polarized). On top of that, electrobioremediation of brewery wastewater using PPB showed ca. 3-fold higher yield than using non-photosynthetic culture (Chapter 3). Furthermore, polarization minimizes or completely prevented methanogenesis. The electroactive PPB genera *Rhodopseudomonas* and *Rhodobacter* outcompeted other genera during growth under polarization and illumination conditions.

The electrode acting as a cathode, served as an extra source of electrons in the cultivation of purple phototrophic bacteria (Chapter 4). By providing extra electrons the electron sinks pathways were activated, specifically carbon fixation and consequently biomass production was maximized. Indeed, PPB biomass production was enhanced 3-fold to 7-fold in presence of cathodic polarization during brewery wastewater treatment.

Finally, Chapter 5 discusses the experimental results of the thesis, putting their implications in context. In addition, some ideas about future work to bring this technology to the market are presented.

Resumen

El impacto de la actividad humana en el medio ambiente ha llevado al planeta a una situación de emergencia climática. La concentración de gases de efecto invernadero en la atmósfera junto con la contaminación de ambientes naturales tiene enormes consecuencias como las intensas sequías, escasez de agua, incendios, incremento del nivel del mar, tormentas catastróficas y reducción de la biodiversidad.

Esta situación requiere un cambio drástico en el modelo de consumo y producción. El sector alimentario es responsable de aproximadamente un 25% de las emisiones de gases de efecto invernadero. Los hábitos de consumo están cambiando, según Boston Consulting Group (BCG), los modelos de predicción señalan que las fuentes alternativas de proteínas representarán entre el 11% y el 22% del consumo de éstas en 2035. Por lo tanto, la búsqueda de nuevas fuentes de proteína es crucial.

Las bacterias fotótrofas rojas, los microorganismos más versátiles de la tierra, se han propuesto como posible fuente alternativa de proteína. Su principal limitación en el mercado es el precio de los compuestos químicos como sustratos utilizados para su cultivo, que representan un alto porcentaje del precio de venta del producto. Por lo tanto, el uso de sustratos alternativos permitiría su implantación en el mercado.

En esta tesis se han explorado dos sustratos alternativos en los que los electrodos son protagonistas: la corriente eléctrica y el agua residual. En el uso de corriente eléctrica como sustrato investigamos como un electrodo actúa como donador de electrones para el cultivo de un consorcio bacteriano dominado por bacterias fotótrofas rojas (Capítulo 2). El uso del segundo sustrato, agua residual de la industria cervecera, se hará en combinación con electrodos como herramienta de control metabólico de las bacterias fotótrofas rojas (Capítulo 3 y 4).

La memoria de esta tesis se organiza en cinco capítulos. En el Capítulo 1 se resume el estado del arte de la electromicrobiolgía, con especial atención a la biología de las bacterias fotótrofas rojas y su interacción con electrodos. Los capítulos del 2 al 4 recogen los resultados de investigación. Finalmente, en el capítulo 5 se discuten los resultados y se comparan con los estudios previos.

Estos microorganismos pueden utilizar electrodos como donador de electrones de la misma forma que utilizan minerales insolubles como el hierro (Capítulo 2). La fijación de carbono asociada a los electrones procedentes un electrodo permite utilizar la electricidad y el dióxido de carbono como sustrato para cultivar bacterias fotótrofas rojas. El electrodo, actuando como cátodo (-0.6 V *vs*. Ag/AgCl) sirve para cultivar un consorcio bacteriano dominado por estos microorganismos. Los análisis electroquímicos y el estudio de la comunidad microbiana apuntan a que el género *Rhodopseudomonas*, una bacteria fotótrofa roja, es el actor principal en la interacción con el electrodo, mediando entre éste y el resto de la comunidad de microorganismos.

Utilizar el agua residual como sustrato es más un servicio que un gasto, ya que muchas industrias necesitan tratar sus efluentes para ajustarse a la legislación. Además, utilizar un residuo como son las aguas contaminadas por la industria para generar un producto tiene un impacto ambiental muy positivo. Aunque el agua residual se ha utilizado como sustrato para cultivar bacterias fotótrofas rojas, la composición del agua determina el comportamiento metabólico de los microorganismos. El electrodo, tanto actuando como donador como aceptor de electrones, puede ayudar a controlar el metabolismo bacteriano, "domesticando" las bacterias fotótrofas rojas.

Las bacterias fotótrofas rojas cultivadas con polarización anódica duplican la eficacia del tratamiento de agua residual de la industria cervecera con respecto al tratamiento sin polarización. Además, la producción de biomasa se triplica con respecto al tratamiento con microorganismos no fotosintéticos (Capítulo 3). El electrodo minimiza o elimina completamente la producción de metano en el reactor. En cuanto a la ecología microbiana, los géneros de bacterias fotótrofas rojas electroactivas *Rhodopseudomonas* y *Rhodobacter* predominan cuando se utiliza de forma simultanea polarización e iluminación.

El electrodo actuando como cátodo, sirve como fuente adicional de electrones en el cultivo de bacterias fotótrofas rojas (Capítulo 4), lo cuál activa las rutas "sumideros de electrones", en concreto la fijación de carbono y, en consecuencia, se maximiza la producción de biomasa. De hecho el rendimiento celular de las PPB se vio incrementado entre tres y siete veces durante el tratamiento de agua residual de la industria cervecera en presencia de polarización catódica.

Finalmente, el Capítulo 5 recoge la discusión de los resultados experimentales de esta tesis, poniendo sus implicaciones en contexto. Además, se presentan algunas ideas para acercar la tecnología al mercado.

Chapter 1:

Introduction

[1] The illustration of the chapter cover has been generated with the wombo.art artificial intelligence exclusively for academic and educational purposes.

Chapter 1: Introduction

CHAPTER 1:

Introduction

1.- Socioeconomic framework

The planet earth and all living beings are facing a global crisis that puts the survival of most species at risk. Environmental data indicates that human activity, mainly through environmental pollution and the emission of greenhouse gases, have drastically changed the natural dynamics of the earth and have led us to a climatic emergency situation (IPCC). Scientists around the world have declared the need for joint action in order to mitigate the environmental damage. Some of these solutions involve a change in the current lifestyle and consumption habits. A new fundamental variable has appeared in the global market: sustainability.

Globally, 80% of wastewater is discharged into the ecosystem without being treated, which poses an environmental and public health risk (UN). Even in cities where wastewater is treated, wastewater treatment is inefficient and entails a high energy cost, thus hindering its possible reuse (UN). One out of three people does not have access to safe drinking water, two out of five people do not have a basic water facilities (UN). Therefore, the treatment and reuse of water is a global challenge that requires a radical change at the political, management and technological levels.

Figure 1. Ardern (first from the left in the back row) and Lockett (first from the right in the first row).

Engineers Edward Arden and William T. Locket, in 1914, were the inventors of the activated sludge system, the main technology for treating wastewater throughout the world (Fig. 1). This system is based on suspended microorganisms that oxidize wastewater pollutants in a process that requires the presence of oxygen (Adern & Lockett, 1914). This oxygen requirement is specifically one of the biggest drawbacks of the technology, since it accounts for more than 50% of the energy consumption of a wastewater treatment plant (Gandiglio et al., 2017). The high energy requirements entail an environmental impact due to the use of non-renewable energy sources, which are associated with greenhouse gas emissions. In addition, energy expenditure represents a high economic cost that must be assumed by the administration. The economic infeasibility to cover these expenses means that wastewater treatment is not carried out in most of the world.

Another of the main drawbacks of activated sludge technology is that it is focused on removing contaminants from wastewater. Wastewater is rich in organic and nitrogenous compounds that can be recovered, generating products that can be used in other industrial processes. Nutrient recovery goes beyond economic sustainability, as it promises to generate added value to pay for wastewater management, making the management model economically viable.

Since the discovery of activated sludge technology and its widespread use back in the 1930s, researchers have worked to reduce the energy consumption of treatment and to recover resources from wastewater. This doctoral thesis is framed within this vision of sustainability and circular economy. This work focuses on how electrodes can be used to domesticate one of the most versatile groups of microorganisms on earth, the purple phototrophic bacteria (PPB), in order to achieve sustainable wastewater treatment and maximize nutrient recovery. In order to enlighten the reader, the state of the art on the interaction between microorganisms and electrodes, called microbial electrochemistry or electromicrobiology, and the main findings on purple phototrophic bacteria are detailed below.

2.- Electroactive bacteria

Electroactive bacteria are able to interact with electrodes. The ability to accept and donate electrons with insoluble species has meant a paradigm shift in the world of microbiology and has led to the emergence of a novel scientific field: microbial electrochemistry.

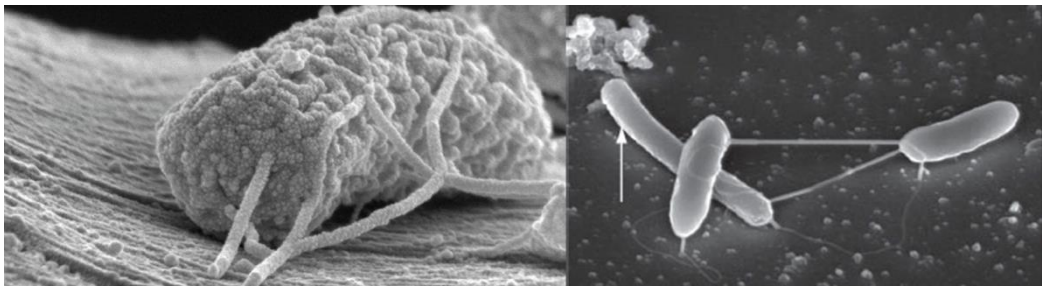

Figure 2. Electroactive bacteria micrographs. Left: Electroactive microorganism attached to a carbon filament (Source: Xing Xie, Stanford Engineering). Right: *P. thermopropionicum* and *M. thermoautotrophicus* (arrow) in methanogenic cocultures showing nanowires connecting the two genera, as reported by Ishii *et al*.

2.1.- History of microbial electrochemistry

The first observations of the production of electric current by microorganisms date back to the end of 1911. Michael Cressé Potter, professor of botany at Cambridge University, first suggested the production of microbial electric current from the degradation of organic compounds (Potter, 1911). For some decades, some other researchers worked exploring this topic (Berk and Canfield, 1964; Hees, 1965), but it was not until the late 20th century that the scientific community understood the importance and implications of this finding.

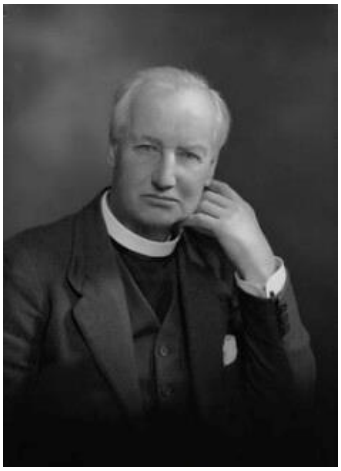

Figure 3. A. Michael Cressé Potter (from National Portrait Gallery).

In the late 1980s, two new discoveries emerged that laid the foundations for the beginnings of microbial electrochemistry. In 1988, two US research groups published the discovery of two species of bacteria capable of using solid metal oxides as final electron acceptors (Myers and Nealson, 1988 , Lovley and Philips, 1988). C. R. Myers and K. H. Nealson, from University of Wisconsin-Milwaukee, isolated the species finally named *Shewanella putrefaciens* MR-1, a facultative aerobe able to reduce manganese oxides present in Oneida Lake, N.Y. From the deep sediments of the Potomac River, Lovley and Phillips isolated a bacterium of the genus *Geobacter*, capable of using iron oxides as the sole electron acceptor. Although these authors did not report the generation of electric current, the reduction of solid materials, understood as a transfer of electrons from the bacteria to the material, is crucial to understand the basis of microbial electrochemistry.

In 2001, Reimers *et al.* demonstrated the generation of electric current by connecting an embedded electrode in the anoxic zone of marine sediment and the surface oxygenic zone of seawater (Reimers et al., 2001). These authors then proposed that the harvested energy came from the microbial oxidation of organic compounds in marine sediment. This finding was the starting point for a frenzied decade of exploration of microorganisms, molecular mechanisms of extracellular electron transfer, and biotechnological applications that made it possible to understand and domesticate electroactive bacteria. Since then, researchers have discovered new species of electroactive microorganisms, such as *Geobacter* spp. and *Shewanella* spp.,with the ability to reduce metal oxides (Koch and Harnisch, 2016). These advances provided the tools for the scientific community to continue deepening this research field, and to work on its application.

Between 2001 and 2010, researchers in the field of microbial electrochemistry were mainly focused on the catalysed production of energy by electroactive microorganisms (Logan and Regan, 2006). The objective was to achieve a clean technology capable of transforming organic waste (such as wastewater or soil pollutants) into electrical energy. Under this premise, the concept of Microbial Fuel Cell (MFC)[1] was developed (Logan, 2008). In this device, the oxidation of organic compounds (at the anode) was spatially separated from the reduction of oxygen (at the cathode) through an external circuit. The circulation of electric current through the external circuit was considered a promising source of clean energy from waste. Despite being a promising technology, the technical limitations that MFC presents at

[1] A detailed description of the differences between Microbial Fuell Cells (MFC), Microbial Electrolysis Cells (MEC) and Microbial Electrochemical Snorkel (MES) can be found in Section 2.5 (Chapter 1).

full scale (such as ohmic losses) and the low energy production compared to other renewable energies, have led researchers to explore other applications in which the production energy is not the main objective (Erable et al., 2011).

Some research groups understood that the MFC concept could be applied in environments in which the electron acceptor is a limiting factor for bacterial metabolism (Zhang et al., 2010). In other words, this technology allows spatially separated environments to be connected through an external circuit. In this way, some scientists used the electrodes to favour the oxidation of organic pollutants, both in water (Angosto et al., 2015; Kelly and He, 2014) and in soils (Zhang et al., 2010), instead of trying to produce electrical energy.

In 2011, during this new trend, in which degradation prevailed over energy production, Erable *et al.* propose a new concept "Microbial electrochemical Snorkel" (Erable et al. 2011). Based on the idea of connecting two spatially separated environments, as was being done with MFC, these authors proposed that a single electrode could connect an anoxic zone, in which organic matter could be oxidized, with an oxygenated zone, in which oxygen would act as the final electron acceptor. In other words, the electrons generated in the TEA-limited environment could reach the electron acceptor without an external circuit. This new concept has received great interest since it has allowed the application of full-scale microbial electrochemistry for soil bioremediation (Viggi et al., 2015) and for wastewater treatment (Aguirre-Sierra at al., 2016). Specifically, in the topic of wastewater treatment, the concept of snorkel has been used to combine microbial electrochemistry with constructed wetlands technology, giving rise to METland® (Aguirre-Sierra et al., 2016). This technology was the demonstration that the microbial electrochemistry allow the wastewater treatment at zero energy cost, putting the microorganisms in contact with the electron acceptor without external energy input.

At some point, some scientists specialized in electrochemistry became part of the microbial electrochemistry field, bringing with them some of the techniques classically used to study oxidation and reduction processes in electrodes (Hamelers et al., 2011). These techniques allowed a much deeper understanding of extracellular electron transfer and the optimization of microbial electrochemical systems. Furthermore, the scientists observed that when thermodynamics and/or kinetics limit the process of interest, control of the electrode potential could be crucial. And this is how microbial electrolysis cells were conceived, in which an external energy source is applied, thus favouring the processes of interest (Liu et al., 2005).

In the beginning, the role of the electrode was only conceived as an electron acceptor or donor. In recent years, researchers have observed important effects of the polarization of the electrode that are not reflected in the current intensity produced. In other words, the scientists observed that the electrode could be conceived as a metabolic regulatory element. This process was called electro-fermentation (Moscoviz et al., 2016, Gong et al., 2020) and it is attracting increasing interest.

2.2.- Extracellular electron transfer

Extracellular electron transfer (EET) is the process by which cellular metabolism is connected with the oxidation/reduction of a species outside the cell. Insoluble species, such as minerals or electrodes, cannot be incorporated into the cell, so bacteria need molecular mechanisms that allow the transfer of electrons to the outside of the cell (Hernandez and Newman, 2001).

The EET mechanisms are classified into Direct Extracellular Electron Transfer (DEET) and Mediated Extracellular Electron Transfer (MEET). DEET refers to the electronic transfer between bacteria and electrode by direct contact with the surface, normally the bacteria are arranged on the electrode forming a biofilm (Bond and Lovely, 2003), although the interaction of planktonic cells with the electrode has been demonstrated (Tejedor-Sanz et al., 2017). In MEET, on the other hand, electron transfer is carried out by redox mediators, which are low-molecular weight compounds that can be oxidized and reduced, carrying electrons between the bacteria and the electrode, the most commonly used mediated transfer is by hydrogen (Schröder, 2007), but other redox mediator such us quinones has been widely used (Kotloski and Gralnick, 2013; Voordeckers et al., 2010). Bacteria can use both mediators self-produced, and mediators present in the environment.

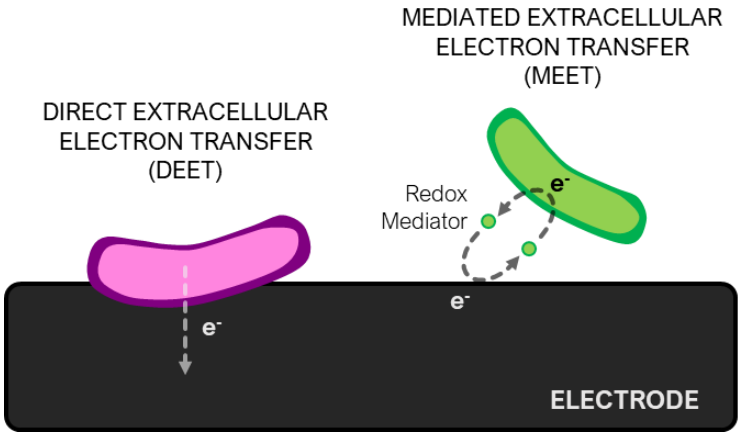

Figure 4. Extracellular electron transfer mechanisms. Direct Extracellular Electron Transfer (DEET) and Mediated Extracellular Electron Transfer (MEET). Red particles represents redox mediators.

In 2010, Summers et al. demonstrated that these EET mechanisms could take place, not only between bacteria and electrodes, but also between different species of bacteria (Summers et al., 2010). This exchange of electrons allows microorganisms to establish symbiotic relationships with each other (Rotaru et al., 2021). This "electric" syntrophy can take place through cell-to-cell contact (DIET, Direct Interspecies Electron Transfer) or

through electrically conductive materials (CIET, Conductive-particle-mediated Interspecies Electron Transfer)[2].

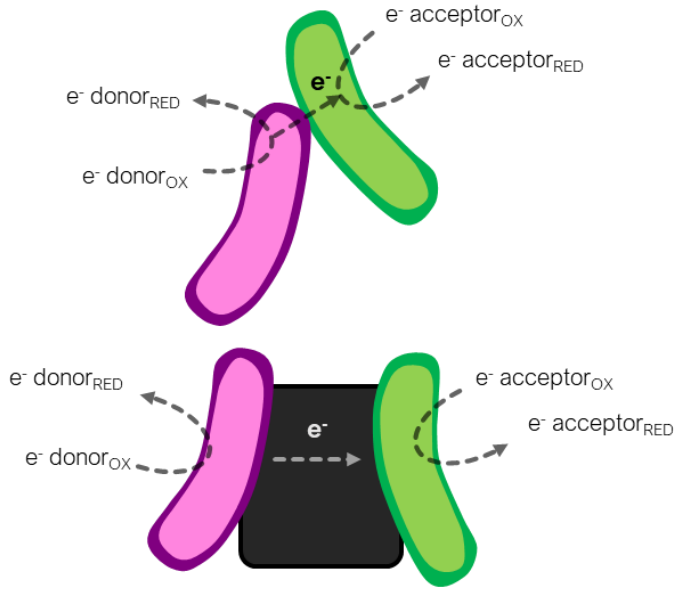

Figure 5. Interspecies electron transfer mechanisms. Up: direct interspecies electron transfer (DIET). Down: Conductive-particle-mediated Interspecies Electron Transfer(CIET).

Specifically, CIET has a proven importance in methanogenesis (Holmes et al., 2021). So much so, that electrically-conductive materials in the absence of electron acceptor, can serve to select CIET-partners (Xu et al., 2019).

2.3.- Evolution and diversity

The surprising ubiquity of electroactive bacteria and their role in biogeochemical processes and their importance in syntrophic metabolism in the environment is increasingly evident (Koch and Harnisch, 2016). Every discovery about molecular mechanisms, their impact on the environment, from the co-production of methane via DIET to the long-distance connection of cable bacteria, sheds light on the evolution of electroactive microorganisms (Lovley and Holmes, 2022). How can a microorganism that has evolved in a world without electrodes produce electrical energy? Random mutations and natural selection are the forces that drive the appearance of new characters (Darwin, 1859). Several natural processes in which EET is fundamental help to understand the scenario in which electroactive bacteria evolved.

[2] The DIET / CIET processes have been extensively reviewed by Rotaru et al. 2021

Most electroactive bacteria are capable of reducing metal oxides, which suggests that the mechanisms of EET are metal-reducing evolutionary mechanisms. This hypothesis would mean that the evolutionary process of bacteria in TEA-limited anoxic environments would have given rise to adaptations that would serve, not only to reduce metals, but also to interact with electrodes (Lovley, 2012). On the other hand, the behaviour of microorganisms in the reduction of metals differs from the interaction with electrodes. In metal reduction, the bacteria are in a planktonic state (Childers et al., 2002, Lovley, 2011), so that they can search for new sources of metal once they are depleted. An electrode, on the other hand, consists of an inexhaustible electron acceptor, and it dominates the formation of biofilms (Franks et al., 2008, Nevin et al., 2009). In addition, the kinetics of electron transfer with metals is considerably slower compared to bacteria-electrode EET kinetics (Bond, 2010). These physiological differences between the two processes do not indicate that this hypothesis is wrong, since the physiological differences do not necessarily indicate a different evolutionary origin. Even so, other authors have proposed other processes that may have led the evolutionary process.

One of the processes that attracts the most attention is the idea of geobatteries. Geobatteries are huge natural electrochemical cells (Bigalke and Grabner, 1997, Bigalske et al., 2003). These formations are composed of subsurface graphite deposits that electrically connect different redox environments. Some authors hypothesized that the geobattery could function as an inexhaustible electron acceptor or donor (Leung and Xuan, 2015). This characteristic (inexhaustibility) is shared with the electrodes. In contrast, geobatteries are not ubiquitous and hardly explain the large number and diversity of electroactive bacteria discovered so far.

More recently, interspecies electron transfer has been proposed as a driver of the evolutionary process of electroactive bacteria (Shrestha and Rotaru, 2014). This hypothesis would explain the ubiquity of electroactive bacteria, since the interaction with the materials would be nothing more than a very interesting coincidence, being the syntrophy between different microorganisms the true driver of the evolution of these incredible microbes.

It is difficult to assess whether metal respiration, interspecies electron transfer, or geobatteries have driven the evolutionary process. Most likely, all of them have played a fundamental role.

2.4.- Microbial electrochemistry from a theoretical point of view

Since the beginning of microbial electrochemistry, scientists have discovered methods and techniques that allowed us to understand and domesticate electroactive bacteria. Some authors, such as Derek Lovley (University of Massachusetts, USA) or Falk Harnicsh (Helmholtz Centre for Environmental Research, Germany) have focused on the knowledge of microorganisms. Other authors, such as Bruce Logan (Penn State, Pennsylvania) with engineering contributions have provided tools to the scientific community to work on the microbial electrochemistry field. This section aims to make a compendium of the main techniques and systems of microbial electrochemistry.

2.4.1.- Classification based on modus operandi: electrode arrangement

As the scientific discipline evolved, experts have classified systems according to different criteria. In this thesis, systems were classified according to whether or not they are externally polarized. In an non-externally polarized system two redox environments are electrically connected. On the contrary, in a externally polarized system, an energy source is used that allows (1) non-spontaneous reactions to occur or (2) enhances the kinetics of spontaneous reactions.

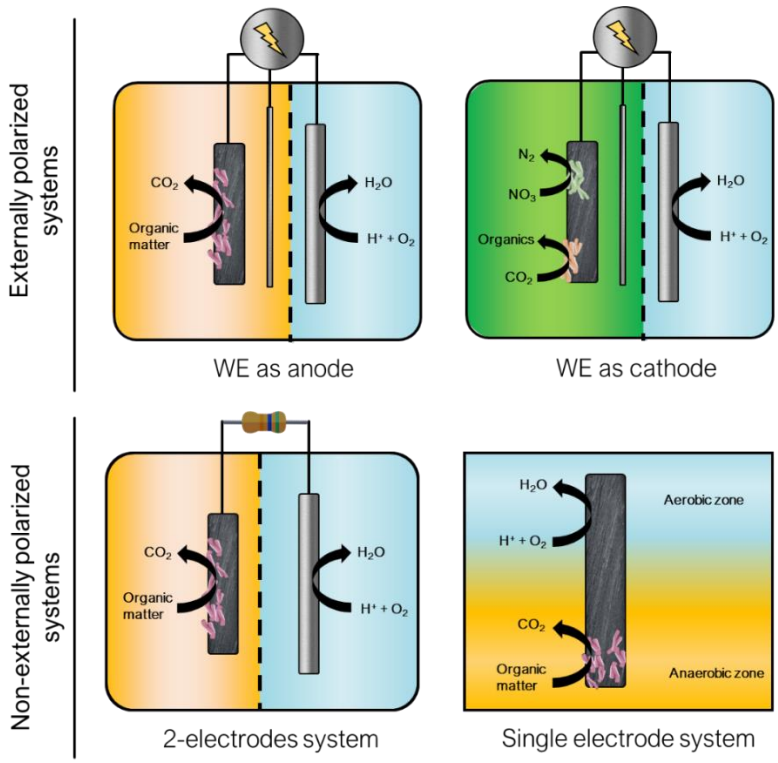

Figure 6. Scheme of the main microbial electrochemical system configurations.

Externally polarized systems

The most simplified version of an externally polarized system consists of an anode and a cathode between which a potential difference is fixed with a power supply. At scientific level, this approach is obsolete, since it is difficult to control the potential of the electrodes (since only the potential of the cell is controlled). This option is only suitable for applications where precise control of electrical potential is not required.

In most scientific works and also in this thesis, externally polarized systems are formed by three electrodes (Working Electrode (WE), Counter Electrode (CE) and reference electrode (RE)). The working electrode is where the microorganisms are located and where the process of interest takes place. Its electric potential is controlled by an electronic device called a potentiostat. The potentiostat allows the electric potential of the working electrode to be controlled based on a fixed and stable potential of another electrode, the reference electrode. The counter electrode is where the counter reaction takes place.

Depending on the electric potential that is fixed in the working electrode, it can act as an anode or as a cathode. Anodic electric potentials are classically used to promote the oxidation of organic compounds (VFAs (Yang et al., 2015), xenobiotics (Yang et al., 2022), antibiotics (Rodrigo Quejigo et al., 2019) and recently, anode-dependent anaerobic ammonium oxidation (Shaw et al., 2020) has also been described. The cathodic electrical potentials have been used to promote the reduction of compounds such as nitrate (Tejedor et al., 2020), and even to favour the production of biohydrogen and methane (Perona-Vico et al., 2020; Ning et al., 2021). One of the newest applications of cathodic polarization is bioelectrosynthesis (Finkelstein et al., 2022), in which electrons are provided for the synthesis of organic compounds through carbon dioxide fixation. Electro-fermentation, the process in which polarization affects bacterial metabolism, but low current densities occur, would also be classified as MET (Gong et al., 2020).

Non-externally polarized systems

These systems are made up of an anode and a cathode connected by an external circuit with an electrical resistance. Spontaneous redox reactions occur at both electrodes, oxidation at the anode and reduction at the cathode.

Classically, both redox environments are separated by an ion exchange membrane. In the anodic chamber, electroactive bacteria catalyse the oxidation of organic compounds and transfer the electrons produced to the anode. The electrons produced circulate through the external circuit to the cathode (in the cathodic chamber), where the reduction (generally) of oxygen takes place (**Fig. 6**). Electricity circulating through the external circuit, understood as renewable energy, has been the focus of microbial electrochemistry during the early years.

A non-externally polarized system that is attracting more and more attention is the snorkel. In this approach, a single electrode is used that connects two different redox environments.

In this way, catalysis is prioritized over energy generation, since in the absence of an external circuit, the electric current cannot be used (Fig 6).

2.4.2.- Chronoamperometry and Coulombic Efficiency

In electric potential controlled systems (3-electrodes), a potential is set in the working electrode and the intensity of electric current (I) is measured over time, this is called chronoamperometry (Bard and Faulkner, 2001). Any potential other than the open circuit potential (OCP) can potentially promote redox reactions. Current intensity is related to oxidation (I> 0) or reduction (I <0) at the electrode surface.

Electroactive bacteria on a polarized electrode would catalyse the redox reactions that would translate into electrical current (I). Therefore, at an electrode under abiotic conditions, zero electrical current would be measured. In summary, the intensity of current produced, and therefore chronoamperometry is the first step to study the electroactivity[3] of microorganisms. (Hamlers et al., 2011).

To evaluate whether the measured current intensity is related to the redox reaction studied, the transferred charge (Q) must be correlated with the reduction or oxidation of the species in solution. In other words, in an anode in which organic compounds are biologically oxidized, the electrons coming from the oxidation should be measured in chronoamperometry. The ratio between the theoretical electrons (calculated by the disappearance of the organic compound) and those measured by the potentiostat is the CE (Eq. 1).

$$CE = \frac{\int I \, dt}{\Delta n_s z F} \quad (1)$$

Where I is current is time, Δn_S is amount of consumed substrate, z is number of electrons in the substrate, F is the Faraday constant (96485.3 C mol^{-1}).

2.4.3.- Cyclic Voltammetry

Cyclic voltammetry (CV) is an electrochemical technique in which a cyclical sweep of potentials is performed and the intensity of the current produced is recorded. CV allows to study in detail the redox reactions that occur on the surface of the electrode (Marsili et al., 2010). The identification of redox pairs can allow to know the potential window in which the EET occurs and therefore to identify the optimal working potential for each specific case. Furthermore, the midpoint potential of the redox couple can be informative of the bacterial species that interacts with the electrode (Fricke et al., 2008). The midpoint potential can be calculated as the average potential between the oxidation peak and the reduction peak, it can be calculated mathematically or graphically as shown in figure 7.

