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Performance Evaluation of Microbial Fuel Cells in the Winery Effluent Treatment

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Abstract

Microbial fuel cells (MFCs) are a sustainable way to produce energy since they combine electricity generation and wastewater treatment. This technology is a promising alternative to the traditional wastewater treatment systems, which require a high input of energy and maintenance costs. MFCs oxidize the organic matter present in the effluent by microorganisms under anaerobic conditions, releasing electrons and protons. Winery industries worldwide produce billion of liters of wine every year and consequently high quantities of winery wastewater (WW) must to be pre-treated before disposal. Most of the winery wastewaters have a high organic charge with an average chemical oxygen demand (COD) of 11 250 mg O₂/L, considerable ratios of organic acids (acetic, malic, lactic, etc.), sugars (glucose and fructose), alcohols (ethanol, glycerol, etc.), esters, polyphenols and commonly an acid pH. In general, the concentrations of these different compounds in the WW exceed the limits established by law for their application in irrigation water for agriculture. To avoid environmental issues related to WW disposal, biological winery wastewater treatments, such as anaerobic digestion and activated sludge reactors, are crucial to allow the use of these effluents in agricultural soils as fertilizers, however, this kind of treatments represent high costs to the winery industry. For that reason, finding new lower cost technologies is assumed as a major challenge. In this context, the use of a technology to accomplish this treatment without high energy necessities such as MFCs appears as a promising idea. In this work, *Acetobacter aceti*, *Gluconobacter oxydans*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Bacillus cereus* and *Escherichia coli* were analyzed through their cell culturability and COD removal rate in a synthetic winery wastewater (SWW). These microorganisms were evaluated as potential candidates to act as inoculum in the treatment of the winery effluent through MFC technology based in their resilient behavior in acid pH environments and due the fact that most of them were identified during diverse stages of wine production. Among these seven microorganisms, *Z. bailii* and *B. cereus* presented a high adaptation to the SWW and for that reason were selected and properly characterized, in terms of their growth phase curve with the objective of being further inoculated in a single-chamber MFC (SC-MFC). Both MFC's performance were evaluated in terms of power density achieved, cell culturability, COD, total organic carbon (TOC) and polysaccharides removal rate. Moreover, quantification of the total solids, total volatile solids and fixed solids of the biofilms formed in both MFCs was performed at the end of each experiment. MFC inoculated with *B. cereus* (MFC2) presented better results regarding to the maximum COD removal rate ($65 \pm 4\%$) and to the maximum power density obtained (8.35 mW/m^2) in relation to the MFC inoculated with *Z. bailii* (MFC1), which demonstrate a maximum COD removal rate of $41 \pm 1\%$ and a maximum power density of 6.21 mW/m^2 . MFC1 and MFC2 presented a biofilm quantification very similar, 6.80 g/L and 6.95 g/L , respectively.

Resumo

As células de combustível microbianas (CCMs) baseiam-se num mecanismo sustentável de produção de energia, uma vez que combinam simultaneamente a produção de eletricidade e o tratamento de águas residuais. As CCMs são consideradas uma alternativa promissora aos tratamentos de águas residuais convencionais. Os tratamentos atualmente aplicados exigem elevadas necessidades energéticas e custos de manutenção. As CCMs são capazes de oxidar a matéria orgânica através da atividade metabólica dos microrganismos em condições anaeróbias libertando eletrões. Atualmente a indústria vinícola produz anualmente biliões de toneladas de vinho à escala global, e conseqüentemente grandes quantidades de efluente vinícola têm que ser previamente tratadas antes de serem destinadas à irrigação. A maioria das águas residuais das estações vinícolas possui uma alta carga orgânica com uma carência química de oxigénio (CQO) média de 11 250 mg O₂/L, ácidos orgânicos, açúcares, álcoois, ésteres, polifenóis e, um pH ácido. Por norma, as concentrações dos diversos constituintes do efluente vinícola excedem os limites estabelecidos pela legislação. Para evitar problemas ambientais relacionados com a descarga do efluente vinícola, os tratamentos biológicos anaeróbios e aeróbios são fundamentais para permitir a aplicação deste efluente em solos agrícolas como fertilizante, no entanto, este tipo de tratamento representa elevados custos económicos para a indústria. O desenvolvimento de novas tecnologias sustentáveis e de baixo custo é assumido como uma das prioridades neste sector. A aplicação e desenvolvimento da tecnologia associada às CCMs constitui um conceito bastante promissor, uma vez que as CCMs permitem executar o tratamento do efluente vinícola sem elevadas necessidades energéticas e sem afetar negativamente o ambiente. Nesta experiência laboratorial, analisou-se a culturabilidade e a taxa de remoção da CQO no efluente vinícola sintético (EVS) de *A. aceti*, *G. oxydans*, *S. cerevisiae*, *Z. bailii*, *Z. rouxii*, *B. cereus* e *E. coli*. Os microrganismos foram escolhidos com base no seu comportamento resiliente ao pH ácido e devido ao facto de terem sido identificados durante as diversas etapas da produção do vinho. Entre os sete microrganismos descritos, os dois que apresentaram o maior crescimento celular associado com a maior taxa de remoção da matéria orgânica no EVS foram selecionados e caracterizados, através da sua curva de crescimento, com o objetivo de serem inoculados numa CCM. O desempenho dos microrganismos na CCM foi avaliado em termos da densidade energética obtida, viabilidade celular, taxa de remoção da CQO, taxa de remoção da totalidade dos carbonos orgânicos e taxa de remoção dos polissacarídeos. A quantificação dos sólidos totais (g/L), sólidos voláteis totais (g/L) e sólidos fixos (g/L) dos biofilmes formados em ambas as CCMs foi realizada no final de cada experiência. A CCM inoculada com *B. cereus* (CCM1) apresentou melhores resultados associados à taxa máxima de remoção da CQO (65 ± 4%) e à máxima potência elétrica obtida (8.35 mW / m²) em relação à CCM inoculada com *Z. bailii* (CCM2), a qual evidenciou uma taxa máxima de remoção da CQO de 41 ± 1% e uma potência elétrica máxima de 6.21 mW / m².