[3] During this doctoral thesis, Antonio Berná, PhD laid the foundations for the study of the electroactive microorganisms. As a source and reference book for electrochemical techniques we used Electrochemical Methods (Allen J. Bard)

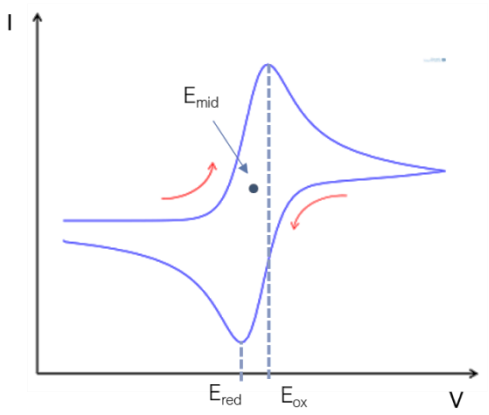

Figure 7. Cyclic voltammetry. E_{ox}: oxidation peak potential. E_{red}: reduction peak potential. E_{mid}: midpoint potential.

Although classically it has been possible to study the electrode by means of CV, in some cases in which the electrode is formed by a bed of conductive material, it is necessary to use other approaches i.e. connect a single granule of the bed using titanium or gold and carry out the CVs in a separate cell (Rodrigo Quejigo et al., 2018).

2.5.- Microbial electrochemical technologies: from science to reality

The interaction between bacteria and electrodes has led to the development of what has been called microbial electrochemical technologies (MET). MET applications have been extensively reviewed in recent years (Wang and Ren, 2013). The biotechnological exploitation of electroactive bacteria began with the study of microbial fuel cells (2.5.1) and the idea of obtaining electrical energy from the chemical energy contained in organic compounds. In recent decades, a bottleneck has been identified in the scaling of technologies aimed at obtaining electrical energy. During the development of this field of research, new applications have emerged that have proved more promising and more easily scalable, such as, monitoring microbial activity (2.5.2), stimulating the degradation of organic compounds in water and soils by providing an inexhaustible electron acceptor and allowing exchanging of electrons between bacteria (2.5.3) and feeding electrons to microorganisms for obtaining new products or directing fermentation pathways for getting valuable products (2.5.4).

2.5.1.- Microbial fuel cell: first but not the best

MFC are non-externally polarized systems (2.4.1) whose function is the production of energy by uncoupling oxidation and reduction reactions (Allen and Benetto, 1993; Park and Zeikus, 2000). This technology is mainly based on the biological oxidation of organic compounds, so it has been applied to wastewater (Torres et al., 2008), even to contaminated soils (through the so-called sedimentary MFC or sMFC) (Reimers et al, 2001; Domínguez-Garay et al., 2018; Domínguez-Garay et al., 2013).

2.5.2.- Microbial electrochemical sensors

Another use of electroactive bacteria is their application to monitor natural environments through microbial electrochemical sensors. These devices base their operation on the fact that the electrical current produced depends directly on microbial activity and therefore allows the measurement or detection of biodegradable compounds (Estevez-Canales et al., 2015, 2017). These devices detect and quantify common urban wastewater organic compounds (Kumlanghan et al., 2008; Di Lorenzo et al., 2009; Corbella et al., 2019) and even toxic compounds (Dávila et al., 2011).

2.5.3.- Bioelectroremediation: enhancing decontamination with electrodes

After the development of MFC and after focusing on organic pollutants, some researchers developed systems whose main objective was decontamination (and not energy generation). For this purpose, the common feature of all systems is that the microorganisms are provided with an electron acceptor (or donor in some cases) that allows decontamination.

The systems applied to decontamination cover the entire plethora of systems explained in 2.4.1: non-externally polarized systems, snorkels and externally polarized systems. Due to its importance in the world and in this thesis, only snorkel systems and externally polarized systems (3-electrodes) for wastewater treatment will be deepened[4].

Many types of wastewater are produced every day in the world, each with a different composition and different physical characteristics (Sikosana et al., 2019). The general components of wastewater are organic matter and nitrogen.

Organic matter

Organic matter refers to dissolved organic compounds in wastewater such as fatty acids, saccharides, amino acids and aromatic compounds. Organic matter in wastewater is typically quantified by Chemical Oxygen Demand (COD)(Sikosana et al., 2019) In microbial electrochemical systems, the removal of organic matter occurs by oxidation (anode) of the compounds catalysed by electroactive bacteria. Some of these compounds are directly degradable by electroactive bacteria such as volatile fatty acids (Coppi, 2005) or some saccharides (Borole et al., 2011) and aromatic compounds (Zhang et al., 2013). To degrade more complex substances, electroactive bacteria often need to establish syntrophies with microbial partners. These syntrophies have been identified by studying the microbial communities of the electrodes (Dubé and Guiot, 2015). One of the most illustrative examples of this process was described by our laboratory, in which we studied the distribution of species in a biofilm (Tejedor-Sanz et al., 2018). In the external part of the biofilm we found the heterotrophic species whose function was to metabolize the more complex polymers into more easily metabolizable substances (such as acetate) (**Fig. 8**). In the inner-layer, we

[4] There is a huge bibliography that deals with bioelectroremediation of soils and sediments but it is out of the scope of this thesis.

found a predominance of *Geobacter* species, capable of oxidizing these simple substances and transferring electrons to the electrode.

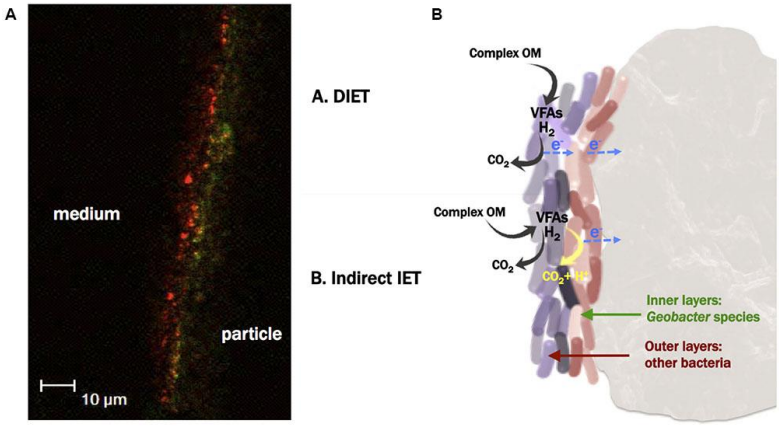

Figure 8. A: FISH image from the section of the biofilm developed on an activated carbon particle. B: Proposed microbial electron transfer mechanisms among the different microbial communities colonizing the particles and toward the fluidized anode. (From Tejedor et al. 2018).

Microbial electrochemistry has been successfully applied to wastewater with very different kinds of organic matter: urban wastewater (Ren et al., 2014; Rozendal et al., 2008), brewery wastewater (Wang et al., 2008; Dong et al., 2015, Yu et al., 2015), wastewater from the cheese industry (Kelly and He, 2014) and wastewater from the wine industry. (Cercado-Quezada et al., 2010).

Nitrogen

Wastewater generally contains nitrogen-rich compounds, especially organic compounds, ammonium (NH_4^+) and nitrite (NO_3^-). (Sikosana et al., 2019). Wastewater treatments use biological processes of nitrification and denitrification to achieve nitrogen removal. The nitrification process consists of transforming ammonium into nitrate and usually occurs in the presence of oxygen. Denitrification is the reduction of nitrate to nitrogen gas and this reaction requires an electron donor. The biological reactions of the nitrogen cycle have been extensively studied and reviewed (Kuypers et al., 2018).

The first report of nitrate reduction with electroactive bacteria was in 2004. In this study, *Geobacter metallireducens* catalysed nitrate to nitrite reaction using the electrode (cathode) as the sole electron donor (Gregory et al., 2004). After this first publication, the complete denitrification process has been demonstrated with both pure and mixed cultures (Tejedor et al.; Rodríguez-Arredondo et al., 2015; Puig et al., 2011).

For several years, the oxidation of ammonia with electroactive bacteria had not been demonstrated. In 2018, the laboratory of Sebastiá Puig (LEITAT, Girona, Spain) obtained a first approximation (Vilajeliu-Pons et al., 2018). Finally, in 2020 the anode-dependent

anaerobic oxidation of ammonium, also called "electroannamox", was published for the first time (Shaw et al., 2020).

Fluid-like electrodes and METland® as promises for the wastewater treatment of the future

Finally, two ingenious examples are detailed in which electroactive bacteria efficiently remove contaminants from wastewater: Microbial electrochemical fluid-like bed reactors (ME-FBR) and METland®.

Microbial electrochemical fluid-like bed reactor: wiring in motion

Fluidized beds are used in electrochemical catalysis processes such as cathodic reductions, electrowinning of precious metals cathodic reductions and energy storage systems (Huh, 1985). This type of electrode architecture consists of a fluid-like bed of continuously moving electrically conductive material. The electrode volume (or its expansion) is controlled by the recirculating flow of the electrolyte. The main advantage of these electrodes compared to conventional electrodes is the large electrode surface.

This technology was successfully used to perform electrode-driven bacterial reactions. Our research group developed and patented the microbial electrochemical fluidized bed reactor (ME-FBR). In these reactors, electroactive microorganisms catalyse redox reactions in which the fluid-like electrode acts as an electron acceptor or donor (**Fig. 9**) (Tejedor-Sanz et al., 2016, 2017; Borsje et al., 2021).

The performance of ME-FBR was first evaluated using the model electroactive microorganism, *Geobacter sulfurreducens* (Tejedor-Sanz et al., 2017). In this microorganism, the network of c-type cytochromes that connects the metabolism with the electrode acts as a capacitor, storing the electrons until they come into contact with an acceptor (Liu et al., 2011; Malvankar et al., 2012). This mechanism allows planktonic cells to discharge at point contacts with the fluid-like electrode. This bacteria-electrode interaction mechanism allows harvest 5-fold charge collection than with a static bed (Tejedor-Sanz et al., 2017).

Despite the potential of the planktonic interaction with the electrode, it is also possible to promote biofilm formation on the bed particles. The fundamental parameter that determines the attachment of bacteria is the electrically conductive material used. Non-porous and smooth materials, such as glassy carbon, favour planktonic growth (Tejedor-Sanz et al., 2017). Other materials, more porous and hydrophilic, such as activated carbon, allow bacteria to attach and form a biofilm on its surface (Tejedor-Sanz et al., 2018).

Fluidized electrodes can be operated as anodes to promote organic material and nutrient removal. In this case, the fluidized electrode acts as an electron acceptor reaching coulombic efficiencies of 91% (using *G. sulfurreducens*). This approach has been applied to brewery wastewater treatment (Tejedor-Sanz et al., 2018; Asensio et al., 2021) and pharmaceutical industry wastewater treatment (Asensio et al., 2022).

Fluid-like polarized electrodes as cathodes have been less deeply studied. In 2020, we demonstrate the use of a fluid-like cathode as an electron donor for the biological reduction of nitrate (Tejedor et al., 2020). In addition, fluid-like cathodes can serve as an electron donor in autotrophic carbon fixation into bacterial biomass (Llorente et al., 2023, unpublished).

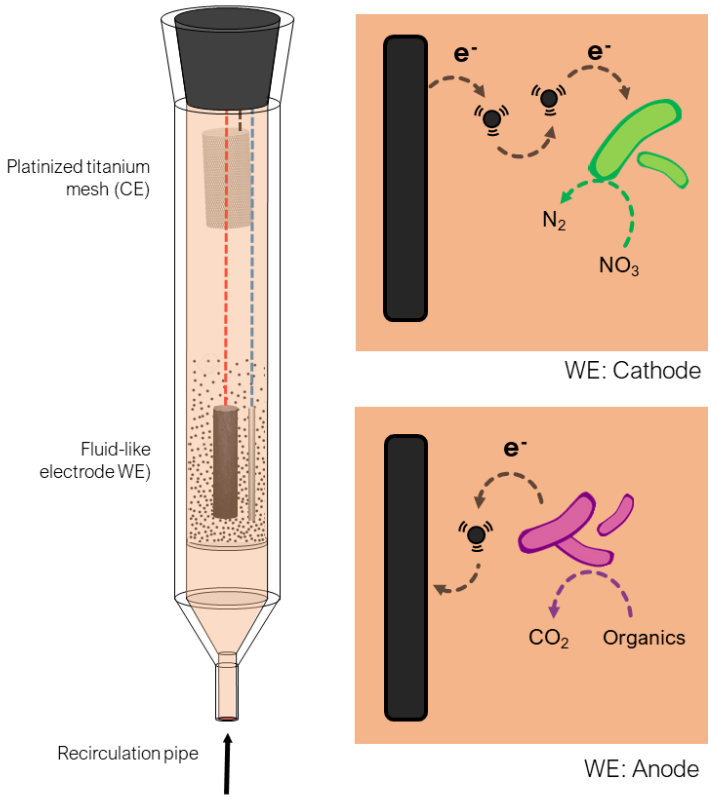

Figure 9. Scheme of a microbial electrochemical fluid-like bed reactor following a three electrodes configurations. Working electrode can be operated as anode or cathode.

METland®: a successful case of snorkel application

METland® are systems that combine wetlands treatment with microbial electrochemistry (Fig. 10). In these devices, the snorkel concept has been ingeniously adapted, substituting the inert material of the bed for electrically conductive material (Aguirre-Sierra et al., 2016).

This nature-based technology has been widely explored for wastewater treatment (Aguirre-Sierra et al., 2016; Prado et al., 2020, 2022), removal of emerging pollutants (Pun et al.,

2019) and sustainability (Peñacoba-Antona, Gómez-Delgado, et al., 2021; Peñacoba-Antona, Senán-Salinas, et al., 2021). In addition, the internal functioning of the system has been deepened at the level of bed material (Prado et al., 2019), increase in the electron sink (Prado et al., 2020) and the effect of vegetation (Prado et al., 2022).

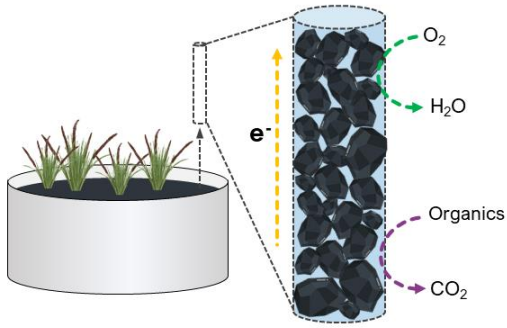

Figure 10. Scheme of METland.

2.5.4.- Microbial electrosynthesis

Microbial electrosynthesis seeks to use the electrons provided by a biocathode to synthesize value-added compounds, generally volatile fatty acids and alcohol like acetate, propanol or butyrate (Nevin et al., 2010; Ganigué et al., 2015; Izadi et al., 2021), but also other compounds such as bioplastics (Nishio et al., 2013; Chen et al., 2018) or microbial biomass as protein source (Xu et al., 2021) .

After a decade of effort and scientific advances (Nevin et al., 2010), microbial electrosynthesis is still far from the technical and economic competitiveness of current industrial processes (Prévoteau et al., 2020). Microbial electrosynthesis requires engineering improvements such as electrode material (Nie et al., 2013; Jourdin et al., 2014) and reactor design (Kantzow et al., 2015; Jourdin et al., 2018) but also in the selection of the microbial community (Prévoteau et al., 2020). The resilience and versatility of the microbial community will determine the range and rate of products obtained as well as the robustness of the synthesis process.

Recently, we have described the production of acetate and microbial biomass with a fluid-like cathode as the sole electron donor (Llorente et al. 2022, unpublished). This approach facilitates nutrient recovery through nutrient-partitioning, unlike a biofilm-based system in which physical methods would be required to recover biomass.

3.- Purple Phototrophic Bacteria

Purple phototrophic bacteria (PPB) is considered one of the most versatile group of microorganisms. The PPB has been historically classified in two big groups: Purple sulphur bacteria (PSB) and Purple non-sulphur bacteria (PNSB) attending to the capability to use sulphur compounds in their metabolism. PSB are able to habit in high concentration of sulphide, and use this sulphide as electron donor. Once the sulphide is oxidized to elemental sulphur, it forms granules in the cytoplasm of the cell. PNSB, despite not being able to tolerate such high amounts of sulphur compounds, they tolerate and metabolize low concentrations. Currently, it is considered that the physiological difference between PSB and PNSB is the accumulation of elemental sulphur granules inside the cell (Madigan, Michael. Bender, Kelly. Buckley, Daniel. Sattley, W. Stahl, 2018).

3.1.- History of purple phototrophic bacteria research

In the investigation about PPB, several stages are distinguished according to the focus of the research. Colored sulfur bacteria were discovered and described by Ehrenberg in 1835 (Gorlenko, 2004) and the understanding of their metabolism and ecology has allowed the development of numerous biotechnological applications. During the first stage (1835-1932) researchers were focus on the observation of PPB ecology and morphology. In 1932, Van Niel *et al*. described for first time that this group of bacterium present a specific type of photosynthesis so-called anoxigenic photosynthesis (Van Niel, 1932). This discovery marked the trend of the new stage (1932-1957) that was focused on the investigation of the photosynthetic apparatus of the bacteria, the functioning of the electron transport chain, the metabolism of nitrogen, in summary, the metabolism and biochemistry of PPB. N. Pfennig began the third stage, the domestication of PPB, with the use of enrichment methods that allowed the isolation of PPB species and began to use them and study them in more depth (Pfenning, 1967).

The last stage in the PPB research, which could be included in domestication, is electrification, in which the electrodes are combined with the metabolism of the PPB (see section 4).

3.2.- Metabolic versatility

The most potentially exploited characteristic of PPB, especially PNSB, is the different modes of metabolism: phototrophy or lithotrophy. In the presence of light, these microorganisms are capable of generating energy through the cyclic flow of electrons, in a process known as phototrophy (Madigan, Michael. Bender, Kelly. Buckley, Daniel. Sattley, W. Stahl, 2018). Under phototrophy, PPB can use organic compounds (photoheterotrophy) or inorganic carbon as a source of carbon (photolitotrophy), fixing the inorganic carbon by means of a Calvin cycle. The growth of PPB in the dark has been demonstrated, but it has been considered of little importance in biotechnology (Hülsen et al., 2016; Capson-Tojo et al., 2020).

3.3.- Anoxygenic photosynthesis

PPB are phototrophic organisms, they carry out the conversion of light energy into chemical energy, which is stored as ATP. This process takes place in the bacterial internal membrane and pigment-protein complexes are involved. The process begins in the light harvesting systems: LHI (P875) and LHII (P800-850), which collect and transfer the excitation energy to the Reaction Centre (RC), where photochemistry and electronic transport occur. The energy of light gives rise to a cyclical flow of electrons, in which the RC and the cytochrome bc1 complex are involved, which generates a proton gradient. Finally, this proton gradient is used to catalyse the synthesis of ATP, through ATP synthase (Fig. 11)(Boyle, 2005; Madigan, Michael. Bender, Kelly. Buckley, Daniel. Sattley, W. Stahl, 2018).

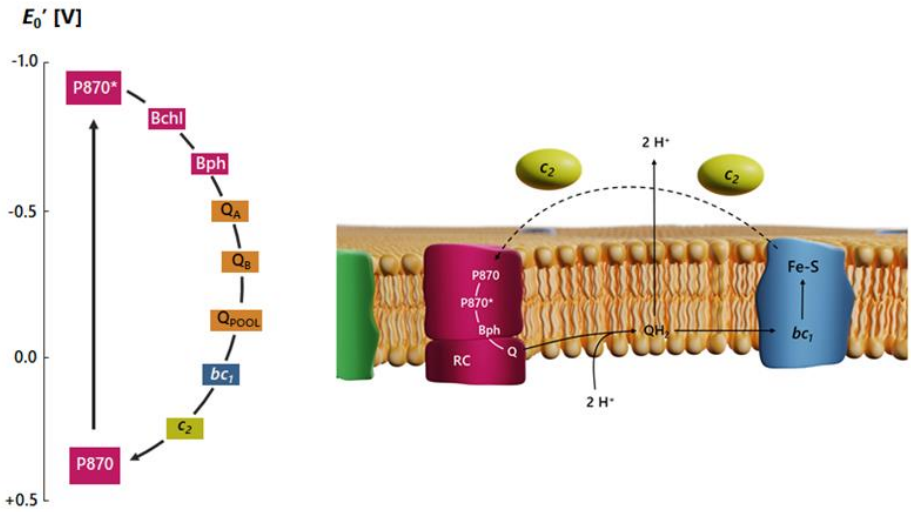

Figure 11. Cyclic electron flow system and redox potential of the components (3D by Cristina Villar).

3.4.- Redox balance mechanisms

The bacterial cell performance is based on electron flow from electron donor to electron acceptor to generate ATP. Microbes must maintain cellular homeostasis by balancing redox power-consuming and -producing reactions. In PPB, maintaining homeostasis is especially challenging under photoheterotrophic growth with highly reduced carbon sources. As a simplification, highly reduced carbon sources are considered those with a degree of reduction higher than biomass (4 mol e^- · mol^{-1} C).

PPB use metabolic mechanisms to face redox unbalanced situations, so-called “electron sinks”. These mechanisms consist of metabolic pathways in which excess reducing power is consumed. In 2010, McKinlay et al. described the main "electron sinks" in PPB: CO_2 fixation (Calvin cycle), nitrogen fixation, and hydrogen production (McKinlay and Harwood, 2010). More recently, alternative metabolic pathways such as production of PHA or

isoleucine have been described (Bayon-Vicente et al., 2021, 2020; Montiel-Corona and Buitrón, 2021).

Alloul *et al.* thoroughly reviewed the stoichiometry and thermodynamics of PPB metabolism in order to domesticate them. It has not been extensively studied, but the scientific understanding so far indicates that the degree of carbon source reduction primarily affects carbon fixation, which often exploited to channel the excess of reducing power to the synthesis of biomass (proteins), bioplastics or H_2 (Alloul et al., 2022).

In short, the redox state of the PPB determines the metabolic pathways that will be active, being able to channel the electrons towards the pathways of interest. In this thesis the electrode is explored as a redox control tool.

4.- Electroactivity in purple phototrophic bacteria

It is necessary to clarify the concept of electroactivity. This work details the PPB species capable of interacting with an electrode, either by means of redox mediators (Mediated Extracellular Electron Transfer, MEET), or by direct contact with the electrode (Direct Extracellular Electron Transfer, DEET). Despite some authors consider electroactive bacteria as those capable of carrying out DEET, this thesis also includes those species that perform MEET for their possible biotechnological applications.

4.1.- Electroactive purple phototrophic bacteria

So far, only a few species have been described as electroactive: *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and only some strains of *Rhodopsudomonas palustris* has been shown to interact directly with an electrode (Table 1).

Table 1. Species of PPB described as electroactive.

Species	Potential (*vs.* Ag/AgCl)	CE	CD	EET mechanism	References
Rhodobacter capsulatus	0.35 V (Anodic)	-	12.25 $\mu A \cdot cm^{-2}$	MEET	Hasan 2015
Rhodobacter capsulatus	- (Anodic)	-	-	MEET	Grattieri 2019
Rhodobacter capsulatus	0.2 V (Anodic)	-	5.5 $\mu A \cdot cm^{-2}$	MEET	Hasan 2013
Rhodobacter capsulatus	Telurium	-	-	MEET	Borghese 2020
Rhodobacter capsulatus	0.36 V (Anodic)	-	4.8 $\mu A \cdot cm^{-2}$	MEET	Grattieri 2019
Rhodobacter sphaeroides	0.2 V (Anodic)	-	1 $\mu A \cdot cm^{-2}$	MEET	Cai 2002
Rhodobacter sphaeroides	DCBQ	-	-	MEET	Kasuno 2009

Rhodopseudomonas palustris RP2	MFC (Anode)	46.7%	305 $mA \cdot m^{-2}$	DEET	Venkidusamy 2016
Rhodopseudomonas palustris RP2	Iron	-	-	DEET	Venkidusamy 2015
Rhodopseudomonas palustris DX-1	MFC (Anode)	-	30 $mW \cdot m^{2}$	DEET	Xing 2008
Rhodopseudomonas palustris TIE-1	100 (SHE)	-	-100 $nA \cdot cm^{-2}$	DEET	Guzman 2019
Rhodopseudomonas palustris TIE-1	100 (SHE)	-	-1.5 $\mu A \cdot cm^{-2}$	DEET	Bose 2014
Rhodopseudomonas palustris TIE-1	100 (SHE)	-	-5.6 $\mu A \cdot cm^{-2}$	MEET	Rengasamy 2018
Rhodopseudomonas palustris TIE-1	100 (SHE)	-	27.9 $\mu A \cdot cm^{-2}$	DEET	Ranaivoarisoa 2019
Rhodopseudomonas palustris TIE-1	-0.220 V	-	7.23 $\mu A \cdot mL^{-1}$	MEET	Doud 2014

R. capsulatus is one of the most studied purple bacteria and is considered the most versatile prokaryote (Madigan and Gest, 1979). Although DEET has not been demonstrated in *R. capsulatus* so far (Hasan et al., 2013), some authors have described that this bacteria is able to perform MEET by using redox mediators such us p-benzoquinone (Hasan et al., 2015), lawsone (Borghese et al., 2020), Fe-NTA (Wong et al., 2016), TMPD, $Fe(COOH)_2$ and Menadione (Cai et al., 2002). In all this studies, anodic potentials were used (0.22 V to 0.35 V (*vs.* Ag/AgCl)) depending on the mediator redox potentials (Hasan et al., 2013, 2015).

Some authors have also described MEET in *R. spheroides*. In fact, they have studied in depth how redox mediators, such as TMPD, menadione and TEMPO interact with the bacteria based on hydrophobicity (Cai et al., 2002). Furthermore it has been described that 2,5-dichloro-1,4-benzoquinone (DCBQ) can act as exogenous electron acceptor in *R. sphaeroides* (Kasuno et al., 2009)

Rhodopseudomonas palustris is one of the first species of electroactive PPB discovered. Specifically, the *R. palustris* DX-1 strain was isolated from an MFC electrode by Xing in 2008. In the same publication, it was also shown that another collection strain (*R. palustris* ATCC 17001) was not able to interact with the electrode. These results indicate that the molecular mechanisms involved in electron transfer are perfectly regulated and encoded in the bacterial genome and are not present in all PPB species (Xing et al., 2008).

Since then, some laboratories, such as the Arpita Bose in Washington, have worked with *R. palustris* as a model for its versatility and easy of genetic manipulation. The importance of *R. palustris* as a model microorganism lies in the fact that (1) it performs direct electron transfer (DEET), which allows studying the molecular mechanisms of EET and also (2) is capable of capturing electrons from the electrode. For these reasons, studies carried out with this microorganism have served to investigate in depth the molecular mechanism behind the

transfer of electrons and how it is connected with bacterial metabolism, which is presented in the following section (4.2)

4.2.- Extracellular electron transfer in purple phototrophic bacteria

The first reports of extracellular transfer in PPB are through redox mediators and therefore the researchers focused on it (Cai et al., 2002). The extracellular electron transfer mechanism is still unclear in some species such as *R. capsulatus*. Although, some models have been proposed. Cytochrome C reductases in the electron chain could play a role in the dissimilatory iron reduction (Dobbin et al.,1996).

Direct extracellular electron transfer is the most important from a biotechnological point of view. Therefore, most of the existing scientific knowledge is based on the extracellular electron pathway of *Rhodopseudomonas palustris*.

The studies carried out with electroactive strains of *R. palustris*, indicate that there is a group of proteins that plays a fundamental role in DEET (Bose et al., 2014). This proteins are coded in the pioABC operon: pioA (mtrA homologue in *Shewanella oneidensis*) is a periplasmic decaheme cytochrome c protein, pioB (mtrB homologue in *S. oneidensis*) is an outer membrane porin and pioC is a periplasmic high-potential iron-sulfur protein (Gupta et al., 2019). The pioAB complex is located in the outer membrane and interacts with the exogenous electron donor, the pioC is considered present in the periplasm and would be in charge of connecting with the electron chain, made up of the reaction center and the bc1 complex (Fig. 12). The mechanism of electronic transfer from pioC to the electron transport chain is still unclear, although the most supported possibility is that the connection occurs at proton-translocating protein bc1 (Guzman et al., 2019). This author, used Antimycin A, a quinone anolog that blocks bc1 Q site, showing that electron uptake decrease. (Rosenbaum et al., 2010).

Despite this mechanism has been proposed for the electron uptake process, some authors affirmed that this type of protein could intervene in the bidirectional transport of electrons (Gong et al., 2020).

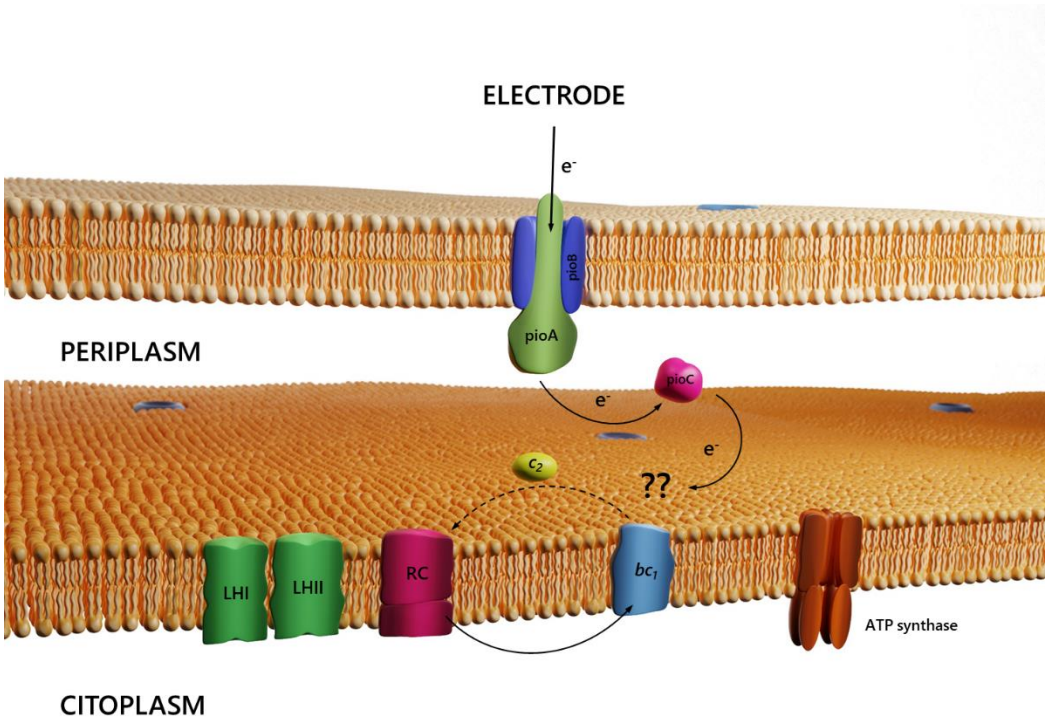

Figure 12. Cellular structure involved in extracellular electron transfer in *Rhodopseudomonas palustris* proposed by Bose et al. 2014 (3D by Cristina Villar).