Declaração

Declara, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

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Nomenclature

A – Proton exchange membrane area

AGA SA – Álcool e Géneros Alimentares SA

AL - Aerated lagoons

AMBB - Air micro-bubble bioreactor

ANTs - Anaerobic treatments

APA - Portuguese environmental agency

AS- Activated-sludge

ASBR - Anaerobic sequencing batch reactor

ATs - Aerobic treatments

C - Cultaribility

Cd - Cadmium

CFU- Counting forming unit

CNF - Carbon nano fiber

CNTs - Carbon nano tubes

COD - Chemical oxygen demand

Co – Cobalt

CO₂ – Carbon dioxide

Cr - Chromium

Cu - Copper

DET - Direct electron transfer

DC-MFC - Dual-chamber microbial fuel cell

ELV - Emission limit values

FBBR - Fixed Bed Biofilm Reactor

Fe - Iron

Fe(III) - Ferric iron

FePc - Phthalocyanine

FS – Fixed solids

h - Hour

H₂O - Water

I - Current intensity

JLB - Jet-loop bioreactor

kg - Kilogram

kWh - Kilowatt per hour

Pb - Lead

PbO₂ - Lead dioxide

Pt – Platinum

L – Liter

M – Molar concentration

m³ – Cubic meter

Mg - Magnesium

mL - milliliter

MnO₂ - Manganese dioxides

MET - Mediated electron transfer

MBR - Membrane bioreactor

MFCs - Microbial fuel cells

MFC - Microbial fuel cell

NB - Neutral broth

Ni - Nickel

NF - Nickel foam

NF/PANI - Nickel foam with polyaniline

NF/PANI/TC - Nickel foam with polyaniline and titanium carbide

Nt - Nitrogen compounds

OCV - Open circuit voltage

OD – Optical Density

p - significance level

P - Power

PCA - Plate Count Agar

PL - Polysaccharides

Pp - Phosphorus

Pt - Platinum

PTFE - Polytetrafluoroethylene

K – Potassium

r - Pearson correlation coefficient

r^2 - percentage of the variation explained by regression

PEM - Proton exchange membrane

R - external resistance

RWL - Red wine lees

SBR- Sequencing batch reactor

SC-MFC - Single-chamber microbial fuel cell

SO² - Sulfur dioxide

SPSS - Statistical Package for the Social Sciences

SRT - Solid retention time

SWW - Synthetic winery wastewater

CoTMPP - Tetramethylphenylporphyrin

Nafion - Perfluorosulfonic acid

NF/PANI/TC/Chit - Titanium carbide and chitosan

TOC - Total organic carbon

TS - Total solids

TVS - Total volatile solids

U - Current voltage

UASB – Up-flow anaerobic sludge blanket

(v/v %) - Volume percent concentration

WWL - Wine white lees

WW - Winery wastewater

YPD - Yeast extract peptone dextrose

YPD - Yeast nitrogen base

Zn - Zinc

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1. Introduction

Portugal in 2015 was considered the fifth largest wine producer in Europe and the ninth wine exporter worldwide only behind countries like France, Spain, Italy and Germany, which together represent almost 90% of the European market. Portugal has an area of 177 000 hectares destined exclusively to the production of vineyards. The total average volume of wine produced annually in Portugal is 614 million liters.^{1,2} In Estremadura (Portugal) and in Santiago de Compostela (Spain) regions were determined average ratios of 0,7 and 1,8 liters of winery effluent generated per one liter of wine produced.^{3,4} Assuming the average value of these two ratios gives an approximately theoretical estimate quantity of 735 million liters of winery effluent produced every year in Portugal. This theoretical value reflects a high amount of winery wastewater that needs to be pretreated before being destined for irrigation of agricultural soils to prevent possible environmental damages. Winery effluent consist mainly in glucose, fructose and ethanol, and in smaller quantities by organic acids, glycerol and polyphenols^{5,6}.

To not exceed the limits imposed by the wastewater discharge legislation⁷, diverse aerobic and anaerobic biological treatment systems are being applied with the objective of removing the pollutant organic load from the vineyards effluents to rates higher than 99%. However, the great majority of these treatments represent high economical costs to the winery sector associated with the purchase of infrastructures, energy maintenance of the system and biomass disposal.^{8,9} The development of self-sustainable MFC systems to complement and achieve the winery wastewater treatment without high energy needs (as for example aeration energy used in the aerobic treatment) appears as a promising concept for the winery industry. MFCs are bio-electrochemical systems capable of metabolize organic matter into electricity, by using microorganisms under anaerobic conditions.¹⁰ MFCs are composed by an anode, an external circuit, a cathode and a semipermeable proton exchange membrane (PEM) that keeps separated the anode from the cathode.¹¹ Nonetheless, until now, only eight studies have reported the winery wastewater treatment with MFCs and in those studies only *Acetobacter aceti*, *Gluconobacter roseus* and activated sludge were used as anodic inoculum.^{12–15,16,17,18,19,20}

The objective of this project is to contribute to the provision of information and results that allow a greater understanding and contextualization of the potentiality of the MFC technology as a possible complementary alternative to the winery effluent treatment. In the first chapter an introductory and descriptive approach is made to the project outline. The second chapter provides a general description of the main concepts associated with MFC technology, the operating principle, the electron transfer mechanisms, the most used and promising materials, the MFC configurations and the manly operational conditions (such as pH, temperature, etc.). In the third chapter, a characterization of the organic and inorganic constituents of the composition of the

winery wastewater is described (in this chapter it is also reflected the influence of the vinification processes in the winery residues production). The fourth chapter presents an economic, energetic, environmental and performance comparison and analysis between the biological treatments typically applied to the winery wastewater and the MFC technology. In the fifth chapter, a bibliographic research was done to identify which potential microorganisms could be tested as inoculum in the application of MFCs for the efficient treatment of the winery effluent. In the sixth chapter, a state of the art is provided through a bibliographical review of the scientific reports on the winery effluent treatment by using MFC technology. The seventh chapter describes the materials and methods used. The eighth chapter refers to the results and discussion of the laboratorial experiment. The ninth chapter makes the statically relations between the experiment analytical parameters in both MFCs. The tenth chapter mentions the conclusions and the suggestions for future work. The eleventh and twelfth chapters presents the references and the annexes, respectively.

The focus of this project was based on two distinct stages. In an initial phase, the aim was to screen, from a selection of seven microorganisms, the two microorganisms which presented better results associated with the conjugation of cell culturability and organic matter removal rate for the SWW. The microorganisms were chosen with the objective of being inoculated and tested in a SC-MFC. In the next stage, the performance of the two microorganisms in the winery wastewater treatment by using two identical SC-MFC were analyzed and compared. The performance of each inoculum was evaluated based on power density achieved, cell culturability, COD, TOC and polysaccharides removal rate and biofilm quantification in terms of concentration of total solids, total volatile solids and fixed solids.