Studies carried out by Venkidusamy *et al.* in 2016 revealed the presence of conductive nanofilaments in *R. palustris* RP2 that enable electronic transport (Venkidusamy et al., 2016). This type of appendages has been described in other microorganisms such as *Geobacter* or *Shewanella* as a mechanism for EET, in DIET and in electronic transport in communities such as biofilms (Malvankar et al., 2011). In this study, the researchers demonstrated that nanofilament expression is induced in anoxic photoheterotrophic conditions.

Little is known about the relationship between extracellular electron transfer and PPB metabolism. Carbon fixation via the Calvin cycle seems to be the metabolic pathway mainly connected to the electrode, both in pure cultures (Guzman et al., 2019) and in mixed cultures (Vasiliadou et al., 2018; ; Edreira et al., 2021). Although other metabolic pathways could be affected by the electrode depending on the culture conditions.

4.3.- Purple phototrophic bacteria applications

For years, wastewater treatment has been based on the removal of contaminants. Currently, the climate crisis requires a change in the way wastewater is treated, requiring the recovery of nutrients and energy (Batstone et al., 2015). Nutrient partitioning by microbial culture,

specifically, photoheterotrophic bacteria are particularly effective. Purple phototrophic bacteria cultivation allows to maximize the amount of nutrients recovered, since the nutrients are not dissipated as in chemoheterotrophic catabolism (Winkler and Straka, 2019)

Mixed culture of purple phototrophic bacteria are gaining attention for this purpose, being used in the treatment of urban wastewater (Hulsen et al., 2016) and a wide plethora of industrial wastewater (Rai et al., 2012). Some researchers have successfully applied electromicrobiology to the treatment of wastewater with PPB (Vasiliadou et al., 2018; ; Edreira et al., 2021), which has led to the construction of pilot and demo scale reactors in Spain (https://deep-purple.eu), India (https://projectsaraswati2.com) and Australia (Hulsen et al., 2022).

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6.- Objectives and thesis outline

This thesis aims to understand how electromicrobiology can help to cultivate purple phototrophic bacteria. The challenge in culturing these microorganisms is to identify affordable feedstocks. Electrodes can overcome this limitation either directly, by providing electrons as feedstock to microorganisms, or indirectly, by controlling the metabolism of bacteria when alternative feedstocks such as wastewater are used. The following objectives were proposed:

1.- To investigate the cultivation of a purple phototrophic bacteria dominated consortium under autotrophic conditions with an electrode as the sole electron donor.

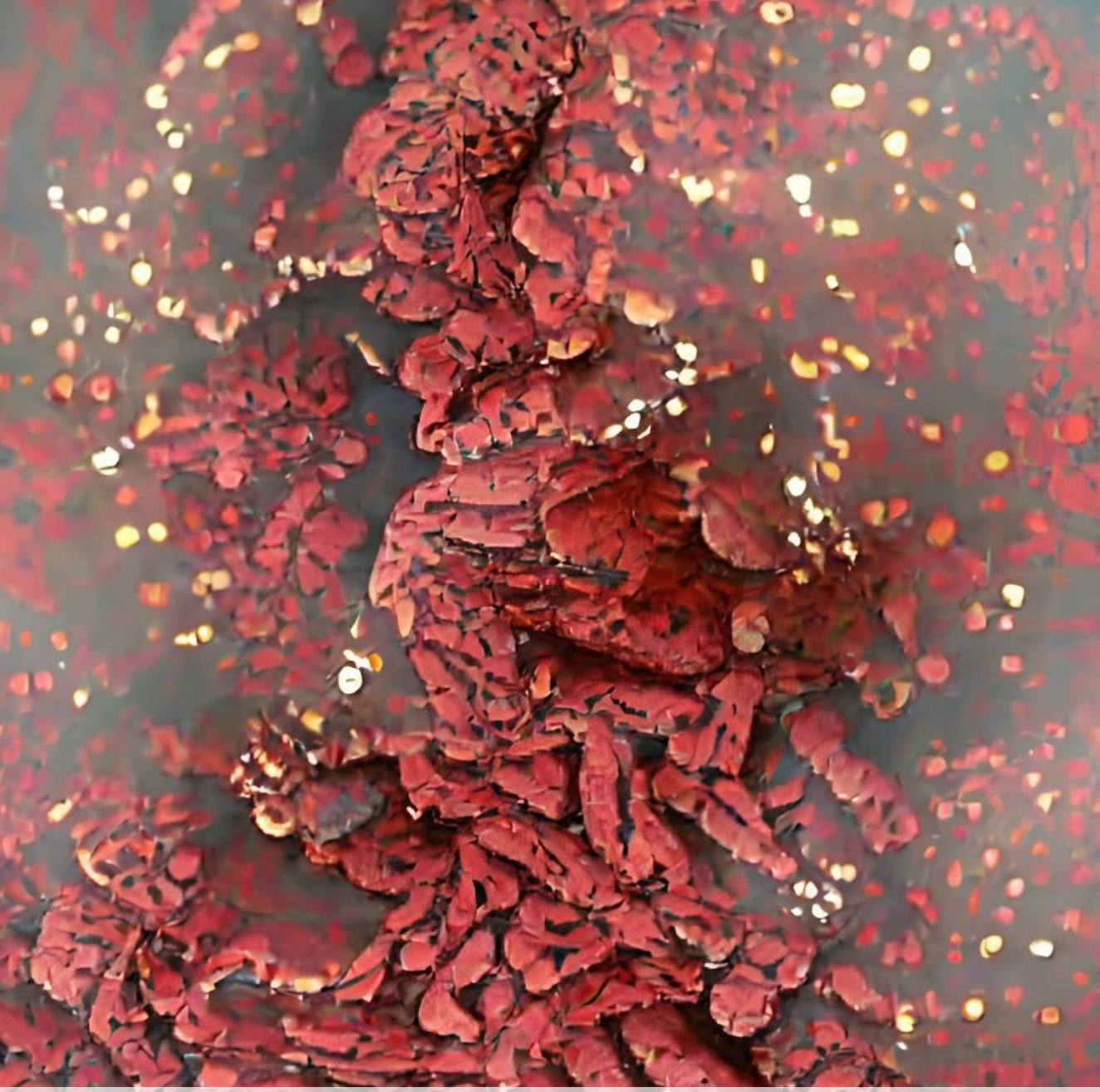
This objective has been addressed in Chapter 2 of this thesis: *Microbial photo-electrosynthesis: feeding purple phototrophic bacteria electricity to produce bacterial biomass*. Purple phototrophic bacteria dominated consortium was grown with a cathode as electron donor. We studied the biomass production and the selected microbial community under these culture conditions.

2.- To study the cultivation of a purple phototrophic bacteria dominated consortium in brewery wastewater with an electrode to maximize treatment efficiency.

This objective was evaluated in Chapter 3: *Fluid-like electrodes overcome the biofilm-based paradigm for growing electroactive planktonic Purple Phototrophic Bacteria*. We used a fluidized anode to evaluate the effect of an extra electron acceptor on the growth of purple phototrophic bacteria when brewery wastewater is used as feedstock. We studied the treatment efficiency, the biomass production and the microbial population.

3.- To explore the cultivation of a purple phototrophic bacteria dominated consortium in brewery wastewater with an electrode to maximize biomass production.

This objective is detailed in Chapter 4: *Fluid-like cathode enhances valuable biomass production from brewery wastewater in purple phototrophic bacteria cultivation*. We use fluidized cathodes to evaluate the effect of an extra electron donor on the growth of purple phototrophic bacteria when brewery wastewater is used as feedstock. We studied the treatment efficiency, the biomass production and the microbial population. Furthermore, we evaluated the effect of the reducing power of wastewater (COD:TOC ratio) on the interaction with the electrode and the microbial community.



Chapter 2:

Microbial photo-electrosynthesis: feeding purple phototrophic bacteria electricity to produce bacterial biomass

This chapter is based on the scientific publication:

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Coauthors: Fernando Muniesa-Merino, María Llorente and Abraham Esteve-Núñez

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CHAPTER 2:

Microbial photo-electrosynthesis: feeding purple phototrophic bacteria electricity to produce bacterial biomass

Abstract

Purple phototrophic bacteria are one of the main actors in chemolithotrophic carbon fixation and, therefore, fundamental in the biogeochemical cycle. These microbes are capable of using insoluble electron donors such as ferrous minerals or even carbon-based electrodes. Carbon fixation through extracellular electron uptake places purple phototrophic bacteria in the field of microbial electrosynthesis as key carbon capturing microorganisms. In this work we demonstrate biomass production dominated by purple phototrophic bacteria with a cathode (-0.6 V *vs.* Ag/AgCl) as electron donor. In addition, we compared the growth and microbial population structure with ferrous iron as the electron donor. We detect interaction between the cathode and the consortium showing a midpoint potential of 0.05 V (*vs.* Ag/AgCl). Microbial community analyses revealed different microbial communities depending on the electron donor, indicating different metabolic interactions. Electrochemical measurements together with population analyses point to *Rhodopseudomonas* genus as the key genus in the extracellular electron uptake. Furthermore, the genera *Azospira* and *Azospirillum* could play a role in the photoelectrotrophic consortium.

1.- Introduction

Microbial anoxygenic photosynthesis is one of the most important processes in the biogeochemical cycle, since it allows light-driven carbon fixation (McKinlay and Harwood, 2010; Bryce et al., 2018; Ozaki et al., 2019). Chemolithotrophic carbon fixation that occurs in subsurface dictates carbon fluxes in environments such as soils and groundwater (Taubert et al., 2021). One of the main actors in this process are purple phototrophic bacteria (PPB), which fix CO_2 using a wide range of electron donors such as H_2, $S_2O_3^{2-}$ or S^{2-}. Surprisingly, some microorganisms of this type are also capable of accepting electrons from solid phases such as ferrous minerals (Widdel et al., 1993) or even graphite electrodes (Xing et al., 2008). In this context, the mechanisms behind extracellular electron uptake to accept electrons from a cathode have been revealed (Bose et al., 2014). Furthermore, carbon fixation (via the Calvin-Benson cycle) has been recently proved to be strongly linked to phototrophic electron uptake using an electrode (cathode) as electron donor (Guzman et al., 2019) . Furthermore, other metabolic pathways such as nitrogen fixation or hydrogen production could be also affected (McKinlay and Harwood, 2010) under electrode control. Carbon fixation through extracellular electron uptake places purple phototrophic bacteria in the field of microbial electrosynthesis as key carbon capturing microorganisms. Microbial electrosynthesis seeks to use the electrons provided by a biocathode to synthesize value-added compounds, generally volatile fatty acids and alcohol like acetate, propanol or butyrate (Nevin et al., 2010; Ganigué et al., 2015; Izadi et al., 2021), but also other compounds such as bioplastics (Nishio et al., 2013; Chen et al., 2018) or microbial biomass as protein source (Xu et al., 2021).

After a decade of effort and scientific advances (Nevin et al., 2010), microbial electrosynthesis is still far from the technical and economic competitiveness of current industrial processes (Prévoteau et al., 2020). Microbial electrosynthesis requires engineering improvements such as electrode material (Zhang et al., 2012; Nie et al., 2013; Jourdin et al., 2014) and reactor design (Kantzow et al., 2015a, 2015b; Jourdin et al., 2018) but also in the selection of the microbial community (Prévoteau et al., 2020). The resilience and versatility of the microbial community will determine the range and rate of products obtained as well as the robustness of the synthesis process. Purple phototrophic bacteria could bridge the gap to these limitations. Despite discoveries based on pure cultures (Bose et al., 2014), the use of consortium dominated by purple phototrophic bacteria under photoelectroautotrophy still in its infancy.

Therefore, understanding the dynamics of a microbial consortium of purple phototrophic bacteria would allow operation under non-sterile conditions, that despite its limitations such as the purification of products or possible impediments in the food industry, reduce costs and facilitating the operation (Hülsen et al., 2014), while opening the range of potential products. With the aim of bringing electrode-mediated biomass production closer to reality, in this work we have explored the photoelectroautotrophic cultivation of a mixed culture dominated by purple phototrophic bacteria. In addition, we have compared electrode-

dependent biomass production with the most ubiquitous inorganic electron donor, ferrous iron.

2.- Material and methods

Mineral medium and microbial cultivation

In this work, a PPB-dominated microbial consortium was cultured under autotrophic conditions with two electron donors: ferrous iron (Fe^{2+}) and an electrode (cathode). We used a mixed microbial community adapted to electrode interaction (Manchon et al. 2022) as inoculum for all experiments.

Minimal mineral medium was used for all experiments containing $NaHCO_3$ 2,5 g L^{-1}, NH_4Cl 0,5 g L^{-1}, $NaH_2PO_4.2{\cdot}H_2O$ 0,41 g L^{-1}, KCl 0,1 g L^{-1}, a mixed of vitamins 10 mL/L, a mixed of minerals 10 mL/L. Medium was sparged with N_2:CO_2 (80:20) to remove dissolved oxygen. In the Fe^{2+} experiment, Fe^{2+} was added to a final concentration of 20mM under anoxic conditions. All the reactors (culture bottles and H-Cells) were illuminated with IR-lamps (850 nm) to promote the presence and activity of PPB.

Experimental set-up

Photoferrotrophy

PPB-dominated consortium was cultured in serum bottles (n=5) under anoxic, autotrophic and IR-illuminated conditions with ferrous iron (Fe^{2+}) as electron donor (Fig. 1).

Photoelectrotrophy

To evaluate the photoelectroautotrophic growth (cathode as electron donor) of PPB consortium, we explored biomass production in H-cell reactor and we characterized electroactive behaviour in a single chambered reactor.

We used H-Cell with carbon rod (Mersen, Courbevoie, France) was used as cathode (working electrode), a 3M KCl Ag/AgCl reference electrode (Hanna Instruments S.L., Gipuzkoa, Spain) and a platinized titanium mesh (Inagasa S.A., Barcelona, Spain) as the counter electrode. Electrochemical measurements were performed with a potentiostat NEV4 v2.0 (Nanoelectra S.L., Alcalá de Henares, Spain) (Fig. 1).

We used single chambered reactor (100 mL) with a 3M KCl Ag/AgCl reference electrode (Hanna Instruments S.L.) and a platinized titanium mesh (Inagasa S.A., Barcelona, Spain) as the counter electrode. Carbon felt (Mersen, Courbevoie, France) fragment was connected with a gold filament acting as working electrode (Fig. 1).

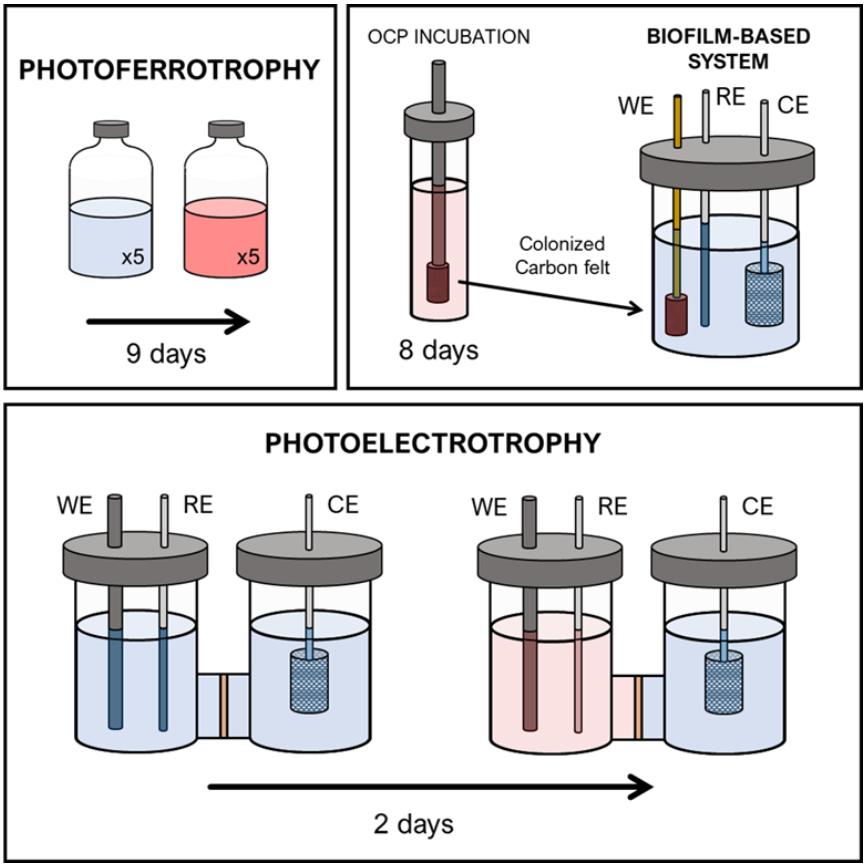

Figure 1. Schematic of the experiment set-up. Top left: Photoferrotrophy cultivation experiment. Top right: Biofilm-based experiment to assess electroactivity of the consortium. Down: Photoelectrotrophy.

Experimental design

First, we studied the consortium growth under photoferrotrophy (Fe^{2+} as electron donor), for which we inoculated serum bottles (1/100) and incubated them at 30ºC with constant stirring and IR-illumination (IR-lamps, 850 nm). We monitored microbial growth by measuring absorbance (λ=400 nm -1000 nm) and ferrous iron concentration as described elsewhere (Carpenter and Ward, 2017). The presence of organic compounds in the process was analysed by HPLC. At the end of the experiment, biomass growth was measured as total organic carbon (TOC) and volatile suspended solids (VVS). We also visualized biomass using scanning (SEM) and transmission (TEM) electron microscopy. The microbial population at the end of the experiment was analysed by 16S Illumina.

Next, we inoculated H-cell reactor (1/100) with the same inoculum and we operated the reactor at -0.6 V (*vs.* Ag/AgCl). During growth, we measured absorbance (590 nm) by spectrophotometer and organic compounds by HPLC. At the end of the experiment we

visualized the biomass produced by transmission electron microscopy and we analysed microbial population (16S Illumina) at the end of the experiment.

Finally, we studied the electroactivity of the consortium in a biofilm-based approach with carbon felt as material. After 200 hours in contact with the consortium under non-polarized autotrophic conditions, a piece of the colonized felt was connected to a gold filament for bioelectrochemical study similar to Rodrigo-Quejigo et al., 2019. After 24 hours of polarization (-0.6 V *vs.* Ag/AgCl) as acclimatation, cyclic voltammetries were performed. Another fragment of colonized carbon felt was analysed by transmission electron microscopy (TEM).

Analytical methods

Volatile fatty acids (VFA) were measured by HPLC (1100 high pressure liquid chromatograph) with a 210 nm UV detector and Supelco C-610H column. H_3PO_4 0.1 % (v/v) was used as mobile phase at 0.5 mL/min flow rate. To rule out the presence of organic compounds not measurable by HPLC in the medium, the total organic carbon (TOC) was analysed by TOC -VCSH analyser (Shimadzu).

We used scanning electron microscope (SEM) and transmission electron microscopy (TEM) to explore cell morphology and biofilm formation both in ferrotrophic and electrotrophic experiments. SEM samples were fixed with 5% (v/v) glutaraldehyde in cacodylate buffer (0.2 M, pH 7.2), and gradually dehydrated with ethanol solutions (25, 50, 70, 90 and 100%, 10 min each step). Then, samples were rinsed with acetone for 10 min and immerse in anhydrous acetone at 4ºC overnight. Finally, the samples were dried in CO_2 and coated with gold. Micrographs were taken using a scanning electron microscope JSM-IT500 (JEOL).

For the TEM assay, 5 mL planktonic cell suspensions were centrifuged at 10000g for 5 min. Primary fixation was carried out by resuspending the cells pellets in 2% formaldehyde and 2.5% glutaraldehyde in sodium phosphate buffer (pH 7.2) for ~ 45 min at room temperature. For second fixation, samples were incubated with osmium tetroxide 1% for 1 hour. After second fixation, cells were subjected to dehydration by acetone and resin infiltration. Ultrathin sections (~50–60 nm) were obtained using ultramicrotome. To obtain TEM micrographs were characterized using a JEM 1400 (National Center of electron microscopy, UCM, Madrid, Spain).

The biomass produced in the reactors was quantified by spectrophotometry and analysis of volatile suspended solids (VSS). Spectrophotometric spectrum (1100 nm to 400 nm) was measured with a UV-1800 spectrophotometer (Shimadzu). The VSS concentration (gVSS/L) was measured according to standard methods (D. Eaton et al., 2005).

3.- Results and discussions

In contrast with previous experiences with pure culture of purple phototrophic bacteria (Guzman et al., 2019), we have explored for the first time the cultivation of a microbial mixed consortium dominated by PPB with a cathode as the sole electron source.

Photoferrotrophic growth: ferrous iron as electron donor

Our first approach to understand autotrophic growth extracellular electron uptake led us to cultivate a mixed community dominated by purple phototrophic bacteria with ferrous iron (Fe^{2+}). For this purpose, we used an enriched culture dominated by anodic purple phototrophic bacteria to inoculate batch reactors with ferrous iron a sole electron donor.

We observed an increase in optical density (OD_{590}) over time, accompanied by a decrease in Fe^{2+} concentration indicating that the consortium was able to fix inorganic carbon into biomass by using Fe^{2+} as electron donor (Fig. 2A). Biomass production was also verified at values concentration of 22.08 ± 2.71 $g \cdot L^{-1}$ for total organic carbon (TOC) and 0.1334 ± 0.0171 $g \cdot L^{-1}$ VSS at the end of the experiment.

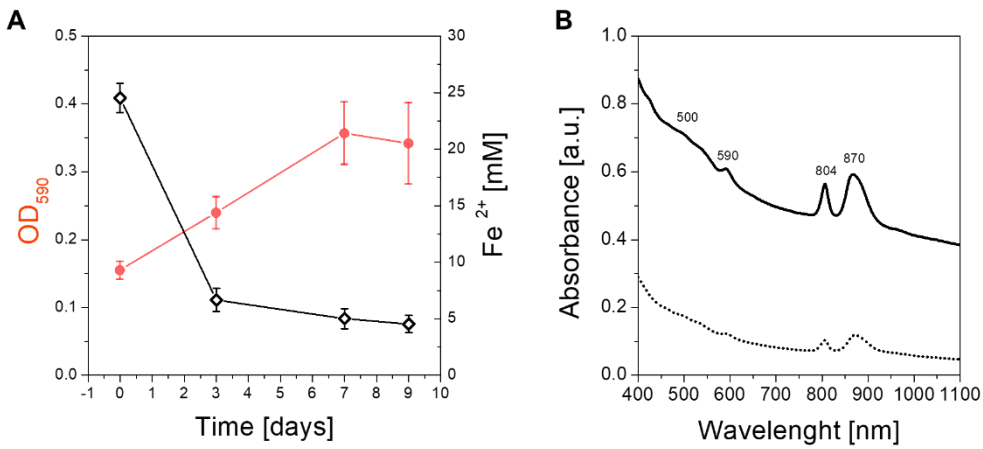

Figure 2. Purple phototrophic bacteria dominated consortium under photoferrotrophic conditions. A: Growth curve. Red circles correspond to optical density (OD_{590}) and white diamonds corresponds to ferrous iron concentration (Fe^{2+}). B: Absorbance spectrum at time 0 (dashed line) and stationary phase (straight line).

The increase in optical density showed a certain delay with respect to the oxidation of ferrous iron (Fig. 2A). This result could indicate that other microorganisms could be playing a role in the process i.e. electroactive acetogens that produce organic compounds that purple phototrophic bacteria can use to grow heterotrophically. We observed two maximum absorption peaks (804 nm and 870 nm) characteristic peaks of PPB bacteriochlorophylls. In addition to several peaks between the wavelengths 500 nm and 590 nm, corresponding to carotenoids. These absorption peaks match to those described in purple phototrophic

bacteria dominated consortia (Vasiliadou et al., 2018). Therefore, the population of purple phototrophic bacteria remains predominant under photoferrotrophic conditions (Fig. 2B).

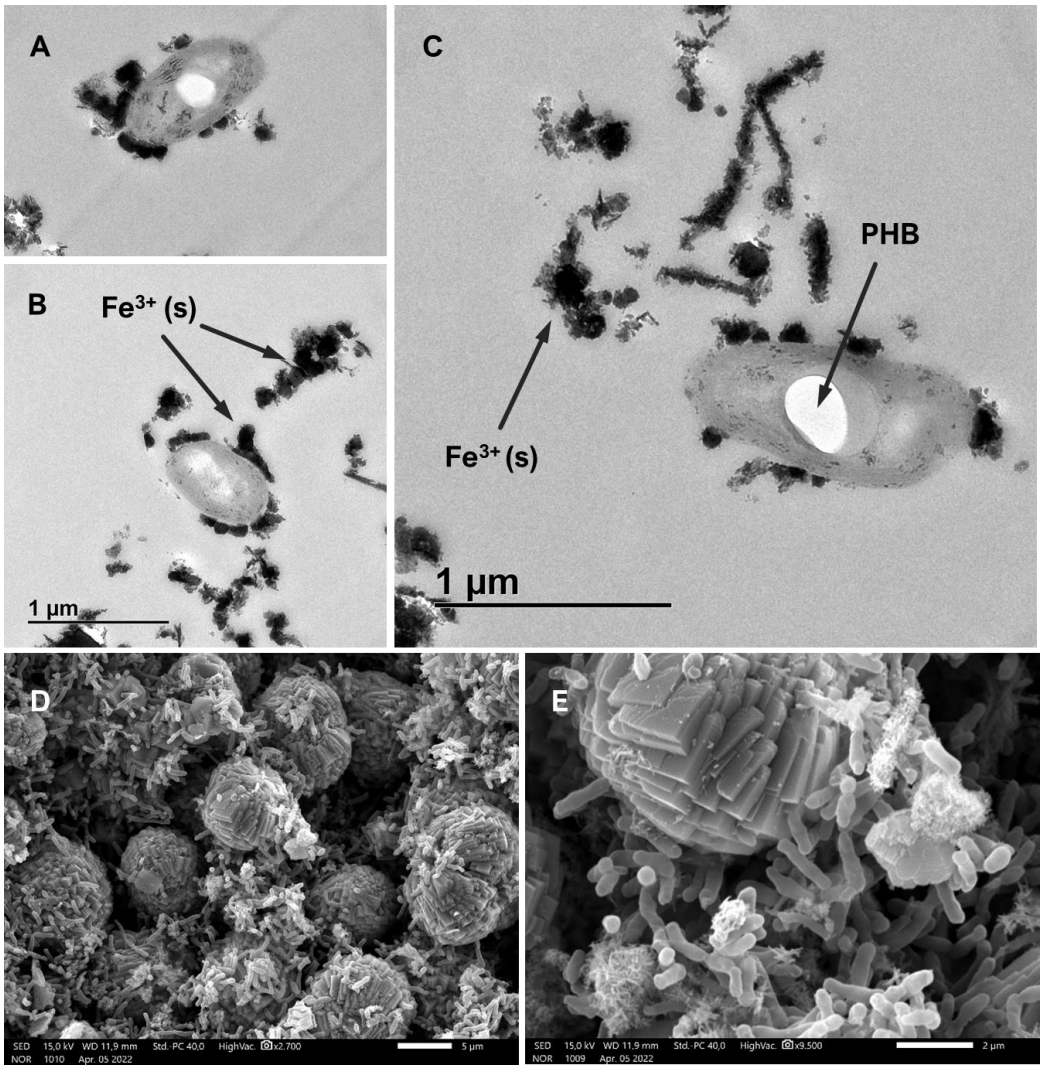

Figure 3. Purple phototrophic bacteria dominated consortium under photoferrotrophic conditions. A: TEM micrograph with type internal membrane structures. B: TEM micrograph with insoluble iron oxides (Fe^{3+}). C: TEM micrograph with cell inclusions. D and E: SEM micrograph with insoluble iron oxides (Fe^{3+}) and bacteria.

At the end of the experiment, we studied photoferrotrophically cultured consortium using transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Fig. 3). The micrographs showed microbial cells associated with different mineral particles classically observed in processes of biomineralization of iron (Oggerin et al., 2013). The solubility of ferric iron (Fe^{3+}) is considerably less than that of ferrous iron (Fe^{2+}). Therefore, the oxidation of ferrous iron to ferric iron (Fe^{3+}) led to the formation of insoluble ferric iron (Fe^{2+}) minerals (Fig. 3B, 3D and 3E). Notably, the images revealed lamellar type internal membrane structures arranged parallel to the cytoplasmic membrane, structures typical of purple phototrophic bacteria (Fig. 3A and 3C)(Ramana et al., 2010; LaSarre et al., 2018). In addition, despite not having analysed in depth the presence of bioplastics produced, we observed granules in the cytoplasm very similar to the polyhydroxybutyrate (PHB) and polyhydroxyalkanoate (PHA) granules described by other authors (Higuchi-Takeuchi et al., 2016) (Fig. 3C).

Furthermore, SEM micrographs showed angular crystal structures of iron oxides with bacteria forming rosette-like clusters previously described by other researchers in the model electroactive purple phototrophic bacteria, *Rhodopseudomonas palustris* (Hougardy et al., 2000) (Fig 3D and 3E).

Photoelectrotrophic growth: cathode as electron donor

After exploring the photoferrotrophic growth of PPB we explored the capacity of such culture to use an electrode (cathode) as the sole electron donor. Our goal was to study both biomass production and bioelectrochemical response. Under these conditions, we carried out two independent assays using i) PPB-planktonic cells and ii) PPB-biofilm cells.

As previously described, cathodic polarization hinders irreversible cell adhesion, due to the electrostatic repulsion between the electrode and the cell wall charges (Bayer and Sloyer, 1990; Busalmen and de Sánchez, 2001). This phenomenon, despite being a problem to study cathodic biofilms, allowed us to accurately quantify biomass production. To assess biomass production, we inoculated a two-chamber reactor with our bacterial consortium (Fig. SI1). In this reactor, planktonic growth outcompeted biofilm-based growth by using a carbon rod as working electrode (-0.6 V *vs.* Ag/AgCl). We monitored electron uptake (current density) and planktonic microbial growth (OD_{590}) (Fig. 4A). We observed an increase in biomass growth (OD_{590}) 24 hours after inoculation; actually, absorption peaks coincided with those typical of PPB (Fig. SI2)(Vasiliadou et al., 2018). This microbial growth was accompanied by electron uptake, visible by the increase in current intensity from values close to 0 µA to almost reaching -100 µA. Even at the end of the experiment, we did not observe biomass attached to the cathode but planktonic growth (Fig. SI1). Some species of purple phototrophic bacteria have been previously shown to uptake electrons from cathodes through redox mediators (Hasan et al., 2013; Borghese et al., 2020). In order to clarify the electron transfer mechanism, we studied the reactor supernatant by cyclic voltammetry and no redox species were detected (Fig. SI3), which suggests that direct extracellular electron transfer was the main electron uptake mechanism. We hypothesize that extracellular

electron transfer could occur through random contacts between bacterial cells and the polarized electrode. In the same way that some electroactive bacteria interact with fluid electrodes (Tejedor et al. 2017), the electrostatic repulsion caused by cathodic polarization could promote planktonic interaction over biofilm interaction.

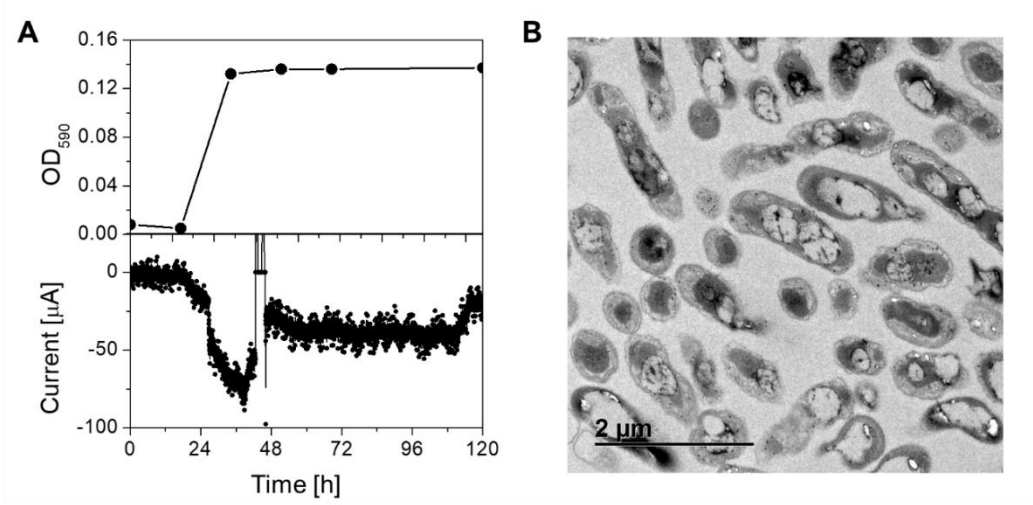

Figure 4. Purple phototrophic bacteria dominated consortium under photoelectrotrophic conditions. A: Growth curve. Up: Optical density (OD_{590}). Down: Current intensity. B: TEM micrograph with type internal membrane structures.

At the end of the experiment, we took TEM micrographs of the planktonic biomass to study the cell structure (Fig. 4B). The bacteria had markedly different cell structures than those observed in the previous experiment (Fig. 3A, 3B and 3C). We identified smaller PHB inclusions (Fig. 4B) compared to the iron-oxidizing consortium (Fig. 3A, 3B and 3C).

These results unequivocally indicate that the PPB-dominated consortium was able to grow with a cathode as sole electron donor. Unfortunately, planktonic cells did not showed electroactivity after cyclic voltammetry analysis (Fig. SI4). An alternative strategy, previously explored (Manchon et al 2022), to acquire electrochemical data from PPB consist of using a biofilm-based approach.

Non-polarized autotrophic conditions allowed biofilm-formation on the carbon felt surface. After ca. 200 hours of inoculation, a fragment of the inoculated felt was analysed by TEM, revealing the formation of the biofilm on the surface, with clustered cells and exopolysaccharide (Fig. 5A and 5B). Some of these cells formed rosette-like clusters like those described in figure 3D and 3E, characteristic of *Rhodopseudomonas palustris*, the model electroactive purple phototrophic bacteria (Hougardy et al., 2000). We observed visible reddish filamentous structures on the surface of the carbon felt (Fig. 5C).

Another fragment of the colonized carbon felt was connected through a gold wire in a single-chamber electrochemical cell to assess the electroactivity of the consortium (Fig. SI5). After

10 hours of polarization (-0.6 V *vs.* Ag/AgCl) cyclic voltammetry analysis revealed a redox couple (E_{mp}= 0.05 V *vs*. Ag/AgCl) and a reductive process below -0.6 V (*vs.* Ag/AgCl) (Figure 5D). The midpoint redox potential was similar to the redox potential of pure cultures of *Rhodosepudomonas palustris* TIE-1 (Bose et al., 2014). The identified redox pair indicates the presence of redox active components that could participate in the extracellular electron uptake. Electrochemical analyses of the supernatant, both in the planktonic-based approach and in the biofilm-based one, did not reveal the presence of redox mediators. Therefore, the electrochemical characterization of the consortium suggests direct extracellular electron transfer in which the redox site (E_{mp}=0.05 V) could be involved. In addition, the similarity of our signal to previous signals reported in *Rhodopseudomonas* sp. suggest the presence of this bacterial genus in our PPB consortium.

The reduction process observed at redox potential lower than -0.6 V *vs.* Ag/AgCl (Fig. 5D, black line) was not observed in the abiotic voltammogram (Fig. 5D, gray line), indicating that it was catalysed by the microbial consortium. Based on our knowledge of purple phototrophic bacteria, the reduction process could correspond to hydrogen bioproduction, as we have previously reported (Vasiliadou et al., 2018). These results pointed to the biohydrogen production potential by PPB-dominated cathodes. To clarify, during all experiments, at the potential used (-0.6 V *vs.* Ag/AgCl) hydrogen production is not thermodynamically possible (-0.63 V *vs.* Ag/AgCl); thus, the H_2-mediated pathway was ruled out.

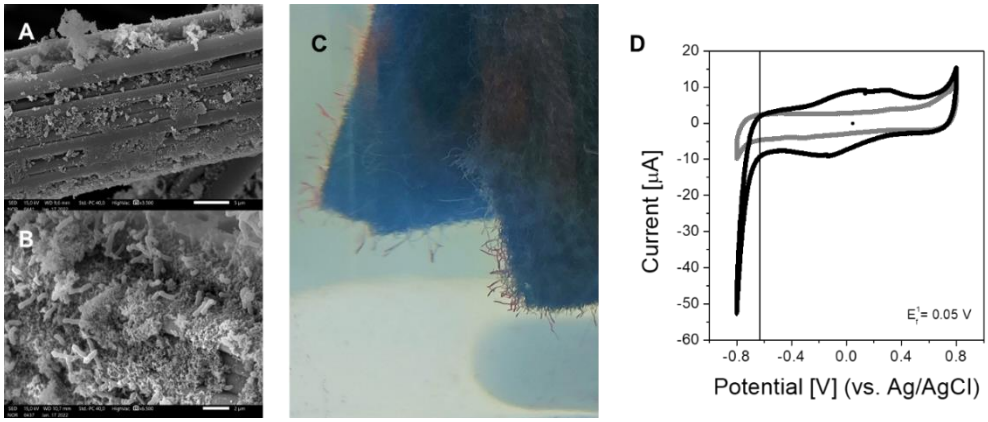

Figure 5. Purple phototrophic bacteria dominated consortium biofilm under photoferrotrophic conditions. A, B: SEM micrograph. C: Carbon felt photograph after inoculation. D: Cyclic voltammograms (5 $mV \cdot s^{-1}$). Gray voltammogram corresponds to abiotic conditions carbon felt. Black voltammogram corresponds to inoculated carbon felt. Vertical line corresponds to thermodinamic potential of hydrogen evolution (-0.63 V *vs.* Ag/AgCl). Mid-point potential (E_{mp}) is indicated by a black point.

Iron and cathodes show similar but different pressure on PPB consortium

The microbial community analysis was performed using 16S Illumina in order to understand how different microbial communities were established under autotrophic conditions and to evaluate the impact of the insoluble electron donor i) ferrous iron and ii) carbon electrode.

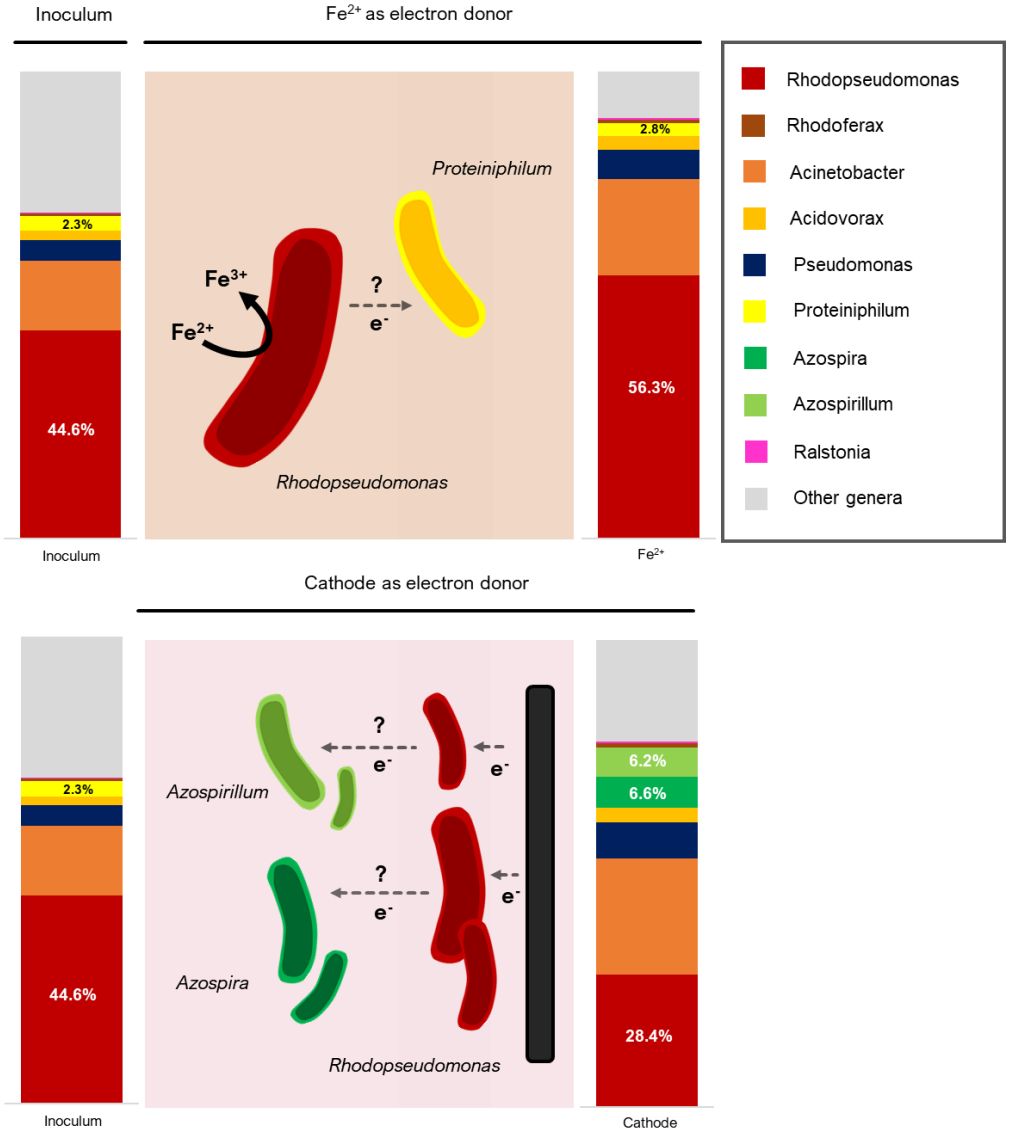

Figure 6. Microbial community analysis and schematic of the genus that might coexist depending on the electron donor. Stacked bars correspond to relative abundance at genus level of inoculum, ferrous iron as electron donor (Fe^{2+}) and cathode as electron donor (Cathode). Left to the bars (Fe^{2+} and cathode) the main genus and their possible interaction for each electron donor are represented.

The predominant genera in common between the two different electron donors are *Rhodopseudomonas*, *Acinetobacter*, *Pseudomonas*, and *Acidovorax* which are considered core genera in electroactive communities (**Fig. 6**)(Xiao et al., 2015). *Rhodopseudomonas*, the most abundant genus in all the samples, is considered the electroactive PPB model (Bose et al., 2014; Guzman et al., 2019). This genus is capable of fixing carbon dioxide into biomass using an extracellular electron donor e.g. ferrous iron (Widdel et al., 1993) or cathodes (Bose et al., 2014; Guzman et al., 2019). *Acinetobacter*, *Pseudomonas*, and *Acidovorax* have been previously found in cathodes (Rabaey et al., 2008; Liu et al., 2014; Rowe et al., 2015) but their electroactivity has not been unequivocally demonstrated. The usual presence of these genera in electroactive consortia indicates a fundamental role. However, the lack of reports demonstrating its electroactivity suggests that *Rhodopseudomonas* is the key genus that performs extracellular electron uptake in our systems, connecting the rest of the community with the extracellular electron donor. Additionally, we observed a strong increase in abundance of *Ralstonia* genus in both ferrotrophy (Fe^{2+}) (2.7 fold) and electrotrophy (cathode) (3.3 fold) compared to the original community used as inoculum. *Ralstonia* was reported in the autotrophic electrode-assisted production of valuable compounds such as PHB (Nishio et al., 2013; Chen et al., 2018) or alcohols (Li et al., 2012). Despite its low abundance in this study, our results indicate that *Ralstonia* could become important in a long-term photoelectrotrophic system.

The structure of the microbial community also revealed differences in terms of the dissimilar abundance and presence of species depending on the extracellular electron donor. First, consortium with Fe^{2+} as electron donor showed lower level diversity (Shannon index: 1.84) respect to the inoculum (Shannon index: 2.266). This result was consistent with the proportion of *Rhodopseudomonas* and *Acinetobacter* genus species that represent more than 75% of the total genera identified in the samples with ferrous iron as electron donor (**Fig. 6**). Both the inoculum and the sample with Fe^{2+} showed a notable presence of the genus *Proteiniphilum*, described as electroactive bacteria (Logan et al., 2019); however, it was not found in cultures with the cathode as electron donor.

In the samples with cathode as electron donor we observed a similar diversity (Shannon index: 2.24) compared with the inoculum. However, the analyses revealed a decrease in the relative abundance of *Rhodopseudomonas* in addition to a drastic decrease in the abundance of *Proteiniphilum* (**Fig. 6**). We discovered that cathode promoted the presence of two bacterial genera: *Azospirillum* and *Azospira*, both considered core genera in electroactive microbial communities (Xiao et al., 2015). *Azospirillum* is a nitrogen-fixing bacterium that has rarely been found in electroactive communities (Pisciotta et al., 2012). *Azospirillum* sp. together with other genera such as *Rhodopseudomonas* and *Ralstonia*, also present in the cathode, have been described as one of the main carbon sequestrants in soils (Yuan et al., 2012). Furthermore, some researchers have reported that *Azospirillum* strains isolated from electrodes are capable of extracellular respiration of anthraquione-2,6-disulfonate (AQDS) (Zhou et al., 2013), a redox mediator classically respirable by electroactive bacteria (Lovley et al., 1999; Dantas et al., 2018). *Azospira*, another genus primarily described as nitrogen-fixing bacteria, has also been detected in electroactive

communities (Sun et al., 2011). Furthermore, some authors have described *Azospira* sp. electron uptake from cathodes using AQDS as a redox mediator (Thrash et al., 2007). Therefore, the decrease in the abundance of *Rhodopseudomonas* together with the appearance of *Azospirillum* and *Azospira* points to the possibility of a syntrophy under photoelectroautotrophic conditions.

The PPB-dominated consortium used as inoculum was previously adapted to perform anodic respiration (Manchon et al. 2022), so the electrode was used as an electron acceptor. In contrast, such microbial consortium successfully adapted to conduct the inverse reaction, using the electrode (now as cathode) as extracellular electron donor. So, our PPB-dominated consortium appeared to have bidirectional electron transfer, being able to both accept and donate electrons from a insoluble material. Some authors have already reported that PPB could carry out transfer in both directions (Xing et al., 2008; Bose et al., 2014). Furthermore, we have previously reported the same behaviour using a PPB-dominated consortia growing on fluid-like electrodes under heterotrophic conditions (Manchon et al 2022).

In summary, our results confirm the *Rhodopseudomonas* genus played the main role in the extracellular electron uptake using Fe^{2+} and with cathode as electron donor. The connection between the cathode and PPB-dominated microbial community still remains unclear. The absence of redox mediators (Fig. SI3) and organic compounds (Table SI1) suggests a direct extracellular electron uptake, as previously reported in pure cultures of purple phototrophic bacteria (Bose et al., 2014). Further studies have shown interspecies electron transfer for methanogenesis (Huang et al., 2022) and carbon fixation (Liu et al., 2021) between purple phototrophic bacteria and other genera. Thus, we hypothesize that *Rhodopseudomonas* strains in our reactors are the link between the electrode and the microbial community.

4.- Conclusions

This work explored for the first time the cultivation of a PPB-dominated consortium under photoelectrotrophic conditions. Using a cathode as a sole electron donor, we have produced biomass dominated by purple phototrophic bacteria without any prior acclimatization period. Electrochemical measurements together with population analyses point to *Rhodopseudomonas* genus as the key bacterial genus in the extracellular electron uptake. Furthermore, the genera *Azospira* and *Azospirillum* could play a role in the photoelectrotrophic consortium.

The cultivation of a mixed culture allows to operate under non-sterile conditions, drastically improving the applicability as a method of bacterial biomass production. Additionally, the biomass composition dominated by purple phototrophic bacteria gives rise to a new exploratory field in the production of protein-rich biomass and value-added products.

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6.- Supplementary information

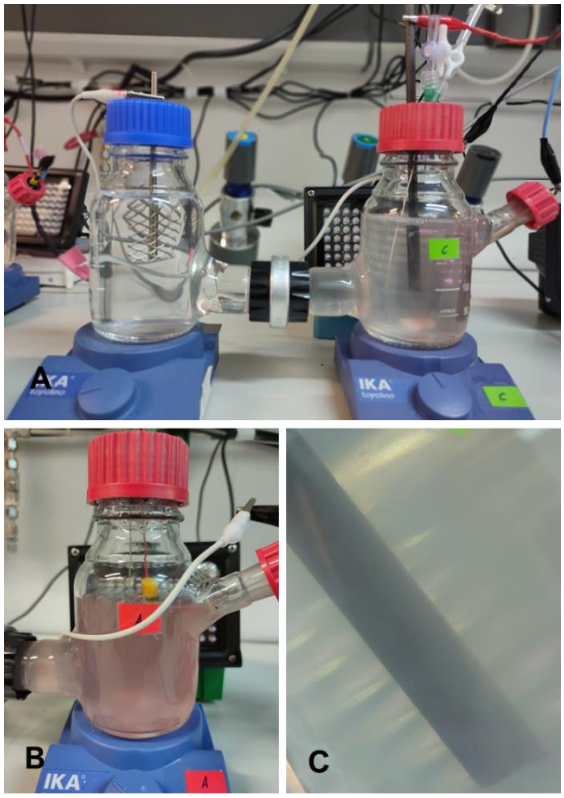


Figure SI1. H-cell used in the photoelectrotrophic growth of PPB-dominated consortium. A: H-cell reactor. B: Cathodic chamber detail. C: Carbon rod with no visible biofilm formation.

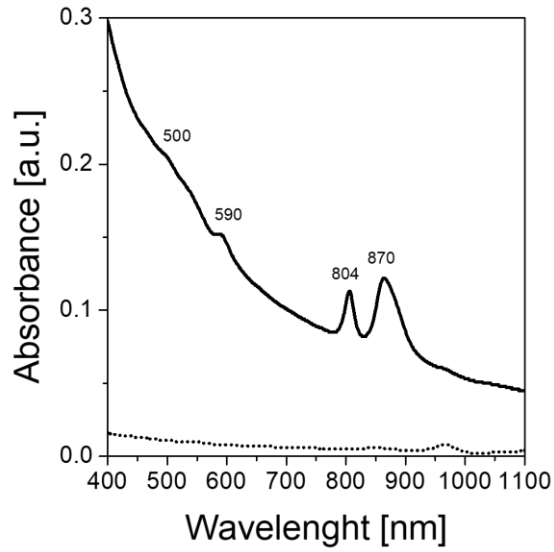


Figure SI2. Absorbance spectrum of purple phototrophic bacteria dominated consortium under photoelectrotrophic conditions. Absorbance spectrum at time 0 (dashed line) and stationary phase (straight line).

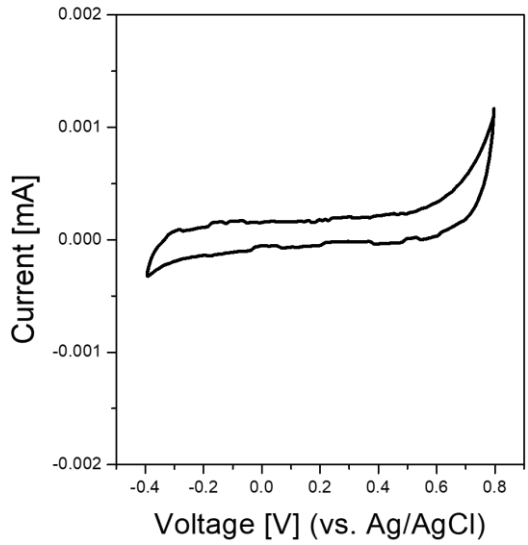


Figure SI3. Cyclic voltammograms (5 $mV \cdot s^{-1}$) in a screen printed electrode (GPHO, Dropsens) in anoxic conditions.

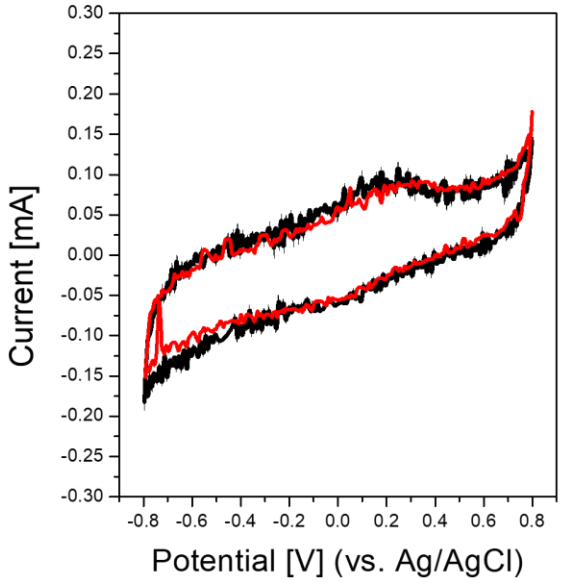


Figure SI4. Cyclic voltammograms (5 $mV \cdot s^{-1}$). Black voltammogram corresponds to abiotic conditions carbon rod. Red voltammogram corresponds to stationary phase. Voltammetries were performed with constant bubbling (N_2:CO_2).

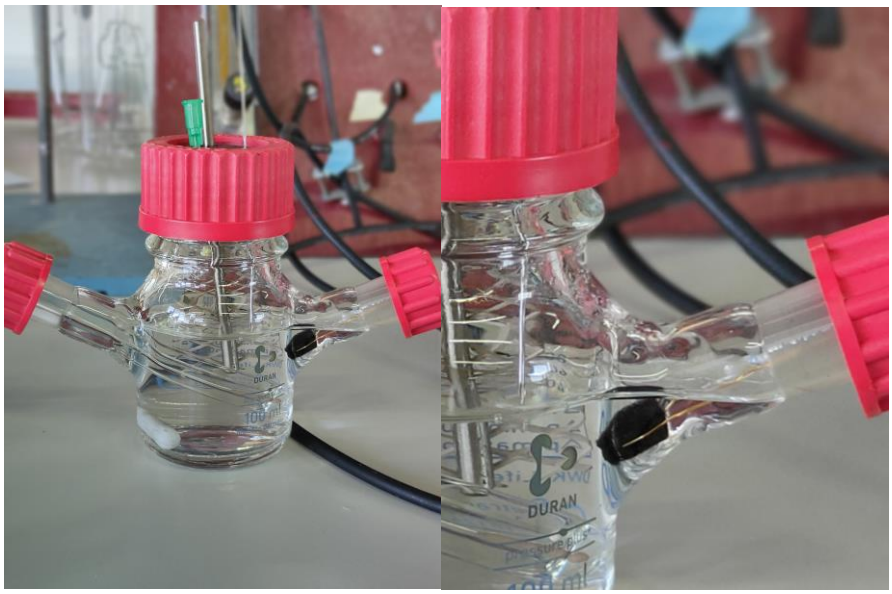
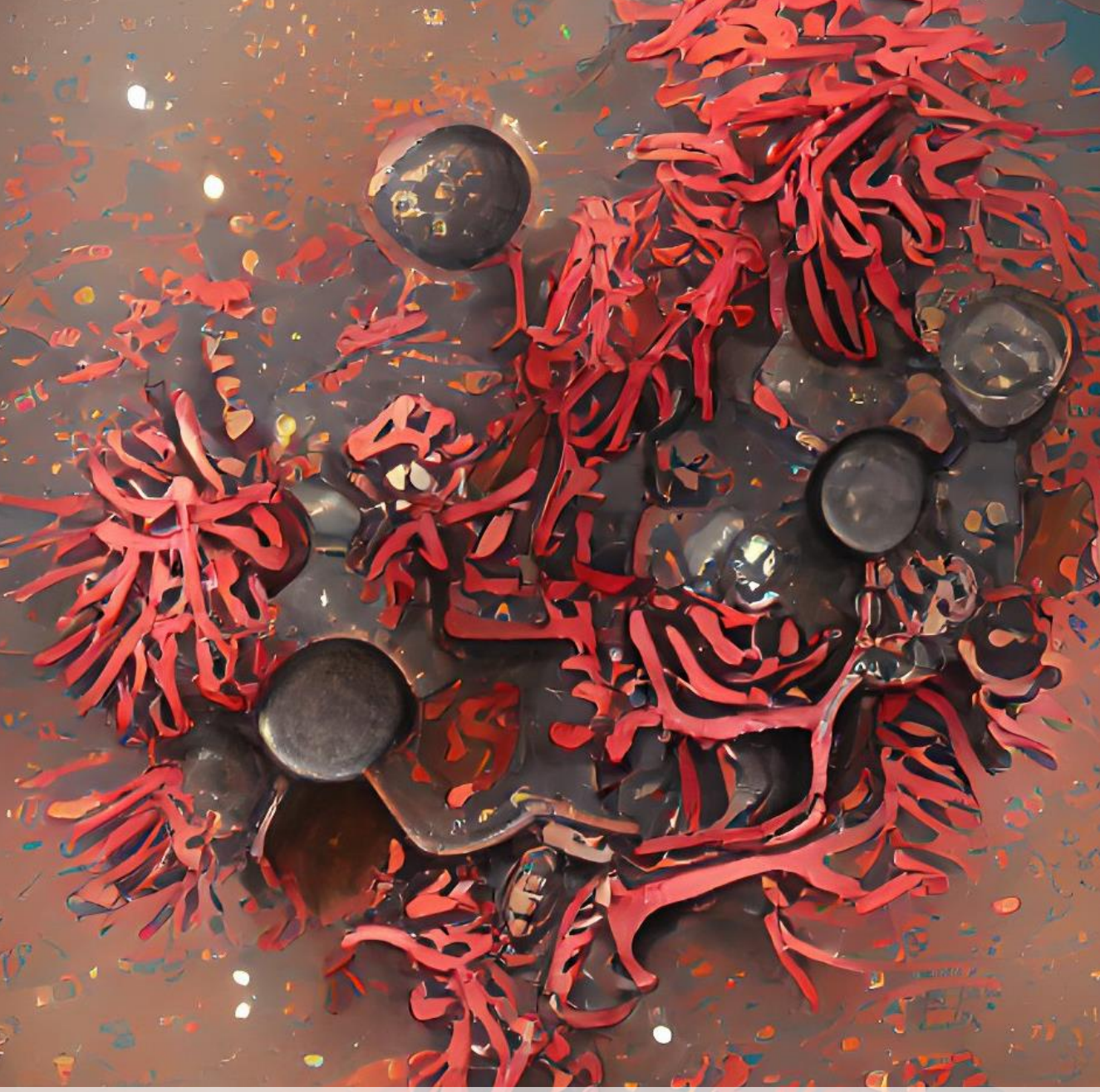


Figure SI5. Single chambered used for cyclic voltammetry analysis.

Table SI1. Volatile fatty acid concentration. The Fe samples correspond to the experiment with Fe^{2+} as electron donor. All 5 reactors showed the same concentrations (below detection limit). The H-cell samples correspond to the experiment with cathode as electron donor.

Reactor	Day	Malate	Pyruvate	Succinate	Acetate	Propionate	Butyrate
Fe	0	0	0	0	0	0	0
Fe	3	0	0	0	0	0	0
Fe	7	0	0	0	0	0	0
Fe	9	0	0	0	0	0	0
H-Cell	0	0	0	0	0	0	0
H-Cell	1	0	0	0	0	0	0
H-Cell	2	0	0	0	0	0	0
H-Cell	3	0	0	0	0	0	0
H-Cell	4	0	0	0	0	0	0
H-Cell	6	0	0	0	0	0	0



Chapter 3:

Fluid-like electrodes overcome the biofilm-based paradigm for growing electroactive planktonic Purple Phototrophic Bacteria

This chapter is based on the scientific publication:

Fluid-like electrodes and Purple Phototrophic Bacteria: bridging the gap in wastewater biorefineries

Coauthors: Fernando Muniesa-Merino, María Llorente, Yeray Asensio, Álvaro Pun and Abraham Esteve-Núñez

Journal: Chemical Engineering Journal (Q1)

Impact factor: 16.7

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Chapter 3: Fluid-like anodes to domesticate PPB

CHAPTER 3:

Fluid-like electrodes overcome the biofilm-based paradigm for growing electroactive planktonic Purple Phototrophic Bacteria

Abstract

Purple phototrophic bacteria (PPB), the most versatile microorganisms on earth, are capable of exchanging electrons with extracellular electron donors and acceptors, such as iron and manganese oxides. Furthermore, PPB are known to form biofilms while respiring upon electroconductive materials like graphite electrodes which constitutes a metabolic limitation due to mass transfer. In contrast with classical biofilm-based techniques in previous electromicrobiology studies, we have demonstrated that a fluid-like electrode can enhance the growth rate by ca. 3-fold over planktonic PPB. Moreover, controlling the electrochemical nature of the extracellular electron acceptor allows for the fine-tuning of the metabolism of a planktonic PPB-dominated microbial community to enhance their biodegradation rate and cell yield while growing on brewery wastewater. For this purpose, a twin set of microbial electrochemical fluidized bed reactors (ME-FBR) were operated in identical conditions, except for illumination conditions (dark *vs*. infrared illumination), to promote the development of purple phototrophic bacteria. Using these reactors operated both without external polarization (open circuit) and at anodic potentials (0.2 and 0.4 V *vs*. Ag/AgCl), the microbial communities, the bioelectrochemical response and the efficiency for removing pollutants were characterized. Illumina sequencing revealed that both infrared radiation and polarization led to changes in the microbial population while producing an electrical current of 7 $A \cdot m^{-3}$. Indeed, electroactive PPB genera like the *Rhodopseudomonas* and *Rhodobacter* outcompeted others under IR illumination and electrostimulation by a polarized fluid-like electrode. Furthermore, the *Proteiniphilum* genus seemed to establish symbiosis with electroactive PPB when fluid-like electrodes are IR illuminated. In this work, we have demonstrated how microbial selection can contribute to the sustainability of an electrobioremediation treatment by avoiding emissions of greenhouse gases such as methane while enhancing, by 2-fold, the removal rate of organic pollutants. In addition, fluid-like bed bioreactors have shown their usefulness in recovering nutrients as PPB biomass, favouring planktonic growth and thus facilitating the recovery of a valuable product: the biomass of purple phototrophic bacteria.

1.- Introduction

For more than a century, activated sludge has treated wastewater. This process has improved worldwide quality of life and health while reducing the environmental impact of eutrophication (Verstraete et al., 2009). The increasing demands of the climate crisis require rethinking wastewater treatment. It is no longer enough only to remove pollutants; to address the coming crisis, places like wastewater treatment plants (WWTPs) also need to recover nutrients and energy into viable products or resources for reuse (Verstraete et al., 2009). To achieve this goal, the underlying concepts of the WWTP need to be reimagined into a wastewater biorefinery concept.

Purple Phototrophic Bacteria (PPB) are considered one of the most metabolically versatile microorganisms on earth (Madigan and Jung, 2009). These microorganisms have been found in very different ecosystems, including those with extreme pH and temperature, or environments polluted by recalcitrant compounds. Its ubiquity is due to its metabolic versatility, using infrared light (IR) and a wide variety of organic compounds as its main energy and carbon source(Madigan and Jung, 2009). These versatile characteristics allow them to grow photoheterotrophically under anaerobic conditions while using light as their energy source. Thus, PPB can simultaneously assimilate carbon and nutrients at high efficiency (Capson-Tojo et al., 2020), facilitating the maximum recovery of these resources as bioplastics (Ranaivoarisoa et al., 2019) , biohydrogen (Vasiliadou et al., 2018) and cellular biomass. The high protein content of PPB biomass makes it a promising product as single cell protein, which has been tested as a feed additive and bulk ingredient in aquaculture (Delamare-Deboutteville et al., 2019; Alloul et al., 2021). Despite its promising potential in recovering nutrients from wastewater, mixed culture products of PPB are not yet commercialized (Hülsen et al., 2022).

PPB are capable of exchanging electrons with extracellular electron donors and acceptors, such as iron and manganese oxides (Ehrenreich and Widdel, 1994). This capacity allows PPB to have a redox interaction with electroconductive materials, like electrodes (Bose et al., 2014a; Vasiliadou et al., 2018; Guzman et al., 2019; Xiao et al., 2022), making them suitable for performing microbial electrochemistry. In processes such as wastewater treatment, where diverse microbial populations meet complex mixtures of organic compounds, electrodes become a control and stabilization element. They can help to overcome metabolic limitations or metabolic imbalances through a process called Electro-Fermentation (EF)(Gong et al., 2020a).

In conventional microbial electrochemistry, solid electrodes (eg. rods, plates, and felts) are typically used as electroconductive materials (Mustakeem, 2015) to support biofilm growth. Under such conditions diffusion and migration processes become a limiting factor for achieving optimal biodegradation rates. In contrast with such static electrodes, an innovative electrochemical configuration called the microbial electrochemical fluidized bed reactor (ME-FBR) uses a fluid-like electrode to minimize mass transfer and energy limitations while simultaneously enhancing the activity of both electroactive planktonic and electroactive

biofilms in the bioreactor (Tejedor-Sanz et al., 2018). Indeed, a fluid-like anode has been shown to be efficient for removing organic pollutants and nitrogen from industrial brewery wastewater (Tejedor-Sanz et al., 2018; Asensio, Llorente, Fernández, et al., 2021) Furthermore, a fluid-like electrode serves as the sole electron donor for promoting microbial denitrification in an organic carbon-depleted medium (Tejedor-Sanz et al., 2020). Additional studies using alternative mobile electroconductive beds have confirmed how bacteria can charge such material with electrons from their metabolism (Deeke et al., 2015; Borsje et al., 2019, 2021).

However, the biotechnological potential of PPB in wastewater treatment and nutrient recovery is limited using current electrochemical tools constrained by the requirement of biofilm growth. Thus, in this work, we have overcome such a bottleneck by demonstrating how a fluid-like electrode stimulates PPB growth under planktonic conditions while enhancing assimilative metabolism and cell yield to electrobioremediate brewery wastewater to generate a valuable product: nutrient-rich PPB biomass. Therefore, our approach combines an efficient treatment of brewery wastewater with the generation of a value-added product, thus bridging the gap in the transition from classical wastewater treatment model to a sustainable biorefinery model.

2.- Material and methods

Experimental Set-up and operating conditions

Two ME-FBR units were built following the design described in Tejedor-Sanz et al, (2018). The reactors were made of borosilicate with a total volume of 0.1 L. Using a three-electrode cell system, the reactors featured a fluidized working electrode (WE), a reference electrode located near the working, and a counter electrode (CE) located at the top of the reactor. Vitreous carbon (20 mL, 0.6 – 1 mm diameter) (Sigradur G, HTW, Germany) was used as fluidized anode (WE) and a graphite rod (Mersen, Spain) was immersed in the bed as a current collector. Platinized titanium mesh (Inagasa, Spain) was used as a cathode (Counter electrode). A 3M KCl Ag/AgCl reference electrode (Hanna) was used as a reference electrode.

The fluidization of the bed was achieved through the recirculation of liquid by means of a peristaltic pump (Heidolph 5006, Germany) at a flow rate of 200 mL·min^{-1} (0.11 cm·s^{-1} linear velocity). A flow distributor was in the lower section of the reactor to ensure optimal flow sharing and fluidization.

One of the reactors was operated under infrared illumination (IR) conditions and the other one was operated under dark conditions as a control (**Fig. 1**). We used the same operating conditions for both reactors (influent (brewery wastewater), inoculum, and polarization potential). The reactors were inoculated (1:10 (v/v)) using sludge from anaerobic digester IWWTP. The reactors were operated with an open circuit potential as the acclimatization stage until steady-state conditions (in terms of COD removal and OD) was reached. The reactors were then operated under two polarization values until current densities were stable

and steady state conditions were achieved. The bed was polarized first at 0.2 V, then at 0.4 V and finally it was operated under open circuit polarization (OCP). The OCP results shown in this work correspond to the experimental period after polarization.

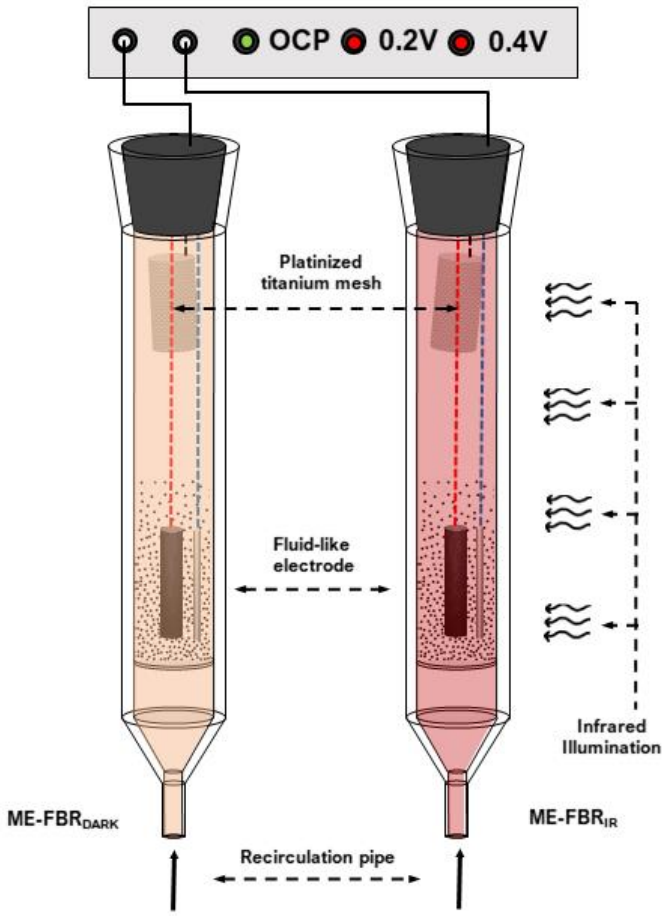


Figure 1. ME-FBR scheme. Left: ME-FBR$_{DARK}$. Right: ME-FBR$_{IR}$. Wavy arrows indicate infrared radiation. The medium was recirculated from the upper part of the reactors to the lower part to fluidize the bed.

We also evaluated the effect of only the electroconductive material on the performance of the ME-FBR$_{IR}$ reactor and biocompatibility regarding PPB growth yield. Thus, two reactors were operated under infrared lighting conditions with synthetic wastewater with acetate (40 mM) as sole electron donor and carbon source (Tejedor-Sanz et al., 2017). The second control reactor was used for growing PPB consortium in the absence of any fluidized material (Fig. 1 SI).

Chemical analyses

Organic contaminants were analyzed using Total Organic Carbon (TOC) and Chemical Oxygen Demand (COD) after filtering (0.22 micrometer). TOC was measured by a TOC-VCSH Shimadzu analyzer. COD was quantified using a commercial kit (Merck Millipore, Germany) as previously described (Tejedor-Sanz et al., 2016; Asensio, Llorente, Fernández, et al., 2021). Acetate was analyzed by HPLC (HP series 1100, UV detector 210 nm and Supelco C-610H column). The theoretical COD for synthetic wastewater was calculated from acetate HPLC determination as previously described (Prado et al., 2019). Ammonium, nitrate, and nitrite were analyzed by ionic chromatography (Metrohm 930 Compact Ion Chromatograph Flex), for which they were filtered at 0.45 µm and later at 0.22 µm with a tangential filter. Methane headspace concentration was measured by gas chromatography (Varian 3350 chromatograph with a packed column, Porapack N 80/100) with nitrogen as the carrier gas (20 $mL \cdot min^{-1}$) and a thermal conductivity detector.

Microbiological analyses

PPB growth was analyzed by measuring the absorption peaks of bacteriochlorophylls and carotenoids (Vasiliadou et al., 2018) in the wavelength range of 1100 to 400 nm (Shimadzu UV-1800 Spectrophotometer). The growth curve data corresponds to the absorbance at 590 nm to minimize measurement error (Mehrabi et al., 2001).

16S Microbial community analysis was performed following the Illumina protocols and Miseq equipment. Illumina Basespace (16S) software was used for data analysis (Autonomous University of Barcelona, Spain).

Electrochemical measurements and characterization

The fluidized reactors were polarized using a potentiostat (NEV-4 v.2, Nanoelectra S.L., Madrid, Spain). Electric current and electric potential were measured every second. Cyclic voltammetry (CV) was carried out in fluidized bed reactors at the end of the experiment with a scan rate of 10 $mV \cdot s^{-1}$ between 0.8 V and -0.8 V (*vs*. Ag/AgCl).

Moreover, to study the electrochemical response of our microbial consortium, we grew a PPB biofilm on a carbon rod using a conventional electrochemical cell in a three-electrode configuration. The electrochemical system was completed by a platinized titanium as a counter electrode, and a leakless reference electrode. The system was polarized at 0.2 V (*vs*. Ag/AgCl) and inoculated with ME-FBR_{IR} planktonic bacteria to promote biofilm formation (3 weeks). The microbial electrochemical cell was fed daily with fresh culture medium with acetate as a carbon source.

3.- Results

Inspired by the potential of purple phototrophic bacteria (PPB) in key environmental biotechnologies we have explored their capacity for interchanging electrons with electroconductive materials. For the first time, we have combined infrared radiation with the operation of microbial electrochemical fluidized bed reactor (ME-FBR$_{IR}$) for growing electroactive planktonic PPB in real brewery wastewater.

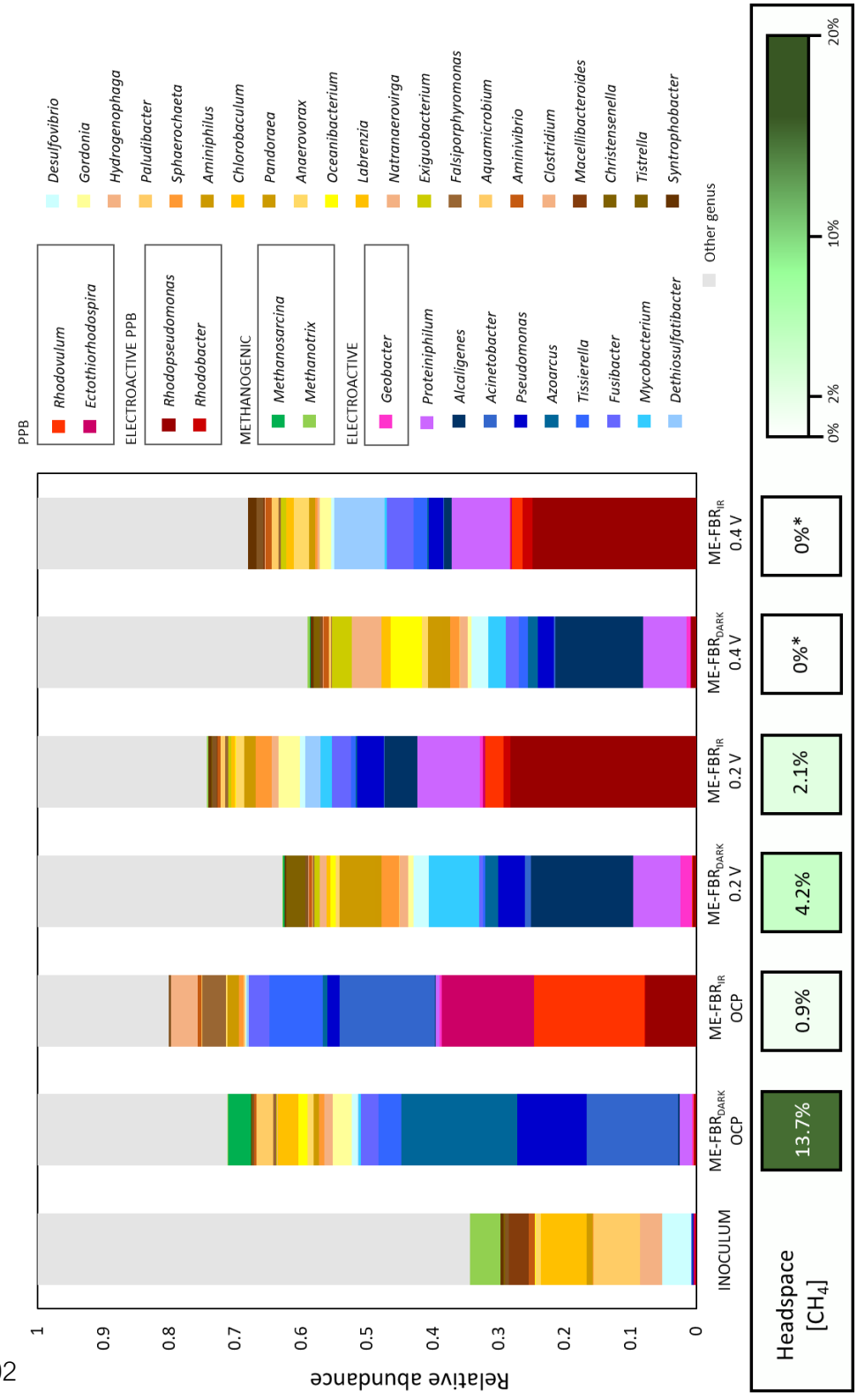
Fluid-like electrode and IR illumination drives changes in microbial population

Environmental conditions determine the structure and activity of a microbial community and the presence of a polarized electrode acting as electron acceptor is no exception. Thus, electrode polarization typically triggers two knock-on effects in microbial processes. First, electrodes select for specific microbial phenotypes leading to a shift in the microbial population composition. Second, polarized electrodes allow for unique metabolic activities, leading to changes in gene expression from individual community members (Moscoviz et al., 2018a; Gong et al., 2020b). This microbial population shift takes place in microbial electrochemical systems (Moscoviz et al., 2018b; Toledo-Alarcón et al., 2021) because microorganisms capable of taking advantage of the extra electrons donors or acceptors will eventually outcompete the others. Therefore, biodiversity analysis using Illumina 16S sequencing was the first stage to explore the structure of our microbial community.

We analysed the microbial community from brewery wastewater sludge used as the inoculum for our reactors. This sludge was characterized by a high content of organic compounds (mainly volatile fatty acids (VFAs)) and nitrogenous compounds but was limited in electron acceptors such as nitrate or sulphate. The analysis showed genera mostly of anaerobic and aerobic fermenters (Fig. 2). Moreover, some methanogenic activity was expected due to the presence of *Methanotrix*, a genus of methanogenic archaea (Noike et al., 1985).

The evolution of this inoculation community was explored while biodegrading real brewery wastewater using twin ME-FBR operated under either dark or IR illumination in the presence of fluid-like polarized anodes (at 0.2 V and 0.4 V *vs*. Ag/AgCl) and non-polarized as an open circuit (OCP). Such six independent operating conditions were grouped into five clusters of variance based on Principal Coordinates Analysis (PCoA) (Fig. 3 SI): 1) wastewater, 2) OCP$_{DARK}$, 3) OCP$_{IR}$, 4) electrode polarization under dark conditions and 5) electrode polarization under IR conditions. These results revealed that both polarization and infrared radiation affected the population structure. In addition, the clear separation of the polarized samples (clusters 4 and 5) indicated that, under illumination conditions, the polarization exerted different selective pressures.

Figure 2. Relative abundance at the genus level. The legend boxes highlight the key genera that play a role in ME-FBRs. The heatmap shows the methane concentration in the reactor headspace for each of the conditions.


PPB
Rhodovulum
Ectothiorhodospira
ELECTROACTIVE PPB
Rhodopseudomonas
Rhodobacter
METHANOGENIC
Methanosarcina
Methanotrix
ELECTROACTIVE
Geobacter
Desulfovibrio
Gordonia
Hydrogenophaga
Paludibacter
Sphaerochaeta
Aminiphilus
Chlorobaculum
Pandoraea
Anaerovorax
Oceanibacterium
Labrenzia
Natranaerovirga
Exiguobacterium
Proteiniphilum
Falsiporphyromonas
Alcaligenes
Aquamicrobium
Acinetobacter
Aminivibrio
Pseudomonas
Clostridium
Azoarcus
Macellibacteroides
Tissierella
Christensenella
Fusibacter
Tistrella
Mycobacterium
Syntrophobacter
Dethiosulfatibacter
Other genus
Relative abundance
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
INOCULUM
ME-FBR$_{DARK}$ OCP
ME-FBR$_{IR}$ OCP
ME-FBR$_{DARK}$ 0.2 V
ME-FBR$_{IR}$ 0.2 V
ME-FBR$_{DARK}$ 0.4 V
ME-FBR$_{IR}$ 0.4 V
Headspace [CH$_4$]
13.7%
0.9%
4.2%
2.1%
0%*
0%*
0%
2%
10%
20%

We observed several differences at all taxonomic levels, but specially at the genus level. We focused on those key genera previously described by other authors regarding their role: classical genera described as electroactive bacteria (Koch and Harnisch, 2016), purple phototrophic bacteria (Madigan and Jung, 2009) and methanogenic bacteria (Garcia, 1990; Madigan and Jung , 2009).

The mere presence of a fluid-like electrode allowed electroactive bacteria (mainly from the *Geobacter* genus) to outcompete other species, even under open circuit conditions where electrical current cannot flow (**Fig. 2**). This process of Conductive-mediated Interspecies Electron Transfer has been recently named as CIET (Rotaru et al., 2021). It is the first time this phenomenon has been described using a fluid-like electrode, although it was previously observed in a fixed electroconductive beds (A. Aguirre-Sierra et al., 2016). Furthermore, in the OCP_{DARK} reactor we also observed the prominent presence of methanogenic bacteria from the *Methanosarcina* genus. The direct extracellular electron transfer between species of the *Methanosarcina* and *Geobacter* genus has been widely demonstrated (Rotaru et al., 2014, 2018). We hypothesize that fluid-like electrode can mediated such extracellular electron transfer, favouring the syntrophic relationship, and therefore promoting the presence of both genera. This was consistent with the high methane concentration detected in the headspace (13.7%), the highest level of all the conditions tested in our study (**Fig. 2**). In contrast, the non-polarized illuminated reactor (OCP_{IR}), had more purple phototrophic bacteria (PPB), specifically from *Rhodopseudomonas*, *Rhodovulum*, and *Ectothiorhodospira* genera. Interestingly, we found a significant lower abundance of those classical electroactive bacteria previously observed in OCP_{DARK}. This result suggested that infrared radiation, as an extra source of energy, promoted the presence of PPB over classical electroactive and methanogenic bacteria.

To polarize or not to polarize the fluid-like electrode

Next, the $ME\text{-}FBR_{DARK}$ was operated under polarized conditions (0.2 V and 0.4 V), revealing a higher abundance of bacteria from *Geobacter* genus compared to OCP_{DARK} (**Fig. 2**). These results confirm previous studies where a fluid-like electrode, acting as a terminal electron acceptor (TEA), promotes the development of electroactive bacteria. This was shown using FISH techniques by the presence of electroactive bacteria at the inner layers of the biofilm, probably acting as a connector between other bacteria genera and the electrode (Tejedor-Sanz et al., 2018). In addition, we observed a significantly lower abundance of methanogenic species when the bed was polarized in comparison to OCP_{DARK}. These results and the low methane concentrations detected in the headspace (**Fig. 2**) suggested that the electrode is a true TEA competing for electrons and minimizing or fully avoiding methanogenesis. We hypothesize that, under dark conditions, *Geobacter* acts as a connector in the electrically conductive fluidized bed, transferring electrons either to i) the electrode when the fluid-like anode is polarized and ii) to the methanogenic partners when the fluid-like anode is non-polarized (OCP).

In the case of the polarized and illuminated bioreactor ME-FBR$_{IR}$, we also observed a high relative abundance of PPB (Fig. 2). In contrast to the OCP$_{IR}$, the majority genera present in the polarized ME-FBR$_{IR}$ were *Rhodopseudomonas* and *Rhodobacter*. These two types of PPB have been reported to be electroactive (Xing et al., 2008; Bose et al., 2014a; Hasan et al., 2015), so their ability to interact with the electrode could justify why polarization selects for them.

We must emphasize the importance of the genus *Proteiniphilum* in polarized reactors, under both dark and light conditions. This genus has been actively selected for in other bioanodes systems fed with complex organic compounds, such as wine industry wastewater (Kiely et al., 2011; Nosek et al., 2020). It is considered a partner of electroactive bacteria, metabolizing complex molecules to oxidizable metabolites for them (Logan et al., 2019). Our results suggest that in polarized ME-FBR$_{DARK}$, *Proteiniphilum* species could establish symbiosis with *Geobacter*, while in ME-FBR$_{IR}$, it would act as a partner of electroactive PPB such as *Rhodopseudomonas* and *Rhodobacter*.

Fluid-like electrode enables photo-electrofermentation

The interaction between the microorganisms and the fluid-like electrode can be monitored through current density, so the actual role of the electrode in the microbial process can be electrochemically explored. Regardless the absence or presence of illumination, electric current production was always detected during the operation of both reactors. The volumetric current density of ME-FBR$_{DARK}$ (max value of 40 A·m^{-3}) was higher than the one measured in the ME-FBR$_{IR}$ (max value of 7 A·m^{-3}) (Fig.3).

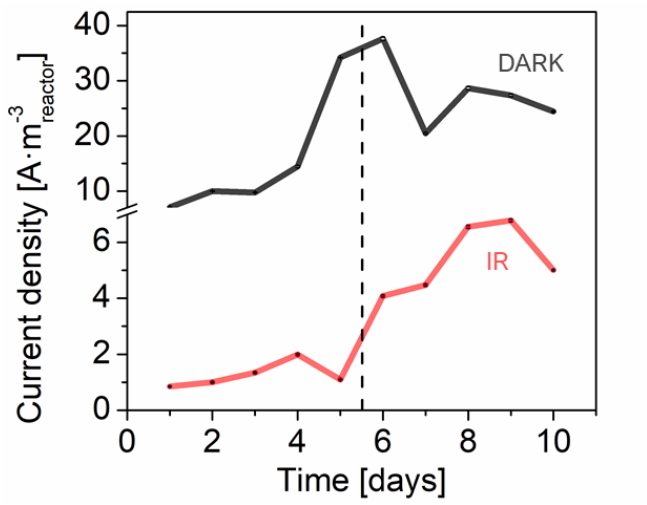


Figure 3. Current density in the fluidized anode polarized to 0.2 V (0-5 days) and 0.4 V (6 to 10 days).

As typically occurs in Microbial electrochemical systems just a percentage of electrons from microbial oxidation of organic pollutants were harvested as electrical current. We observed coulombic efficiencies (CE) of about 22% at 0.2 V and 41.0% at 0.4 V for the ME-FBR$_{DARK}$, confirming that fluid-like anode acts as terminal electron acceptor for bioremediating pollutants. These coulombic efficiency values suggested that the electrode was not acting as the sole electron acceptor in the oxidation processes. The detection of methane confirms the role of alternative electron sinks (Fig. 2). These CE values were similar or even slightly higher than those previously reported using ME-FBR$_{DARK}$ (Tejedor-Sanz et al., 2018).

The illuminated reactor (ME-FBR$_{IR}$) had considerably lower CE values (1.1% at 0.2 V and 4.1% at 0.4 V) in comparison with the ME-FBR$_{DARK}$. The metabolic versatility of PPB, predominant in illuminated reactor, allows them to use alternative metabolic pathways such as nitrogen and carbon fixation or hydrogen production as electron sink (McKinlay and Harwood, 2010). Such electron-sink pathways make PPB less dependent on an extracellular electron acceptor like an electrode, which is consistent with the low CE values.

Two-way extracellular electron transfer in PPB consortium

To understand the role of the electrode on PPB metabolism and how it modulates the activity of such microorganisms, we explored bacteria-electrode interaction by cyclic voltammetry analysis. We carried out the electrochemical analysis in two different situations: i) electron transfer from planktonic PPB to a fluid-like electrode and ii) electron transfer from a PPB-based biofilm to a rod-like electrode. Performing CV directly on the ME-FBR allows for the *in situ* study of the planktonic bacteria-electrode interaction. On the other hand, a biofilm-based system is a more stable scenario for accurately investigating the electrode's role in the regulation of cellular metabolism and for establishing a comparison with previous electrochemical studies using PPB (Guardia et al., 2020).

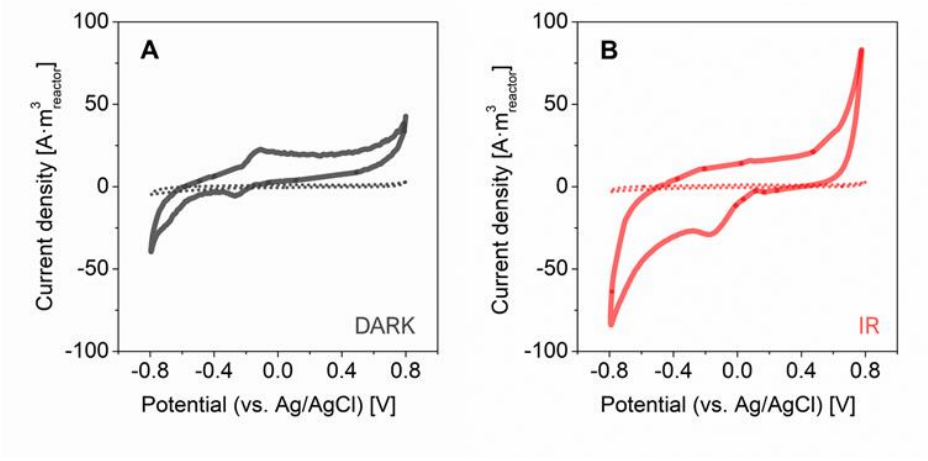


Figure 4. Steady-state voltammograms of ME-FBR at the end of the operation using brewery wastewater: ME-FBR$_{DARK}$ (A) and ME-FBR$_{IR}$ (B). The discontinuous voltammogram corresponds to the abiotic conditions.

The results obtained in ME-FBR showed a different light-dependent electrochemical behaviour (Fig.4). In the ME-FBR$_{DARK}$, the appearance of a redox couple was observed in comparison with the abiotic voltammogram. These redox peaks revealed an electrochemical interaction between the microbial consortium and the fluid electrode. The midpoint potential of the redox pair (-0.19 V *vs*. Ag/AgCl) was similar to those obtained with pure cultures of species of the genus *Geobacter* (Richter et al., 2009). Furthermore, we observed a sigmoidal shape that suggests catalytic biological activity.

The voltammograms corresponding to ME-FBR$_{IR}$ showed a non-reversible reduction peak at -0.16V (*vs*. Ag/AgCl) and, in contrast to the ME-FBR$_{DARK}$ results, we did not observe any oxidation activity. These results were consistent with the low coulombic efficiencies observed and could indicate that in the illuminated reactor, the fluid-like electrode is not acting as a sole electron acceptor. It is worth highlighting that the reduction activity observed in the voltammogram could be related to reduction processes catalysed by PPB reported elsewhere (Bose et al., 2014a, 2014b).

The fluidized bed allows the electrode polarization to be more homogeneous in comparison to fixed bed systems (Tejedor-Sanz et al., 2018; Asensio, Llorente, Sánchez-Gómez, et al., 2021). However, the internal resistance of the bed is still too high to precisely control the potential for performing accurate CV (Rodrigo Quejigo et al., 2018). Therefore, using PPB community grown in the ME-FBR$_{IR}$ as inoculum, we grew a PPB biofilm on a standard rod graphite electrode to give insights into the possible Extracellular Electron Transfer (EET) mechanism, and on metabolic processes.

Three redox pairs (RP1, RP2 and RP3) were clearly identified from cyclic voltammetry in the absence of acetate, under nonturnover (NTO) conditions. These redox-active compounds are possible EET sites, which could connect the electrode with bacterial metabolism to control different bioelectrochemical processes (Fig. 5).

Classically, in microbial electrochemistry, the oxidation of organic compounds has been considered as the main anodic process (Logan, 2008). However, other anodic processes like electrode-dependent anaerobic ammonium oxidation (Shaw et al., 2020) has been recently described. To identify those real EET sites capable of regulating bioelectrochemical anodic processes we proceeded to investigate the metabolism of different substrates: acetate and ammonium. The CVs under turnover (TO) and nonturnover conditions revealed that the Mp^{1} was the EET site related to the oxidation of acetate (Fig. 5B). The electroactivity of the PPB-based biofilm was confirmed by the oxidative catalysis observed in the TO voltammogram, together with an increase in electrical current when medium was spiked with acetate (Fig. 4 SI).

Furthermore, we explored our biofilm-based system under autotrophic conditions regarding ammonium oxidation, and no appreciable shift in current density was detected when medium was spiked with ammonium (Fig. 5 SI).

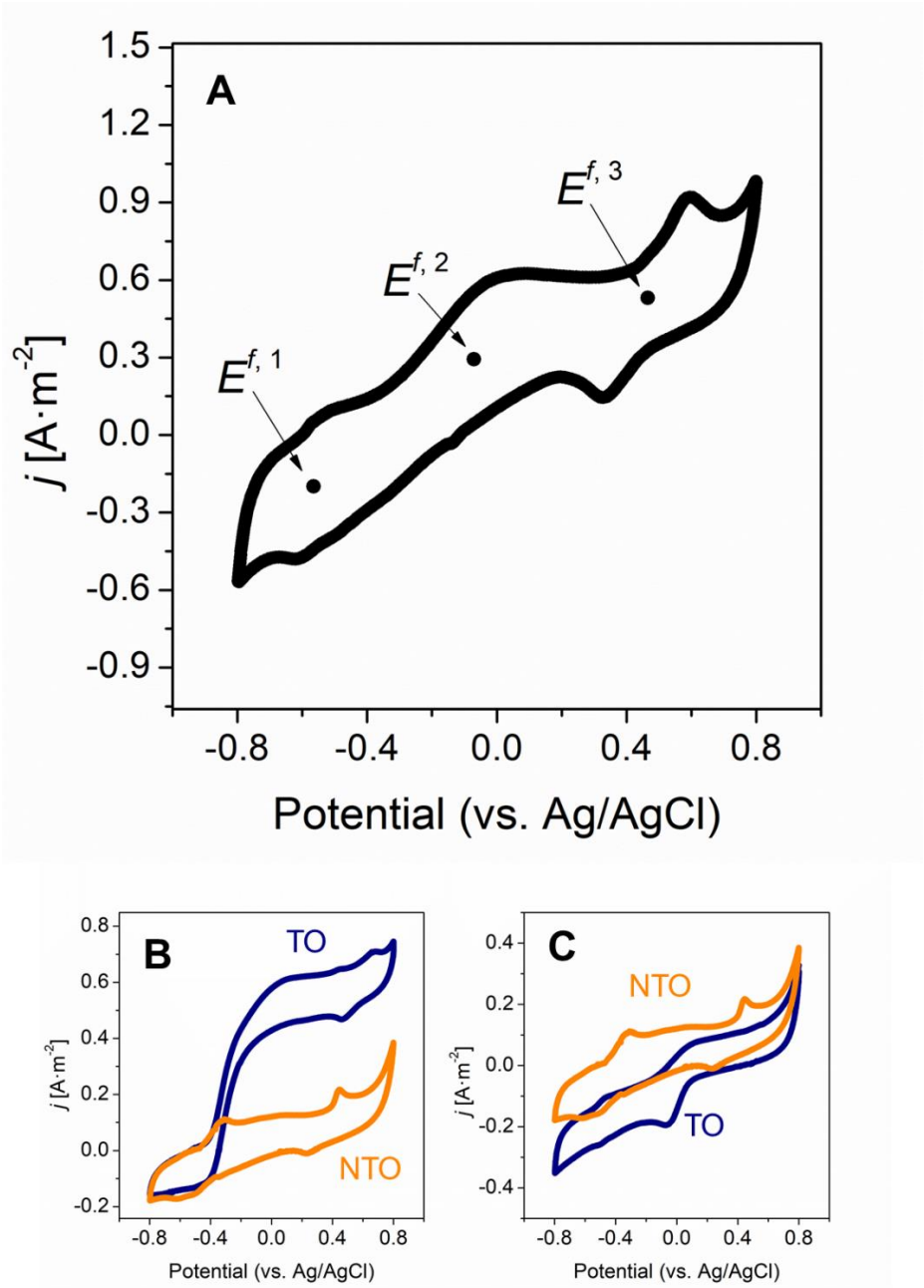


Figure 5. Steady-state voltammograms of the PPB biofilm-based system using mineral medium. A: Voltammogram under non turnover conditions at a scan rate of 10 mV · s^{-1}. Mp^1, Mp^2 and Mp^3 indicate the midpoint potentials of the redox peaks found. B; Voltammogram under non turnover (orange line) and turnover (blue line) with acetate as substrate (Scan rate: 1 mV · s^{-1}). C: Voltammogram under non turnover (blue line) and turnover (orange line) with nitrate as substrate (Scan rate: 1 mV · s^{-1}).

The ability of PPB to uptake electrons from standard graphite electrode has been widely described (Bose et al., 2014a, 2014b). Therefore, to study the cathodic processes that our consortium may catalyse, we used nitrate as a model reducible compound. In the presence of NO_3^-, the voltammogram showed an increase in the cathodic activity at potentials below 0 V. These voltammograms were like those previously reported (Vasiliadou et al., 2018). These results suggested that the PPB consortium might be able to uptake electrons to reduce nitrate in which the Mp^2 site would participate. The similarity of this voltammogram (Fig.5C - Blue line) with the one obtained in the ME-FBR_{IR} (Fig. 4B) suggests that the reduction peak observed could be related to some reduction reaction catalysed by the consortium. Therefore, a fluid-like electrode might also serve as electron donor for PPB allowing relevant applications such as nitrate reduction or bioelectrosynthesis.

Synergic effect between PPB and fluid-like electrodes

We operated both reactors, (Fig. 1) fed with brewery wastewater, to obtain steady state in regards of Total Organic Carbon (TOC) and Total Nitrogen (TN) removal rates.

Under OCP, no significant differences were observed in the TOC removal rate between both reactors. Therefore, the results indicated that just the presence of PPB did not result in a measurable enhancement in biodegradation in a ME-FBR without polarization.

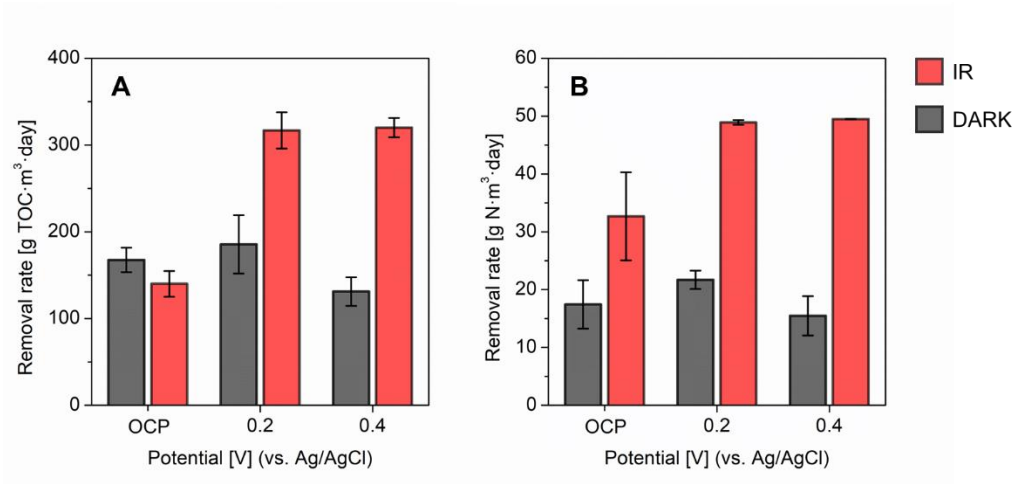


Figure 6. A: Total Organic Carbon (TOC) removal rate ± standard error under Open Circuit Potencial (OCP), 0.2 V and 0.4 V. B: Total Nitrogen (TN) removal rate ± standard error under Open Circuit Potencial (OCP), 0.2 V and 0.4 V. HRT=48 hours.

Other authors have shown that the mere presence of the electrically conductive material accelerates the microbial metabolic processes without the need for external polarization (Aguirre-Sierra et al., 2016; Prado et al., 2019, 2020). This was confirmed by the results obtained in the ME-FBR_{DARK}, in which we observed that there were no significant differences regarding TOC removal in absence of polarization (OCP_{DARK}: 167.5 g TOC $\cdot m^3 \cdot day^{-1}$) and

under different values of polarization (185.5 g ·m^3·day^{-1} at 0.2 V and 131.1 g TOC ·m^3·day^{-1} at 0.4 V) (Fig. 6A). Methane production and microbial community analysis (Fig. 2) suggest that, despite presenting similar biodegradation rates, the microbiological processes in both systems (OCP_{IR} and OCP_{DARK}) are diametrically opposed.

In contrast with a conventional ME-FBR, infrared illumination led ME-FBR_{IR} to a 2-fold increase in TOC removal rate under external polarization conditions at 0.2 V and 0.4 V (316.9 g ·m^3·day^{-1} and 320.1 g·m^3·day^{-1} respectively) compared with non-polarized conditions (OCP_{IR}: 140.0 g ·m^3·day^{-1}). The presence of electrically conductive material under OCP did not stimulate PPB in comparison with the electrode-free control (Fig. 2 SI), and electric current through an external circuit seem to be a strong requirement to enhance the metabolism of the PPB. Furthermore, throughout the experiment, we did not detect concentrations higher than 4% of methane in the ME-FBR_{IR}. This indicates that infrared radiation, by promoting the growth of PPB in the reactor, prevents the activity of methanogenic bacteria. In summary, these results indicated that both IR radiation and external polarization must coexist to achieve a significant improvement in the TOC removal.

Regarding the nitrogen removal rates, ME-FBR_{IR} also outperformed the ME-FBR_{DARK} throughout the experiment (Fig. 6B). In dark conditions, we did not observe any significant effect of external polarization on TN removal. Under OCP conditions, ME-FBR_{IR} achieved rates of 32.7 g ·m^3·day^{-1}, slightly higher than those obtained in the ME-FBR_{DARK} (17.4 g ·m^3·day^{-1}). However, when we applied external polarization to the illuminated reactor (ME-FBR_{IR}) we observed an increase of close to 50% in the removal of TN as compared to the OCP_{IR}. The main nitrogen compound in the influent was ammonium, although low concentrations of nitrate and nitrite were detected. The ability of PPB to assimilate nitrogen as biomass (Hülsen et al., 2016; Delamare-Deboutteville et al., 2019) and our negative results for electrochemically-assisted ammonium oxidation suggested that assimilation is the main nitrogen removal process in the ME-FBR_{IR}.

Fluid-like electrode can modulate PPB biomass production

The use of PPB in wastewater treatment has been successful in nutrient and energy recovery through nutrient partitioning (Hülsen et al., 2014). Biomass growth and yield are key in nutrient recovery so we have studied if electrochemistry and IR radiation can be used as tools to drive biomass production.

The impact of electrode potential on biomass production is reported to be controversial since polarization can both enhance (Korth and Harnisch, 2019) or supress (Scarabotti et al., 2021) cell growth. Thus, biomass production rates and biomass yields were examined in our ME-FBR to determine the role of electrode polarization. The results of both ME-FBR_{DARK} and ME-FBR_{IR} revealed that electrode potential does not have a significant effect on the biomass production rate (Fig. 7A). Regarding the effect of the IR illumination, we measured biomass production rates in the ME-FBR_{IR} 3-fold higher than those from ME-FBR_{DARK}. The additional results (Fig. 2 SI) showed that the biomass production of a ME-FBR_{IR} under OCP was not significantly different from an electrode-free reactor, which suggests that OCP_{IR} behaves like

a conventional PPB culture. The IR illumination promoted the growth of PPB in the ME-FBR$_{IR}$, which present assimilation rates as high as reported elsewhere (Hülsen et al., 2016) and supports why the biomass production rate was higher.

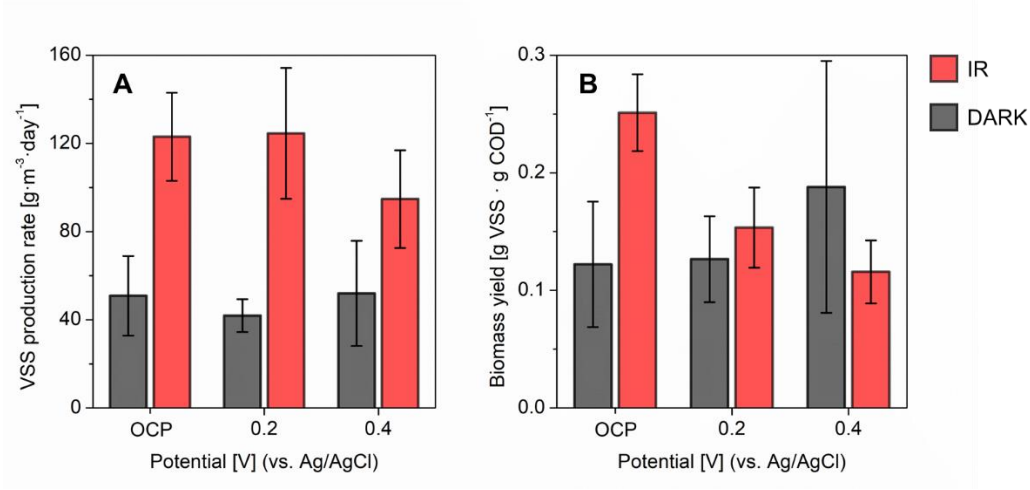


Figure 7. A: Total Organic Carbon (TOC) removal rate ± standard error under Open Circuit Potencial (OCP), 0.2 V and 0.4 V. B: Total Nitrogen (TN) removal rate ± standard error under Open Circuit Potencial (OCP), 0.2 V and 0.4 V. HRT=48 hours.

However, when we explored the conversion of organic pollutants to biomass (yield), we did observe an effect of polarization in the ME-FBR$_{IR}$. In the ME-FBR$_{DARK}$, we observed biomass yields like those described for other anaerobic sludge-based systems (Yoochatchaval et al., 2008). Biomass yield values between 0.1 and 0.2 gVSS · gCOD^{-1} were obtained, apart from the ME-FBR$_{IR}$ without external polarization (OCP$_{IR}$), in which the highest biomass yield was obtained (0.26 gVSS · gCOD^{-1}). This indicated that, despite the completely different metabolism of our electroactive microbial consortium, the biomass yield was similar to conventional anaerobic cultures. However, it is generally accepted that PPB consortia shows higher biomass yields than anaerobic sludge (Nairn et al., 2020), which is consistent with the higher value observed in the OCP$_{IR}$. Carbon fixation as an electron sink has been described as one of the main reasons to justify why values of biomass yield is higher in PPB (McKinlay and Harwood, 2010). However, the PPB consortium biomass yield was reduced (Fig. 7B) under anodic polarization. It has been described that the presence of a polarized electrode (anode) can greatly affect the intracellular redox state (NAD^+/NADH ratio)(Moscoviz et al., 2016). Small variations in this ratio could trigger large metabolic effects (Choi et al., 2014). Reduction in biomass yield may indicate that the Calvin cycle could be inhibited by the action of anodic polarization, favouring other electron utilizing pathways such as hydrogen production or nitrogen fixation which are less sensitive to the redox state of the cell (Dubbs and Tabita, 2004) (Fig. 8).

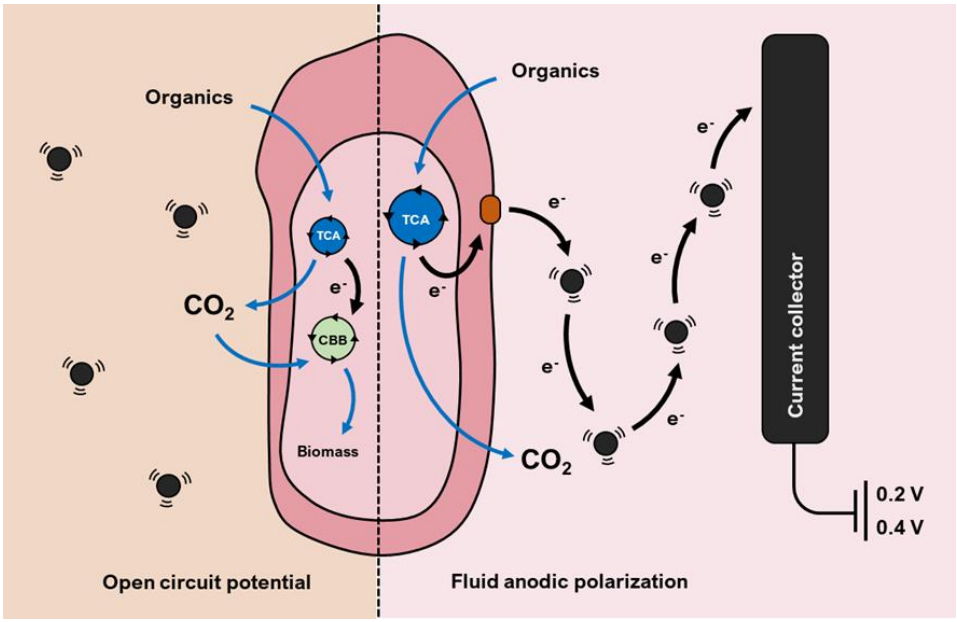


Figure 8. Conceptual model of the photoheterotrophic growth of Purple Phototrophic bacteria under open circuit conditions (left) and anodic polarization conditions in an electrochemical fluidized bed reactor.

PPB Planktonic growth predominates in infrared illuminated ME-FBR$_{IR}$

The biofilm formed on the surface of the fluidized bed particles was examined by scanning electron microscopy (SEM) after the polarization period. The low porosity of the vitreous carbon, together with the stress caused by the collision between the particles in fluidization, makes it difficult for the bed to colonize. In ME-FBR with vitreous carbon, planktonic interaction is favoured (Tejedor-Sanz et al., 2017), which was consistent with the density of the biofilm observed in the reactors (Fig. 9). We found dispersed aggregates of microorganisms and extracellular substances attached to the surface, only in the areas most protected from collisions between fluidized particles. The predominant morphology in the ME-FBR$_{DARK}$ biofilm was short rods with a low presence of exopolymeric substances forming small aggregates. On the other hand, in ME-FBR$_{IR}$, we observed more colonization areas and a vast presence of EPS, in which rod-shape morphologies predominate. It should be noted that in some areas it was possible to observe rosette-like clusters, characteristic of mature cultures of the *Rhodopseudomonas* genus (SIIR6)(Hougardy et al., 2000). Despite certain differences in the colonization of the material between the reactors, most of the microorganisms were planktonic.

4.- Conclusions

In this study, we have demonstrated that purple phototrophic bacteria can be grown planktonically and photoheterotrophically under the control of a fluid-like electrode. This novel method is remarkable by overcoming the limitation of the growth of electroactive PPB at a large scale with the conventional strategy of reliance on biofilm formation. We demonstrated for the first time that fluid-like electrodes can effectively electrobioremediate brewery wastewater using PPB, outperforming traditional electroactive non-photoheterotrophic processes. Additionally, our results indicated that IR illumination and external polarization must be used simultaneously to significantly improve TOC and nutrient removal. Furthermore, we observed that polarization and illumination play a fundamental role in microbiological and phenotypic selection, minimizing or eliminating methanogenic activity by improving the sustainability of the wastewater treatment process. Finally, we conclude that using fluid-like electrodes may accelerate the transition from the wastewater treatment model into the biorefinery model to maximize the recovery and reuse of water, carbon, and nitrogen.

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6.- Supplementary information

Evaluation of the effect of the material on the ME-FBR$_{IR}$

To evaluate the effect of the material on the growth and activity of PPB, two ME-FBR were used, one of them without conductive material and the other with conductive material without external polarization. Both were fed with synthetic wastewater and inoculated with the PPB consortium enriched in the ME-FBR$_{IR}$.

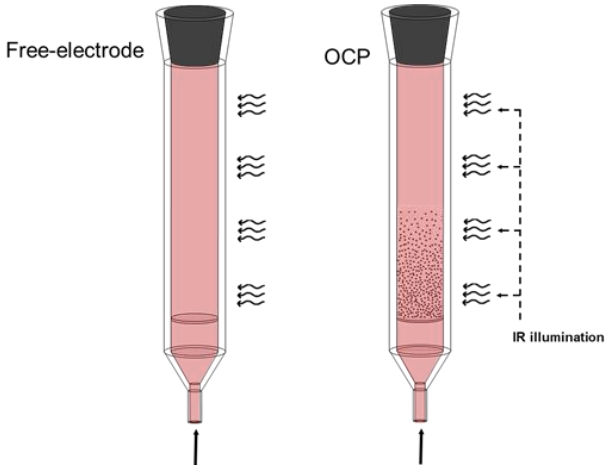

Figure SI1. Scheme of the experimental set-up to evaluate the effect of the material on the behaviour of PPB.

Once steady state was reached, samples for acetate concentration and biomass production analysis were taken. The results are shown below:

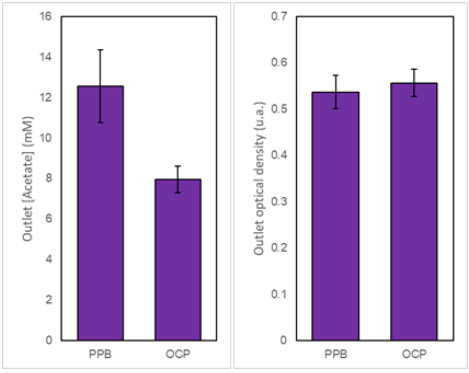

Figure SI2. Analysis corresponding to acetate concentration (left) and biomass production (right).

Metagenomics PCoA clustering

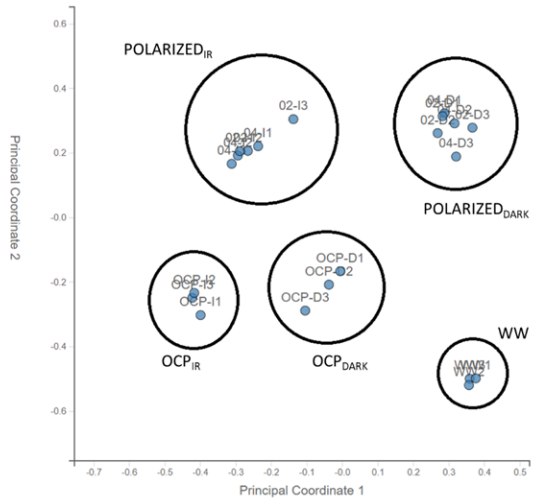

Figure SI3. Metagenomics PCoA clustering of effluent samples from fluid-like bed reactors.

Biofilm-based system to evaluate electroactivity of the PPB-dominated consortium

In the biofilm-based system described in the work, pulse experiments were performed to investigate how the presence or absence relates to the measured electrical current. First, sodium acetate (final concentration 40 mM) was added, which produced an increase in current. The entire cell medium was then replaced by free-acetate medium, causing a decrease in current to the initial levels.

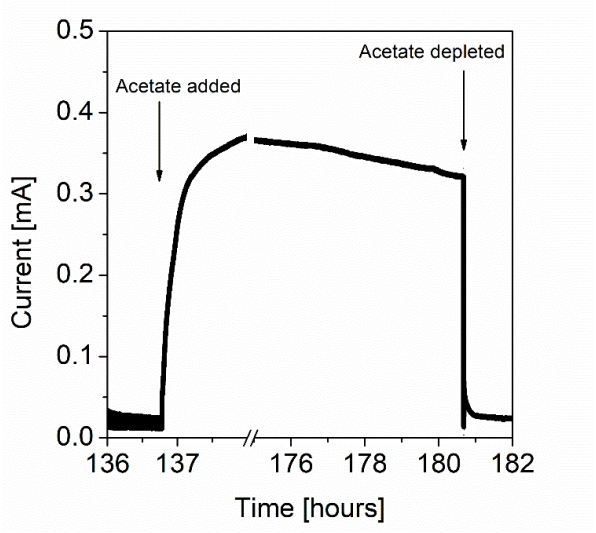

Figure SI4. Acetate pulse carried out in the cell (biofilm-based system).

Finally, to test the possible microbial oxidation of ammonium, a pulse of ammonium chloride (final concentration 60 ppm) was performed, which did not cause any observable change in the measured current.

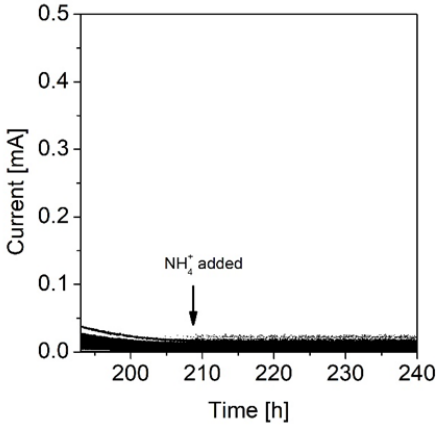

Figure SI5. Ammonium pulse carried out in the cell (biofilm-based system).

$$NRV = I\ [A] / V\ [m^3] \quad \text{(Eq. 1 SI)}$$

Table 1 SI. Main characteristics of the wastewater used in this work.

COD [ppm]	916
TOC [ppm]	407
NH_4^+ [ppm]	40.14
NO_3^- [ppm]	0.00
NO_2^- [ppm]	0.00

Chapter 4:

Fluid-like cathode enhances valuable biomass production from wastewater in purple phototrophic bacteria cultivation

This chapter is based on the scientific publication:

Fluid-like cathode enhances valuable biomass production from wastewater in purple phototrophic bacteria cultivation

Coauthors: Yeray Asensio, Fernando Muniesa-Merino, María Llorente, Álvaro Pun and Abraham Esteve-Núñez

Journal: Frontiers in Microbiology (Q1)

Impact factor: 6.064

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Chapter 4: Fluid-like cathodes to domesticate PPB

CHAPTER 4:

Fluid-like cathode enhances valuable biomass production from wastewater in purple phototrophic bacteria cultivation

Abstract

The climate crisis requires rethinking wastewater treatment to recover resources, such as nutrients and energy. In this scenario, purple phototrophic bacteria (PPB), the most versatile microorganisms on earth, are a promising alternative to transform the wastewater treatment plant concept into a biorefinery model by producing valuable protein-enriched biomass. PPB are capable of interacting with electrodes, exchanging electrons with electrically conductive materials. In this work we have explored for mobile-bed (either stirred or fluidized) cathodes to maximize biomass production. For this purpose, stirred-electrode reactors were operated with low-reduced (3.5 e^-/C) and high-reduced (5.9 e^-/C) wastewater under cathodic polarization (-0.4 V and -0.8 V *vs*. Ag/AgCl). We observed that cathodic polarization and IR irradiation can play a key role in microbial and phenotypic selection, promoting (at -0.4 V) or minimizing (at -0.8 V) the presence of PPB. Then, we further study how cathodic polarization modulates PPB biomass production providing a fluid-like electrode as part of a so-called photo microbial electrochemical fluidized-bed reactor (photoME-FBR). Our results revealed the importance of reduction status of carbon source in the PPB photoheterotrophic culture and how electrodes drive microbial population shifts depending on the reduction status of the carbon source

1.- Introduction

For decades, the strategy to deal with wastewater was aimed at the removal of contaminants. However, nutrients from urban and industrial wastewater represent a valuable source of resources (Hülsen et al., 2016; Puyol et al., 2017; Capson-Tojo et al., 2020). This is how the concept of biofactory was born, in which wastewater treatment plants are upgraded to facilities in which nutrients are recovered from wastewater, greatly improving in economic and environmental terms (Verstraete et al., 2009).

One of the most environmentally friendly and cost effective approach for nutrient recovery is their biological accumulation (Batstone et al., 2015). Specifically, purple phototrophic bacteria (PPB) have been found to be remarkably efficient in recovering nutrients compared to other phototrophic microbes such as algae and cyanobacteria (Puyol et al., 2017; Delamare-Deboutteville et al., 2019). In the framework of nutrient recovery, PPB are used photoheterotrophically where they use infrared light as main energy source and a wide range of organic compounds (Capson-Tojo et al., 2020).

Since the discovery of PPB, researchers have deeply explored the metabolic diversity that allows them to be so ubiquitous (Madigan and Jung, 2009). One of the fundamental pillars of its versatility is the ability to maintain redox homeostasis through the so-called "electron sinks" (McKinlay and Harwood, 2010). These metabolic pathways such as carbon fixation or nitrogen production consume the excess reducing power, ultimately giving rise to other processes such as synthesis of biomass (proteins) or PHA (Bayon-Vicente et al., 2021; Montiel-Corona and Buitrón, 2021).

The activation of the "electron sink" pathways depends mainly on degree of reduction for the carbon source. In short, those carbon sources more reduced than biomass (4.5 e^-/C) require the activation of "electron sink" pathways (McKinlay and Harwood, 2010; Vasiliadou et al., 2018).

Therefore, the optimal growth of PPB requires the control of electron flows by means of artificial addition of electrons to drive metabolism toward different targeted bioproducts (Varfolomeyev, 1992; Vasiliadou et al., 2018). One of the approaches is electrochemistry-based, in which electrodes are used to provide electron acceptors or donors to electroactive microorganism (Lovley and Holmes, 2022). Since the first interaction between a purple phototrophic bacterium and an electrode was reported (Bose et al., 2014), researchers have exploited its potential (Venkidusamy and Megharaj, 2016; Vasiliadou et al., 2018a; Soundararajan et al., 2019; Guardia et al., 2020). These discoveries have demonstrated the malleability of PPB metabolism under the control of an electrode, increasing the biomass production and improving nutrient removal (Guzman et al., 2019; Edreira et al., 2021; Manchon et al., 2023a).

In contrast with conventional electrode-based cultivation of electroactive bacteria (rods, felt, plates) the use of fluid-like electrodes has been demonstrated for growing electroactive planktonic cells (Tejedor-Sanz et al. 2017). Actually, fluid-like anodes can be used for

treating brewery wastewater with both non-photosynthetic bacteria (Tejedor et al., 2020; Asensio et al., 2021) and PPB (Manchon et al., 2023b) as part of a photoelectrofermentation process where pollutant removal is maximized while a valuable product is recovered: the biomass of PPB.

In addition to exhibit an anodic role, fluid-like electrodes may act as electron donor (cathode) in electron uptake processes so microorganism can perform reductive metabolism like denitrification or CO_2 fixation (Tejedor-Sanz et la. 2021; Llorente et al., 2023). In this work we have explored for first time the role of fluid-like cathodes to stimulate the "electron sink" mechanism from purple phototrophic bacteria (PPB) in order to maximize nutrient recovery from brewery wastewater treatment. Furthermore, we have explored how the degree of reduction in carbon sources may impact on PPB electro-fermentation.

2.- Material and methods

Microbial inoculum and microbial growth

As inoculum (1%) for all experiments, we used a microbial consortium selected from an phtotoelectrochemical fluid-like reactor operated under anodic polarization (0.2 V *vs*. Ag/AgCl (Manchon et al., 2023b). The cultivation of PPB was performed under continuous IR-illuminated conditions (850 nm).

Experimental set-up

Screening brewery wastewater photo-electrofermentation

Photo-electrofermentation screening was performed in 250 mL single-chamber reactors fed with wastewater from the Mahou San Miguel brewery (Alovera, Spain). In order to evaluate the effect of reduction status on PPB culture we used two different brewery wastewater: high-reduced wastewater (5.9 e^-/C) and low-reduced wastewater (3.5 e^-/C). In order to explore the effect of polarization on PPB cultivation, different treatments were compared: Open Circuit Potential (OCP), -0.4 V (*vs*. Ag/AgCl) and -0.8 V (*vs*. Ag/AgCl) (Fig. 1).

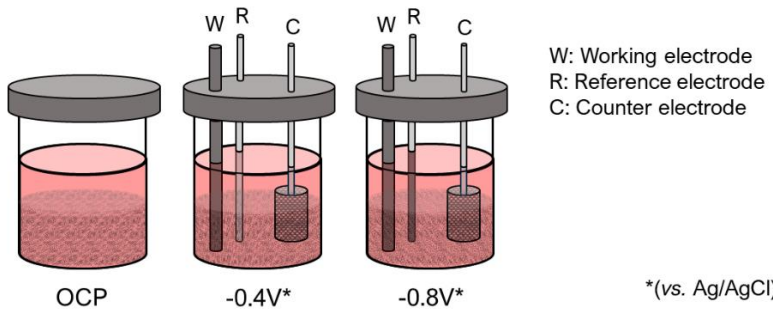

Figure 1. Scheme of the experimental set-up used for the photo-electrofermentation screening with brewery wastewater.

The polarized reactors (-0.4 V and -0.8 V) were made up of three electrodes: a working electrode (WE) whose electrical potential is controlled by a potentiostat with respect to a reference electrode (RE, 3M KCl Ag/AgCl ; Hanna Instruments S.L.) and a counter electrode (CE) in which the counterreaction takes place. In these systems, the working electrode was made up of a stirred bed of vitreous carbon (Sigradur G, HTW, Germany), using a carbon rod (Mersen, Courbevoie, France) as current collector (Liu et al., 2014; Caizán-Juanarena et al., 2020). Moreover, a platinized titanium mesh (Inagasa S.A., Barcelona, Spain) was used as counter electrode. The bed was stirred with a magnetic bar. Electrochemical measurements were performed with a multipotentiostat NEV6 (Nanoelectra S.L., Alcalá de Henares, Spain) and electric current was measured with a Keithley Series 6400 multimeter (Tektronix / Keithley, Cleveland, USA). Additionally, a non-polarized reactor without electrically-conductive material (Electrode-free) was operated as a control.

Giving insight into cathodic photo-electrofermentation

Photo-electrofermentation in presence of a fluid-like cathode was performed in microbial electrochemical fluid-like cathode reactors (Tejedor-Sanz et al., 2020) fed with synthetic wastewater containing $NaHCO_3$ 2,5 g L^{-1}, NH_4Cl 0,5 g L^{-1}, $NaH_2PO_4.2{\cdot}H_2O$ 0,41 g L^{-1}, KCl 0,1 g L^{-1}, a mixed of vitamins 10 mL/L, a mixed of minerals 10 mL/L. Medium was sparged with N_2:CO_2 (80:20) to remove dissolved oxygen. We tested two organic acids as carbon source to a final concentration of 1 g·L^{-1} of total organic carbon (TOC): malate (3 e^-/C) and acetate (4 e^-/C).

Separation of anodic and cathodic is key in electrofermentation so we designed and built a bicameral ME-FBR (Fig 2). Thus, we operated or ME-FBR, with a) non-polarized electroconductive fluid-like bed (OCP); b) electroconductive fluid-like bed polarized at -0.6 V (*vs*. Ag/AgCl); and c) an electrode free system as control. The polarized reactor potential was -0.6 V to ensure cathodic polarization throughout the bed.

The reactors, made of borosilicate, had an inlet at the bottom and an outlet at the top to recirculate the liquid upwards. At the bottom of the reactor, a fritted plate was used as a diffuser. In the polarized reactor, in addition to the main chamber, a second chamber separated by an ion exchange membrane, was designed, to house the counter electrode.

We used a multichannel peristaltic pump (Dinko, Barcelona, Spain) to recirculate the liquid in all the reactors. In the reactors with electroconductive bed both OCP and polarized at -0.6 V) vitreous carbon particles (Sigradur G, HTW, Germany) were used as bed. In the polarized reactor following the same three-electrode scheme explained in the previous section, a current collector (Mersen, Courbevoie, France) was used to polarize the working electrode (fluidized bed). A reference electrode (Hanna Instruments S.L.) was placed immersed in the bed. A platinized titanium mesh (Inagasa S.A., Barcelona, Spain) was placed in the anodic chamber as a counter electrode. The ion exchange membrane between the chambers was provided by Nafion (The Chemours Company FC, LLC).

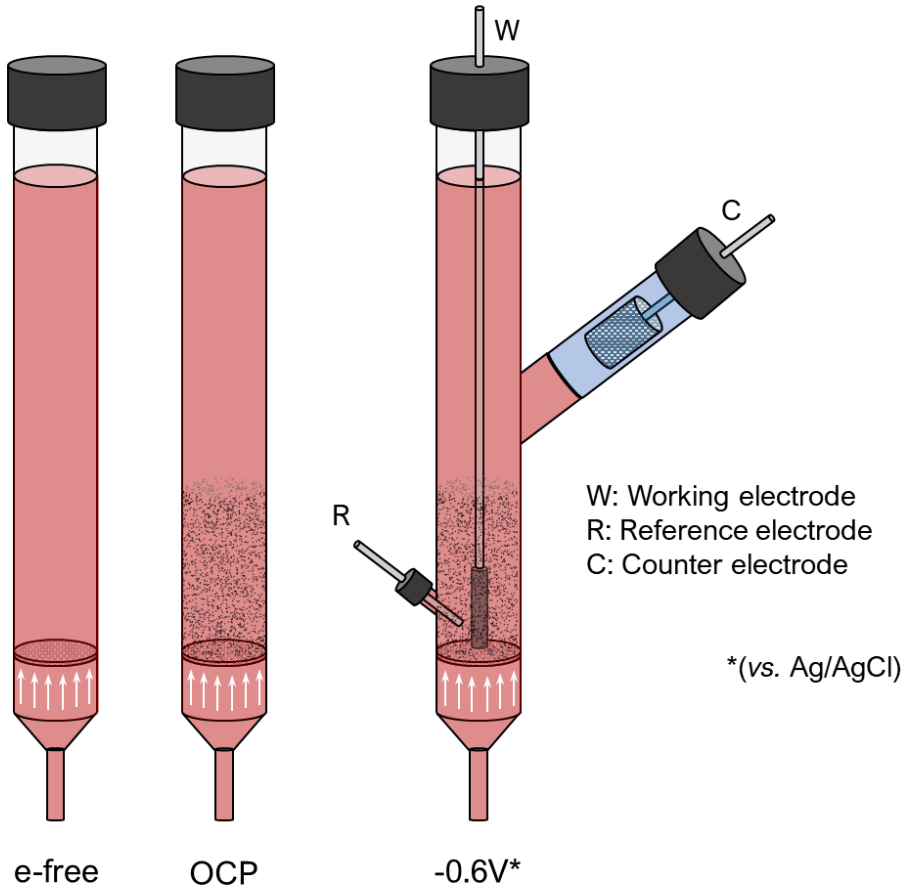

Figure 2. Scheme of the experimental set-up used for the fluid-cathode photo-electrofermentation.

Experimental design

Screening photo-electrofermentation in brewery wastewater

Stirred reactors were operated under IR-illuminated conditions in fed-batch with a hydraulic retention time (HRT) of 5 days during a period enough to reach steady state.. After one week of operation under steady state, samples were taken to assess biomass production and analyze microbial populations.

The OCP acted as non-artificially polarized control. We used two cathodic potentials: -0.4 V and -0.8 V (*vs*. Ag/AgCl).

Giving insight into fluid-like cathodic photo-electrofermentation

Fluid-like bed reactors were operated under IR-illuminated (13 W/m^2) conditions in batch. The systems were operated until the stationary phase of growth was reached. At the end of the experiment, samples were taken to assess biomass production and analyze microbial populations.

Analytical methods

Samples were filtered (0.22 micrometer) and Total Organic Carbon (TOC) and Chemical Oxygen Demand (COD) were measured. COD was quantified using a commercial kit (Merck Millipore, Germany) as previously described (Asensio et al., 2021). TOC was measured by a TOC-VCSH Shimadzu analyzer. Organic acids concentration (malate and acetate) were quantified by HPLC (HP series 1100, UV detector 210 nm and Supelco C-610H column). Inorganic nitrogen compounds were analyzed by ionic chromatography (Metrohm 930 Compact Ion Chromatograph Flex), for which they were filtered at 0.45 µm and later at 0.22 µm with a tangential filter.

3.- Results

Inspired by the performance of fluidized anodes for the metabolic control of purple phototrophic bacteria (Manchon et al., 2023b), in this work we have explored the role of cathodes in the treatment and recovery of nutrients from industrial brewery wastewater. After screening the potential of stirred cathodes in single-chamber reactors (Section 3.1), we have delved into the fluid-like cathode performance using a new bichambered design (Section 3.2).

3.1.- Screening photo-electrofermentation for treating brewery wastewater

Stirred reactors with electrically conductive granular material were operated with brewery wastewater under IR-illuminated conditions to promote PPB growth. We validated the performance of non-externally polarized reactor (OCP) versus reactors whose stirring electrode bed were polarized at -0.4 V (*vs*. Ag/AgCl) to promote direct extracellular electron uptake and at -0.8 V (*vs*. Ag/AgCl) to promote hydrogen-mediated extracellular electron uptake.

As previously reported (McKinlay and Harwood, 2010), the reduction status of carbon source present in wastewater is key for PPB metabolism. Thus, the reactors were fed with wastewater showing different reduction states: high reduction status (5.9 e^-/C) and low reduction status (3.5 e^-/C) (Table 1).

Table 1. Water quality parameters of the two type of brewery wastewater used in the screening. The e^-/C ratio was calculated based on the TOC:COD ratio as shown in SI.

Wastewater	COD [ppm]	TOC [ppm]	TN [ppm]	TOC:COD	e^-/C
High-reduced	232	47	9.9	0.20	5.9
Low-reduced	886	391	30.3	0.44	3.5

Cathodic polarization does not dramatically affect the treatment performance of brewery wastewater

Water quality analysis showed similar COD removal in all treatments with both types of tested wastewater (Fig. 3). Therefore, cathodic polarization did not exert a dramatic effect in organic pollutants removal, except for the apparent lower removal (-20%) in the high-reduced wastewater (5.9 e^-/C) under -0.8 V (*vs*. Ag/AgCl) polarization.

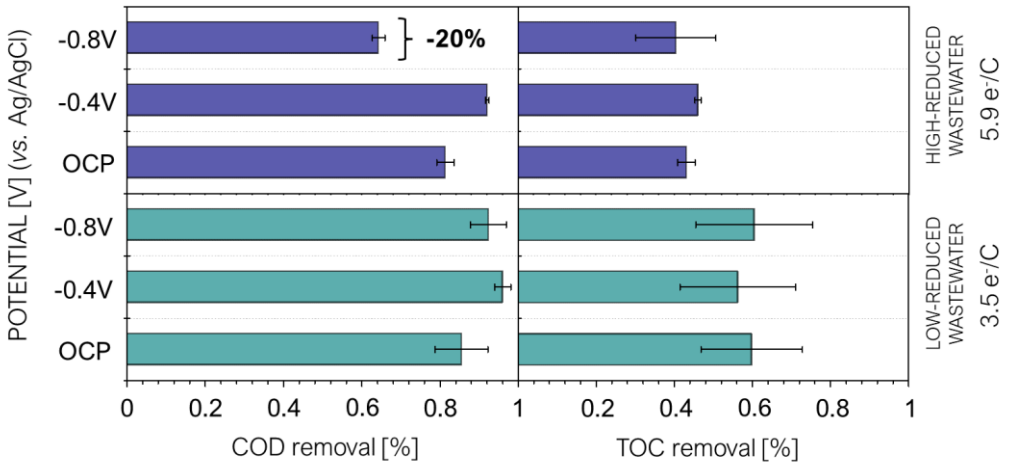

Figure 3. Chemical Oxygen Demand (COD) and Total Organic Carbon (TOC) removals ± standard error under Open Circuit Potential (OCP), -0.4 V and -0.8 V. HRT=5 days. Purple bars corresponds to reactors fed with high-reduced wastewater and blue bars corresponds to the reactors fed with low-reduced wastewater.

The complexity of the compounds in the brewery wastewater, such as sulphur compounds or some metals, may hinder the evaluation of the organic matter removal by COD analysis (Watts and Dean Adams, 1983). The analysis of total organic carbon (TOC), on the other hand, allowed a more accurate quantification of organic matter. Our TOC results revealed that the different COD removal observed in the high-reduced wastewater does not show a negative impact in the biodegradation performance (Figure 3). We hypothesize that the potential -0.8 V *vs*. Ag/AgCl could have favoured electrofermentation processes (Prévoteau et al., 2020), giving rise to the transformation of the organics into other more reduced

compounds. Although this finding requires further exploration, it was observed under hydrogen-mediated conditions (-0.8 V) and not through direct extracellular electron uptake (-0.4 V).

Nitrogen removal showed highly variable results throughout the assays with no significant impact of polarization (Fig. 1SI).

Cathodic polarization effectively promotes biomass production

Extracellular electron uptake from a cathode is strongly connected to carbon dioxide fixation in PPB (Guzman et al., 2019). Therefore, we hypothesized that providing extra electrons to PPB via a cathode in brewery wastewater treatment could maximize carbon fixation and thus increase biomass production. We quantified the amount of biomass produced at the end of each tested condition. The results showed slightly different behaviours depending on the type of wastewater used as growth culture (Fig. 4). In the high-reduced wastewater, we observed a 2-fold increase at -0.4 V and a 3-fold increase for the -0.8 V potential. In contrast, in the low-reduced wastewater there was a 4-fold increase at -0.4 V and a 7-fold increase in the reactor polarized at -0.8 V. These results showed that, unlike the water quality parameters (TOC and COD), biomass production was highly affected by cathodic polarization.

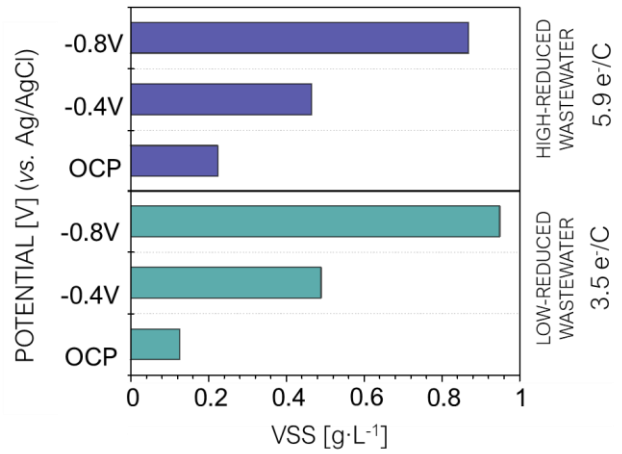

Figure 4. Volatile Suspended Solids (VVS) produced at the end of the experiment under Open Circuit Potential (OCP), -0.4 V and -0.8 V. HRT=5 days. Purple bars corresponds to reactors fed with high-wastewater and blue bars corresponds to the reactors fed with low-reduced wastewater.

The impact in biomass production for both types of wastewaters was consistent with PPB metabolism previously reported in presence of a high-reduced carbon source. Indeed, this conditions activates the electron sinks so the microorganisms become less sensitive to cathodic polarization (McKinlay and Harwood, 2010; Vasiliadou et al., 2018; Guzman et al.,

2019). This may explain why polarization had a greater impact when PPB where grown using low-reduced carbon sources.

Stirred cathodes: high impact but low current

Despite the impact of polarization on biomass production, we observed less consumption of electrical current than expected (ca. -50 to -100 µA) (Fig. 2SI). We hypothesize that the cathode could be acting as a metabolic regulatory element rather than as a true electron donor, as previously reported in PPB electro-fermentation processes (Manchon et al., 2023b). Under polarization conditions where hydrogen should be produced (ca. -0.8 V) we monitor higher current consumption (-80 to -100 µA). We are certainly producing less hydrogen than the one expected with non-carbonaceous materials like platinized titanium; unfortunately we doubt this material can be used fluidized.

Cathodic photo-electrofermentation leads to metabolic and population changes in the microbial community

Electro-fermentation processes using microbial consortia led to changes in microbial population structure and metabolic activity. Therefore, we explored the microbial community using 16S Illumina sequencing to understand the role of polarization in microbial selection. The analyzes revealed differences at all taxonomic levels although we focused on the genus level as it is the most informative regarding functionality.

The mere presence of electrically conductive material has been reported to favour the exchange of electrons between electroactive microorganisms even without external polarization (Rotaru et al., 2021). This phenomenon, which has been described in non-phototrophic bacteria either using fixed bed (Prado et al., 2022) or fluid-like beds (Tejedor-Sanz et al., 2018). In contrast, our stirred reactors showed similar pollutant removal efficiency and biomass production in both free-electrode reactors and stirred-electrode reactors under Open Circuit Potential (OCP) (Fig. 3SIa and Fig.3SIb). However, the presence of electroconductive material had an impact on the microbial population composition (Fig. 4SI). The electrode-free reactors showed a majority presence of microorganisms characteristic of wastewater, with *Pseudomonas* and *Azonexus* genera and a very low presence (<0.5%) of PPB genera such as *Rhodopseudomonas* or *Ectothiorhodospira* and electroactive bacteria such as *Geobacter*. On the contrary, *Rhodopseudomonas* sp., the electroactive PPB model genus (Bose et al., 2014b), was present when stirred electrons were used under OCP (Fig. 5). In addition, other genera such as *Pseudomonas* and *Acidovorax*, considered core genera in electroactive communities (Xiao et al., 2015), were also present. In the OCP-based reactor fed with high-reduced wastewater (5.9 e^-/C), we found *Thauera* genus, which was described as electroactive due to its extracellular respiration of AQDS (Liu et al., 2006). In both OCP reactors, *Propionivibrio* was found, a genus also present in other electro-fermentation systems, capable of producing propionic acid and of interacting with extracellular redox species (Yang et al., 2021).

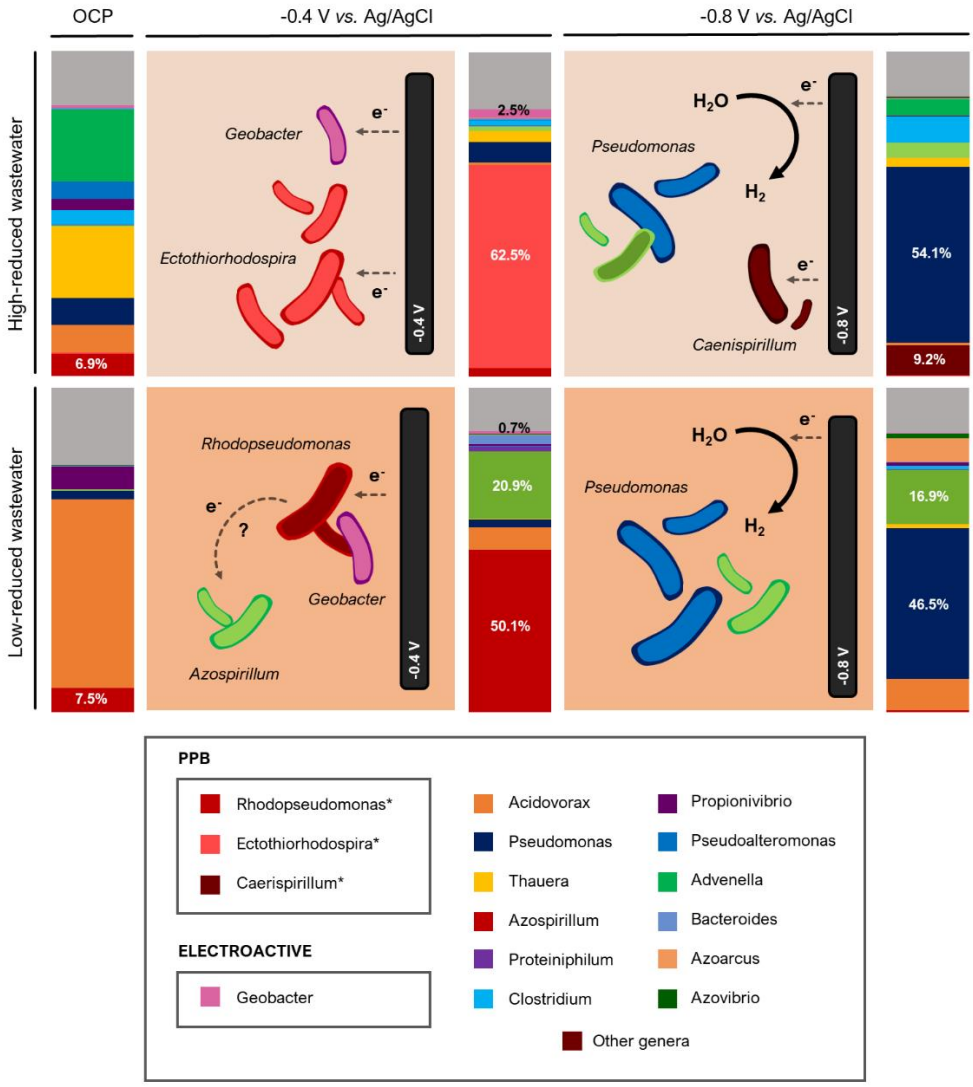

Figure 5. Microbial population structure at the genus level analyzed by 16S Illumina corresponding to screening with brewery wastewater.

Stirred electrode reactors polarized at -0.4 V (*vs*. Ag/AgCl) had a completely different microbial structure, showing maximum predominance of purple phototrophic bacteria genus (Fig. 5). Under polarization at this potential (-0.4 V) the production of hydrogen is thermodynamically not possible (E = -0.63 V *vs*. Ag/AgCl), so extracellular electron uptake was not hydrogen-mediated. Therefore, extracellular electron transfer could only occur through direct electron transfer or through self-produced redox mediators (Logan et al., 2019). This is consistent with the high presence of electroactive genera: classical

electroactive genus such as *Geobacter* (Koch and Harnisch, 2016) and other electroactive PPB genera such as *Rhodopseudomonas* (Bose et al., 2014). In these reactors (-0.4 V) wastewater composition was a key factor in microbial selection (Fig. 5). Thus, using high-reduced wastewater (5.9 e^-/C), we detected a predominance of *Ectothiorhodospira* a purple phototrophic bacteria also found among electroactive communities (Manchon et al., 2023b). On the contrary, *Rhodopseudomonas*, model electroactive PPB genus (Bose et al., 2014; Guzman et al., 2019) predominated in presence of low-reduced wastewater (3.5 e^-/C).

The microbial community analysis revealed a different scenario in the reactors polarized at -0.8 V (Fig. 5). In both reactors, the analyzes showed 50% *Pseudomonas* and only in presence of high-reduced wastewater fed reactor, the PPB *Caenispirillum* was observed. This population structure was, in fact, very similar to the free-electrode reactors used as control (Fig. 4SI). At -0.8 V, hydrogen mediated electron transfer is feasible (E = -0.63 V *vs*. Ag/AgCl). Although another extracellular electron pathway (H_2-mediated) could explain the different community observed at this potential, hydrogen does not inhibit the growth of PPB (Vasiliadou et al., 2018) (Fig, 5SI). These results suggest that -0.8 V polarization inhibits PPB growth and although hydrogen is not involved in such inhibition, further study is needed.

Giving insight into fluid-like cathode photo-electrofermentation

Finally, we further study how cathodic polarization modulates PPB biomass production in a more controlled environment and sophisticated reactor. In this section we analyzed the behaviour of PPB with malate (3 e^-/C) and acetate (4 e^-/C) as carbon source under fluid-like cathode polarization. This experimental approach allowed us to conclude more precisely the effect of the reduction state, eliminating noise due to other parameters not controlled in the experiment with wastewater, such as the carbon-nitrogen ratio, the nature of the organic compounds or the concentration of micronutrients. In addition, our new two-chamber electrochemical fluid-like bed reactor design allowed to study the isolated effect of the cathode on PPB metabolism, eliminating the possible effect of the counter electrode.

Fluid cathodic electrofermentation enhances biomass production

We studied the main parameter in microbial cultivation: biomass production. Biomass analysis revealed higher production under fluid-like cathodic polarization at -0.6 V (*vs*. Ag/AgCl) in comparison with the electrode-free control (Fig. 6). Such enhancement was confirmed for both acetate-feeding (+75%) and malate-feeding (+130%). Therefore, the results of this experiment confirmed our previous observation with stirred-electrode assays. Furthermore, they are consistent with the role of having an extra-source of electrons (cathode) on PPB metabolism, promoting carbon fixation and thus maximizing biomass production (Guzman et al., 2019).

In previous assays with stirred-electrode reactors fed with wastewater, we did not observe an impact in the biomass production (Fig. 4) when the fluid-like electrode was non-polarized (OCP) in comparison with free-electrode conditions. In contrast, our fluid-like electrode (OCP) reactor fed with malate revealed higher biomass production (ca. 50 %) in comparison

with the electrode-free control (Fig. 6). This could indicate that, with oxidized carbon sources (3 e^-/C), the electrically conductive material could specially facilitate syntrophies between microorganisms as reported elsewhere (Rotaru et al., 2014, 2018). These syntrophic relationships seem to favour microbial growth, allowing the consortium to deal more easily with redox stress situations. This hypothesis is consistent with the absence of differences in biomass production when acetate (4 e^-/C), a more balanced electron-carbon ratio (McKinlay and Harwood, 2010) was feeding our free-electrode or OCP reactors.

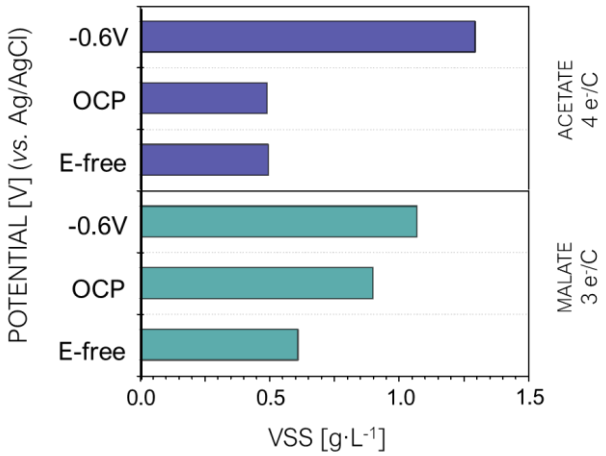

Figure 6. Volatile Suspended Solids (VVS) produced under Electrode-free, Open Circuit Potential (OCP) and -0.4 V (*vs.* Ag/AgCl).

Fluid-like cathodic photo-electrofermentation promotes PPB presence

Once the effect of fluid cathode on biomass production was demonstrated, we analyzed microbial population structure by Illumina 16S sequencing. We focused on the population structure at the genus level as it is the most informative from the point of view of function and metabolism.

As we described in the assays with stirred-electrode reactors fed with wastewater (Fig. 5), the mere presence of conductive material can select for a different microbial community. In this case, the differences were more evident in the malate-fed OCP reactor, with a higher relative abundance of *Rhodopseudomonas* (24.3%) compared to the free-electrode reactor (Fig 7). The acetate-fed reactor, on the other hand, showed a similar abundance of *Rhodopseudomonas* regardless the presence of a non-polarized electrode. These results were consistent with shown in biomass production (Fig 6). Malate is a more unbalanced organic compound, so we hypothesized that the conductive material would promote electroactive bacteria from genus *Rhodopseudomonas* to deal with redox stress through electrosyntrophies (Liu et al., 2021; Rotaru et al., 2021). In the acetate-fed reactor, more redox-balanced substrate, electrically conductive material does not provide a competitive advantage to electroactive bacteria and therefore its effect was less remarkable.

Additionally, we observed a high presence of *Geosporobacter* in all the reactors, including the electrode-free control (Fig 7). Some bacteria of this genus have been described as an anaerobic iron-reducing bacteria (Hong et al., 2015) capable of colonizing electrodes (Miceli et al., 2012; Moscoviz et al., 2018). These results could indicate some type of syntrophic relationship between both genera where *Geosporobacter* , in absence of electron acceptor, may transfer electrons to *Rhodopseudomonas* which is well known for its great ability to dissipate reducing power via electron sinks (Guzman et al., 2019). The possible electrosyntrophy between the genera *Geosporobacter* and *Rhodopseudomonas* requires further study.

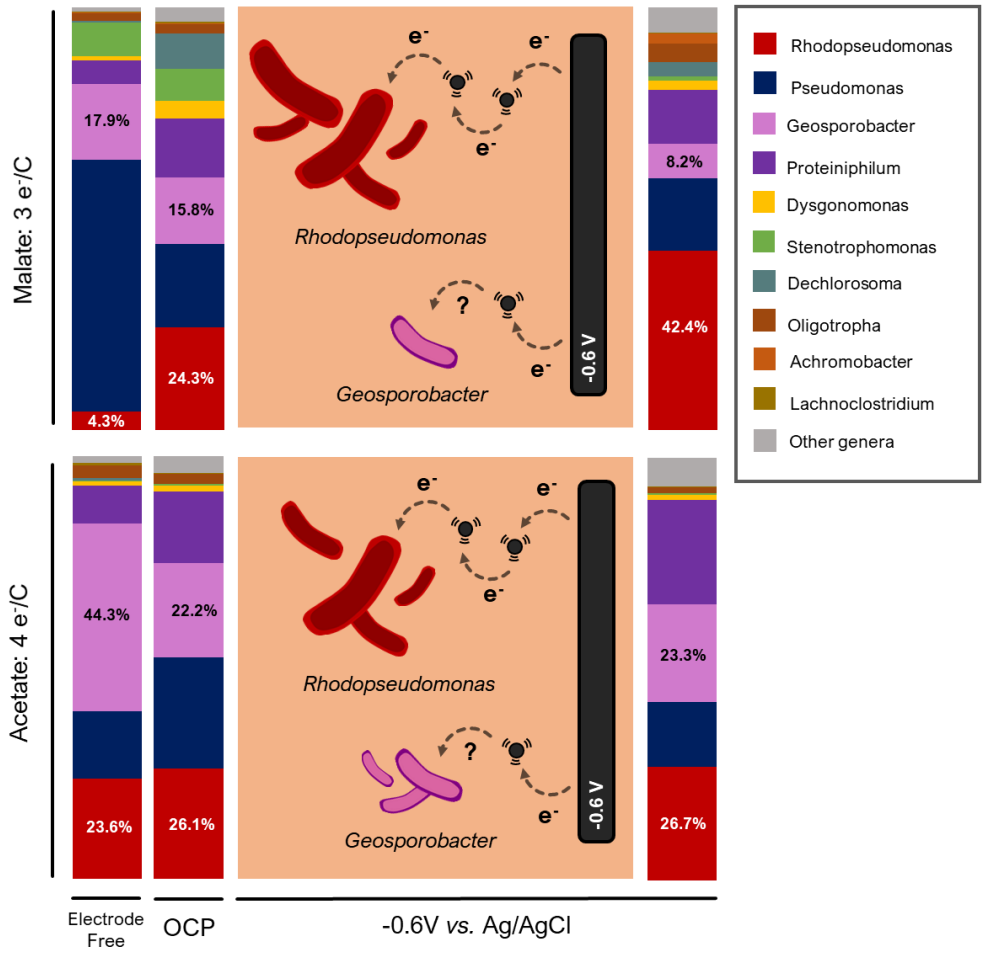

Figure 7. Genus level composition (16S Illumina) of a microbial population grown with synthetic wastewater under fluid-like cathode IR-Irradiated reactor

4.- Conclusions

In this study, we have demonstrated that mobile cathodes (either stirred or fluidized) can shape planktonic microbial communities dominated by purple phototrophic bacteria. We observed that cathodic polarization and IR irradiation can play a key role in microbial and phenotypic selection, promoting the PPB activity (-0.4 V) or minimizing their presence (-0.8 V).

Furthermore, our results revealed the importance of reduction status of carbon source in the PPB photoheterotrophic culture and how electrodes drives microbial population shifts depending on the reduction status of the carbon source. Finally, we conclude that using fluid-like cathodes may accelerate the transition from the wastewater treatment model into the biorefinery model to maximize biomass production.

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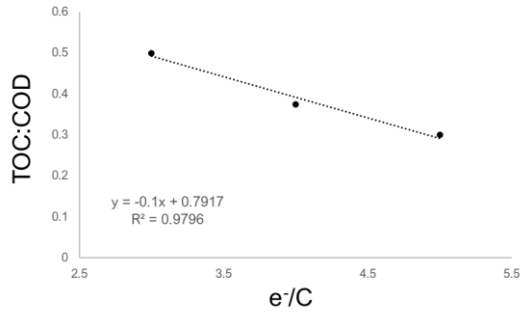
6.- Supplementary information

COD:TOC ratio as indicator for reduction degree in wastewater

The reduction degree of the wastewater used in the Section 3.1 was calculated by using de TOC:COD ratio.

We use classical water quality measures to assess the reduction status of organic compounds in the water. The TOC allows us to assess the amount of carbon in the wastewater. The COD, on the other hand, is a good indicator of the number of electrons contained in the wastewater.

We calculated the electrons per carbon produced in the oxidation to carbon dioxide of three carbon sources (Table 1) and their theoretical TOC:COD values. From the linear regression shown below, we were able to estimate the electrons per carbon of the wastewater used.



Supplementary figures

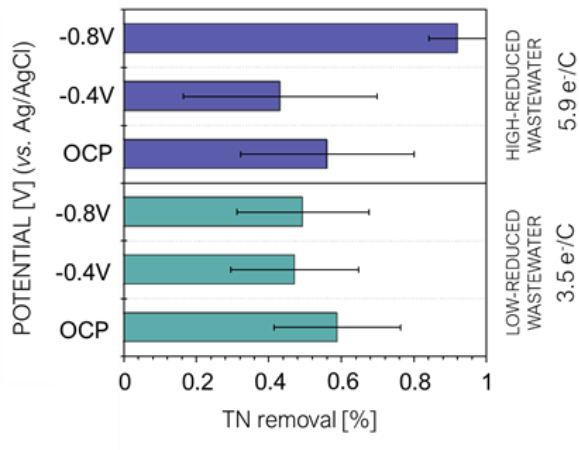


Figure SI1. Total Nitrogen (TN) removal ± standard error under Open Circuit Potential (OCP), -0.4 V and -0.8 V. HRT=5 days. Purple bars corresponds to reactors fed with high-reduced wastewater and blue bars corresponds to the reactors fed with low-reduced wastewater.

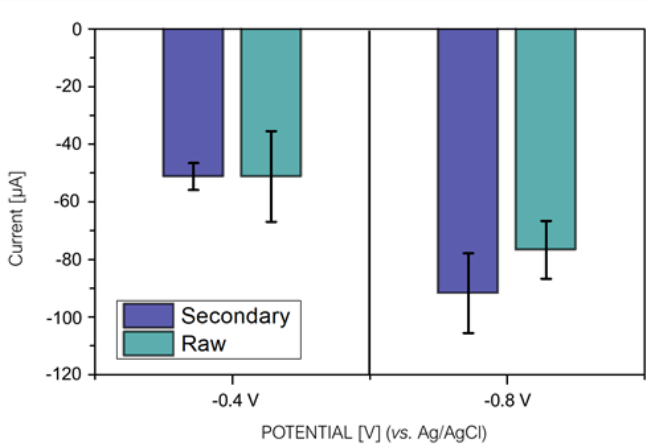


Figure SI2. Current intensity ± standard error under Open Circuit Potential (OCP), -0.4 V and -0.8 V. HRT=5 days. Purple bars corresponds to reactors fed with high-reduced wastewater and blue bars corresponds to the reactors fed with low-reduced wastewater.

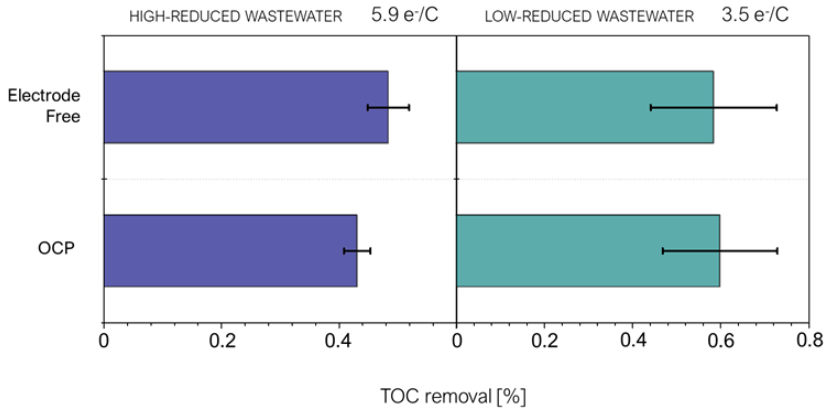


Figure SI3a. Total Organic Carbon (TOC) removal ± standard error under Open Circuit Potential (OCP) and electrode-free. HRT=5 days. Purple bars corresponds to reactors fed with high-reduced wastewater and blue bars corresponds to the reactors fed with low-reduced wastewater.

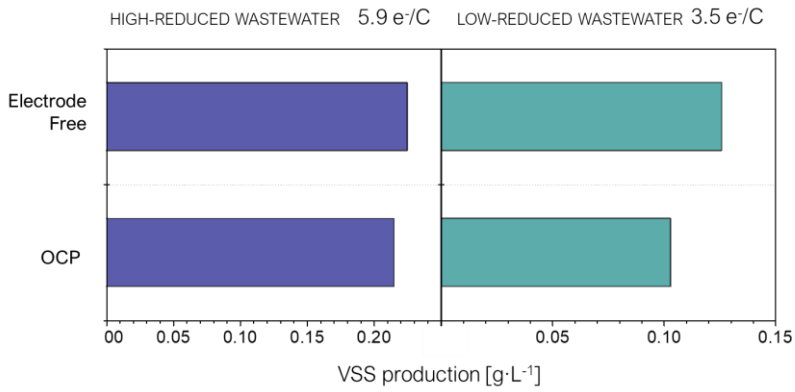


Figure SI3b. Volatile Suspended Solids (VVS) produced at the end of the experiment under Open Circuit Potential (OCP) and electrode-free. HRT=5 days. Purple bars corresponds to reactors fed with high-reduced wastewater and blue bars corresponds to the reactors fed with low-reduced wastewater.

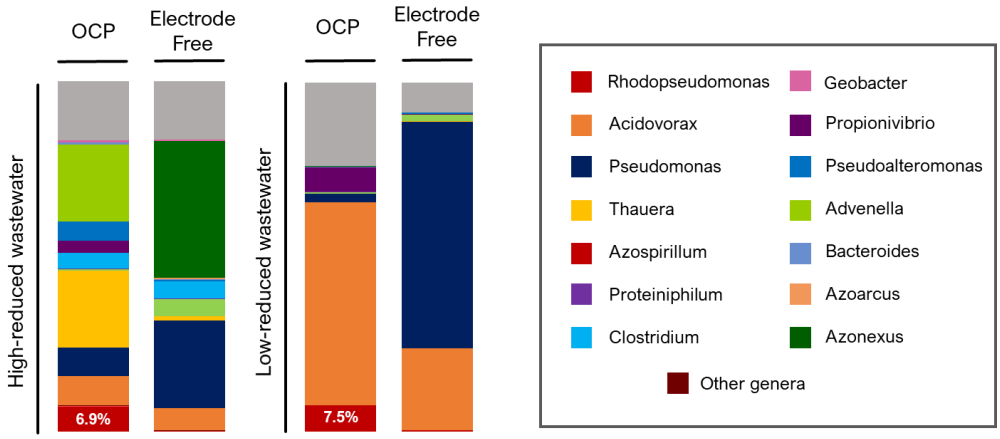


Figure SI4. Microbial population structure at the genus level analyzed by 16S Illumina corresponding to screening with brewery wastewater (OCP and electrode-free).

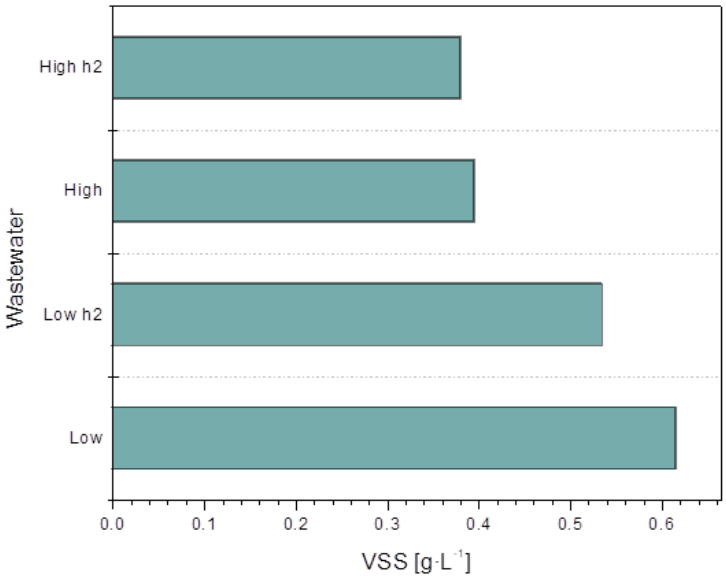


Figure SI5. Volatile Suspended Solids (VVS) produced at the end of the experiment under -0.8 V (*vs*. Ag/AgCl) with and without hydrogen. HRT=5 days. Purple bars corresponds to reactors fed with high-reduced wastewater and blue bars corresponds to the reactors fed with low-reduced wastewater.

Chapter 5:

Discussion, conclusions and future work

Chapter 5: Discussion, conclusions and future work

CHAPTER 5:
Discussion

General discussion, conclusions and future work

The main objective of this thesis was to explore the interaction between purple phototrophic bacteria dominated communities and electrodes. In order to establish the foundations, in this work we have studied electro-autotrophic cultures and electro-heterotrophic cultures, both anodic and cathodic, on a laboratory scale. A general discussion is presented in question-answer format, followed by a number of thesis conclusions and future work.

1.- General Discussion

Why the interaction between purple phototrophic bacteria and electrodes should be studied?

Purple phototrophic bacteria are the most versatile microorganisms on earth (Madigan, Michael. Bender, Kelly. Buckley, Daniel. Sattley, W. Stahl, 2018). Molecular mechanisms for using organic molecules (heterotrophic pathways) and inorganic molecules (lithotrophic pathways) coexist with the ability to transform light into energy (phototrophic pathways) or growth in darkness (chemotrophic pathways). In addition, their ability to interact with electrically conductive materials makes them a key biotechnological piece (Bose et al., 2014; Vasiliadou et al., 2018).

Unlike other heterotrophic microorganisms used in combination with electrodes, purple phototrophic bacteria are easily enriched in mixed cultures using infrared (IR) radiation. IR-radiation is an energy source for PPB, so this competitive advantage over other microorganisms promotes their presence. Therefore, IR radiation in a microbial electrochemical reactor will promote enrichment and predominance over time as we have shown in Chapter 3 and Chapter 4.

In this thesis we demonstrate that these microorganisms, even in a mixed culture scenario, outcompete other non-photosyntetic microorganisms under electrochemical control. In Chapter 3, purple phototrophic bacteria are more abundant and more active when the fluid-like bed was polarized as an anode, maximizing pollutants removal. In Chapter 4, cathodic polarization vastly alter their metabolism, increasing biomass production.

Therefore, purple phototrophic bacteria are easily enriched over other microorganisms both by IR-radiation and electrode polarization. In this way, it is possible to work in non-sterile environments and use wastewater as a substrate for cultivation. In addition, purple phototrophic bacteria are known for their ability to produce value-added compounds as bioplastics and for their high protein content as single cell protein (Alloul et al., 2022).

How purple phototrophic bacteria interact with electrodes?

Throughout the last two decades, microbiologists have used different approaches to understand the interaction between microorganisms and electrodes. In the early days of electromicrobiology, interaction with electrodes was demonstrated by contacting pure cultures and electrodes (Bond and Lovley, 2003). In this way, some of the electroactive

purple phototrophic bacteria such as *Rhodopseudomonas palustris* or *Rhodobacter capsulatus* have been described (Xing et al., 2008; Bose et al., 2014). The democratization of massive sequencing techniques has given rise to other approaches consisting of observational experiments. Instead of subjecting a microorganism to contact with an electrode, some microbiologists have placed electrodes in different environments to analyze the microbial species that colonize the electrode.

In this thesis, we have explored electrode-dwelling communities using electrochemical techniques like chronoamperometry and cyclic voltammetry. Analysis of microbial communities provide a comprehensive and realistic view of those bacteria with a potential participation in the extracellular electron transfer in nature. On the contrary, this approach does not reveal the specific role of each specie in the community. Both approaches should coexist in order to understand the full picture. In this work we observed two possible roles of the electrode regarding PPB activity: i) a role as main electron donor/acceptor or i) a role as regulatory element of metabolism (electrofermentation).

The role in which the electrode acts as the main electron donor/acceptor, is characterized by high coulombic efficiencies and a particular signal which demonstrate the existence of an extracellular electron transfer process. We observed this kind of interaction in biofilm-based experiments, both at the anode (Chapter 3) and at the cathode (Chapter 4). Under anodic conditions, the PPB-dominated community showed oxidative catalysis unequivocally demonstrating the interaction with the electrode. This experiment showed a redox peak (-0.1 V *vs.* Ag/AgCl) involved in the extracellular electron transfer is similar to Vasiliadou et al., (2018). Under cathodic conditions, we identified a redox pair with a midpoint potential at 0.05 V (*vs.* Ag/AgCl) and a cathodic barrier from -0.6 V.

The electrode can also act as a metabolic controller in a process called electrofermentation. PPB-dominated communities perform electrofermentation especially under planktonic state, with both anodes (Chapter 3) and cathodes (Chapter 4). This way of interaction does not generate a robust electrochemical signal in terms of extracellular electronic transfer. However, electrofermentation is the main way of interaction observed in this thesis research and we have demonstrated its direct impact on PPB metabolism

Can purple phototrophic bacteria be cultured with an electrode as sole electron donor?

Electrodes support autotrophic growth in PPB through electron uptake process, in a similar way than natural electron donors such as Fe(II). This mechanism, already described in Arpita Bose laboratory (Bose et al., 2014) using a pure culture, has been demonstrated with a PPB-dominated community in this thesis (Chapter 2).

We identified different non-PPB genera, such as *Azospira* and *Azospirillum* along with other PPB-genera such as *Rhodopseudomonas* or *Rhodoferax*. *Rhodopseudomonas* genus was the key electroactive bacterium found under electroautotrophic conditions, indicating its key role in extracellular electron uptake in our systems, connecting the rest of the

community with the extracellular electron donor. Our results indicate that PPB can be cultured with an electrode as sole electron donor.

Can anode or cathode promote different behavior in purple phototrophic bacteria?

In this thesis we have explored the behavior of PPBs in presence of both anodes and cathodes. Some authors have described that extracellular electron transfer can be bidirectional in many cases (Gong et al., 2020). In mixed cultures, the issue is more complex, since polarization has an impact on metabolic regulation and on the microbial population at the same time. Therefore, the results obtained in this thesis are a mixture of metabolic and population changes.

Even so, we have detected some remarkable peculiarities between anode and cathode polarization under heterotrophic cultivation.

In Chapter 3, we evaluate the behavior of PPBs under anodic polarization. The treatment efficiency analyzes showed a 2-fold increase under polarization (0.2V and 0.4V). We hypothesize that the anode facilitates some metabolic pathways by increasing the kinetics of contaminant removal. Regarding biomass production, anode polarization caused a decrease in biomass yield.

In Chapter 4, we explore the effect of the cathode on the metabolism of PPBs. Under cathodic polarization, the main effect observed was the increase in biomass (3-fold to 7-fold).

Our observations together with current knowledge of PPB metabolism, led us to propose that the electrode acts mainly on some metabolic pathways acting as electron sinks.

How electrodes control electron sinks pathways in PPB?

Electron sink pathways allow to deal with excess reducing power and maintain homeostasis under heterotrophy (McKinlay and Harwood, 2010). These routes are activated or deactivated depending on the requirement of reducing power consumption and are: carbon fixation (via Calvin-Benson cycle), hydrogen production and nitrogen fixation.

The main electron sink is carbon fixation, which has an important implication on biomass yield, that is, its activation increases the carbon converted to biomass. Until the use of electrodes in PPB cultivation, the activation or deactivation of these pathways depended entirely on the substrate used in the culture. Recently, Guzman *et al.* demonstrated that carbon fixation is linked to extracellular electron uptake (Guzman et al., 2019).

In this thesis, especially in Chapter 3 and Chapter 4, we have demonstrated the effect of the electrode on biomass production. Under anodic polarization, PPB showed lower biomass yield, which is consistent with the inhibition of carbon fixation. The anode, acting as an electron acceptor, allows the bacteria to remove excess reducing power, thus inhibiting carbon fixation. Under cathodic polarization, instead, we observed an increase

in biomass production. The cathode, acting as an extra source of electrons, increases the excess intracellular reducing power, promoting the activation of the electron sink, specifically carbon fixation.

Under the experimental conditions used in this thesis, we have not observed an impact on alternative electron sinks like nitrogen fixation or hydrogen production. These pathways are often activated in environments with unbalanced carbon-nitrogen substrates. Therefore, we hypothesized that the use of alternative substrates may enhance the connection between the electrode and these metabolic pathways.

What is the best strategy to cultivate purple phototrophic bacteria? Fluid-like electrodes or conventional static electrodes?

Extracellular electron transfer has classically been located at the electrode-biofilm interface. Fluid-like electrodes meant a paradigm shift, allowing extracellular electron transfer to occur in planktonic cells (Tejedor-Sanz et al., 2017). In this thesis, electron transfer in both classical biofilm-based and in planktonic-based systems have been explored.

As we demonstrated in Chapter 3, PPB can easily form biofilm on carbon-based static anodes. Even when the IR-light source comes from outside the reactor, PPB form biofilm on the electrode and not on the reactor surface. Furthermore, under fluid-like electrode polarization, PPB grow planktonically but under electrode control. Under cathodic polarization, the scenario is different since planktonic growth is favored over biofilm growth. Therefore, planktonic growth under anodic polarization requires fluidization of the electrode, whereas under cathodic polarization, simple polarization is sufficient to favor planktonic growth. This is consistent with the electrostatic repulsion generated in cathodic polarization and has been previously reported (Vasiliadou et al., 2018).

Despite this, the fluidization of the electrode allows maximizing planktonic growth and influencing a much larger culture volume than biofilm-based systems. In applications where biomass partitioning is the strategy to recover nutrients, fluid-like electrodes are the most appropriate choice. However, in treatment-focused applications, anodically polarized biofilm-based systems would facilitate operation, generating biomass-free effluent suitable for reuse.

Electrode impact in microbial communities dominated by PPB: who and when?

In this thesis, the electroactive microbial community in reactors illuminated with infrared radiation has been studied in depth for the first time. Although these results are very valuable for understanding the relationships between electroactive PPBs and other microbial partners, these results are not generalizable and will vary depending on the conditions used.

In Chapter 3, the exploration of the community in fluid-like anodes revealed that polarization is crucial in the selection of certain PPB genera. Indeed, when the electrically

conductive material was not polarized, the predominant genera were *Ectothiorhodospira* and *Rhodovulum*. In contrast, when the electrically conductive bed was anodically polarized, *Rhodopseudomonas* genus together with *Proteiniphilum* genus were predominant.

In addition, in Chapter 4 of this thesis, the microbial community showed a fundamental parameter to consider: the redox state of the carbon source. We observed that cathodic polarization promotes the presence of different genera of PPB depending on the substrate redox state. The genus *Ecthiorhodospira* was the predominant PPB genus (62.5%) in combination with the genus *Geobacter* (2.5%) when high-reduced substrate was used. In contrast, with low-reduced substrate, the predominant PPB genus was *Rhodopseudomonas* (50%) in partnership with the *Azospirillum* genus. Therefore, our results indicate that the effect of the electrode on the microbial community strongly depends on the substrate used.

In Chapter 2, we discovered that under electroautotrophic growth, *Rhodopseudomonas* genera play the main role in the extracellular electron uptake. In addition, the genera *Azospirillum* and *Azospira* could have a role in electron uptake or establish some kind of syntrophic relationship with electroactive purple phototrophic bacteria.

2.- Conclusions

The main conclusions drawn from this thesis are the following:

- The combination of IR-radiation and electrodes promotes the selection and maintenance of PPB-dominated microbial communities.

- PPB-dominated communities can interact with electrodes by forming biofilms or planktonically.

- The biofilm-based interaction occurs with conventional polarized static electrodes polarized as anodes, showing high coulombic efficiencies and a major role of the electrode in oxidative catalysis.

- Fluid-like electrodes promote planktonic growth of PPB-dominated bacteria in which coulombic efficiency is low and the electrode has a regulatory role in metabolism (electro-fermentation).

- PPB-dominated community can use the electrode as a sole electron donor.

- The electrode under heterotrophic conditions show an impact on the electron sink pathways, specifically carbon fixation.

    The anode, acting as an electron acceptor, inhibits carbon fixation, thus decreasing the biomass yield.

    On the contrary, the cathode, acting as an extra source of electrons, activates carbon fixation, increasing biomass production.

- *Rhodopseudomonas* genera is the key player in both anodes and cathodes in the PPB-dominated bacterium. Despite this, depending on the redox state of the substrate, other genera such as *Ecthiorhodospira* may outcompete.

- Some genera such as *Proteiniphilum* in anodes and *Azospira* and *Azospirillum* in cathodes could play a role in the PPB -dominated microbial community.

3.- Future outlook

Despite the fact that the findings described in this thesis and those reported by other authors in recent years announce a promising application of PPBs in wastewater treatment and nutrient recovery, some challenges must be faced:

Scaling Up: big problems require big sizes

The full-scale application of purple phototrophic bacteria to wastewater treatment has recently been explored (https://deep-purple.eu and https://incover-project.eu). Its use through electrochemical strategies, on the other hand, has only been demonstrated on a laboratory scale. Despite this, the reactors used in this thesis (fluid-like microbial electrochemical reactors) were probe to be scalable till TRL6 (http://life-answer.eu) for treating brewery wastewater open a new scenario that will be explored through the project PHOTOELECTRA in the following years. Current challenges consist of improving the adaptation of PPB to new electroconductive materials, searching for configurations to maximize IR lighting systems.

Economic viability

The economic viability is given by the relationship between the price of the final product and the cost of production. The economic assessment showed that the price of feedstock to grow PPB is a considerable fraction of the overall operational expenditure (Alloul et al., 2022). In this thesis two alternative approaches have been used to address this limitation: using waste feedstocks (Chapter 3 and Chapter 4) or using electricity as the main feedstock (Chapter 2).

Therefore, in order to achieve an economically viable technology, it is crucial to identify waste feedstocks that can be used for cultivation. Regarding electricity as feedstock, the implementation of electroatutotrophic cultures in combination with renewable energy sources could be a solution. In addition, sources of carbon dioxide from industries could be used in these processes.

Product valorisation and public opinion

The other important variable for economic viability is the valuation of the product. The added value compounds produced by PPB have been widely described: its protein-rich biomass, biopolymers such as PHB (Alloul et al., 2022). Evaluation of the number of full-scale value-added products in combination with electrodes is necessary to determine the feasibility of the technology.

Furthermore, research needs to be done on the food safety of products derived from PPB biomass.

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Abbreviations

CE: Coulombic Efficiency
CIET: Conductive-particle-mediated Interspecies Electron Transfer
COD: Chermical Oxygen Demand
CV: Cyclic Voltammetry
DEET: Direct Extracellular Electron Transfer
DIET: Direct Interspecies Electron Transfer
EET: Extracellular Electron Transfer
EF: Electrofermentation
HPLC: High Performance Liquid Chromatography
HRT: Hydraulic Retention Time
IR: Infrared
MEET: Mediated Extracellular Electron Transfer
ME-FBR: Microbial Electrochemical Fluidized Bed Reactor
MET: Microbial Electrochemical Technologies
MFC: Microbial Fuel Cell
OCP: Open Circuit Potential
OD: Optical Density
PHA: Polyhydroxyalkanoate
PNSB: Purple Non-sulfur Bacteria
PPB: Purple Phototrophic Bacteria
PSB: Purple Sulfur Bacteria
RC: Reaction Centre
RE: Reference Electrode
SEM: Scanning Electron Microscope
sMFC: Sedimentary Microbial Fuel Cell
TEA: Terminal Electron Acceptor
TEM: Transmission Electron Microscope
TN: Total Nitrogen
TO/NTO: Turnover/Non-turnover
TOC: Total Organic Carbon
VFA: Volatile Fatty Acids
VSS: Volatile Suspended Solids
WE: Working Electrode
WWTP: Wastewater Treatment Plant

"Life is nothing but an electron looking for a place to rest"

Albert Szent-Györgyi
Nobel prize 1937

Microbial life is a key piece to understand the history of the earth, from the origin of species to the most advanced biotechnological applications. The challenge of learning to coexist with microbial communities can solve some of the challenges of this century, such as water treatment and new food resources.

Purple phototrophic bacteria, the most versatile microorganisms on earth, are highly effective for sustainable wastewater treatment and nutrient recovery as cell protein. One of the most innovative approaches for applying these bacteria in the wastewater sector is electromicrobiology, using their capacity for interchanging electrons with electroconductive materials.

This PhD thesis explores the use of electrodes to domesticate purple phototrophic bacteria in order to maximize pollutant removal from wastewater or production of microbial biomass.

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