2. Microbial Fuel Cell technology review

2.1. MFC principle

MFCs are a sustainable way to produce energy that combines the energy production and wastewater treatment. MFCs are electrochemical biocatalyst systems capable of transforming biodegradable organic and inorganic matter into energy in form of electricity current. MFCs are constituted by an anode, an external resistor, a cathode and a proton exchange membrane (PEM). At the anode side (negative terminal) the substrate is oxidized by the microbial metabolism activity under anaerobic conditions, releasing electrons and protons. The electrons can be transferred directly by the microorganisms or indirectly through mediators to the anode surface. The electrical circuit, which is connected to an external resistor, is responsible for the transference of electrons from the anode side to the cathode side, generating a positive current flow. At the cathode side (positive terminal), the electrons are released and transferred to a final electron acceptor with high reducing potential (usually oxygen but also nitrate, sulfate, ferricyanide, etc.). Simultaneously, the semipermeable membrane facilitates the protons diffusion to the cathode side. In the cathode side a reduction reaction occurs among the electrons, protons and the final electron acceptor, producing water (H₂O) as final product.^{21,9,22}

Figure 1 presents a schematic illustration of a dual-chamber-MFC (DC-MFC) using oxygen (O₂) as final electron acceptor.

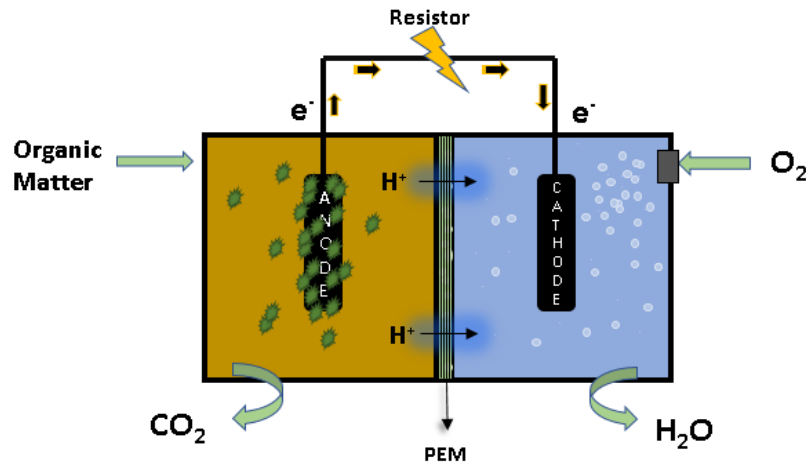
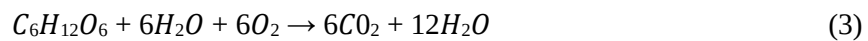
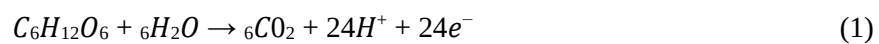


Figure 1: Schematic illustration of a dual-chamber MFC configuration and principle.

The following equations represent the reactions involved in the glucose oxidation process of a MFC.²³



First equation represents the glucose oxidation and the production of carbon dioxide, protons and electrons in the anode chamber. The second represents the oxygen reduction in cathode side, and the third represents the overall balance between the two previous equations.

2.2. Electron transference mechanisms

The two best known possible electron transfer mechanisms in MFCS are direct electron transfer (DET) and mediated electron transfer (MET).²⁴

DET is based in the transport of electrons promoted by the physical contact of the microorganisms with the anode surface through proteins located in their outer membranes (such as cytochromes) or through nanowires.²⁵ Nanowires (also called conductive pili) are filaments that establish the connection between the outer membrane proteins of the microorganism and the electrode surface, letting not only the microorganisms that are closer to the anode efficiently transfer electrons but also the microorganisms that are at the external layers of the biofilm adhered to the anode, allowing the increment of the electricity current.²⁶ DET mechanism was verified in some microorganisms capable of reducing solid terminal electron acceptors such as ferric iron (Fe(III)) and electrodes in the absence of oxygen. *Geobacter sulfurreducens*,²⁷ *Shewanella putrefaciens*,²⁸ *Shewanella oneidensis*,²⁹ *Rhodospirillum rubrum*,³⁰ and *Desulfobulbus propionicus*³¹ are some of the principal microorganisms associated to the DET mechanism. Despite of the capacity of directly transfer electrons to the electrode surface, this type of microorganisms is metabolic limited, being in general only capable of using products of fermentative processes (such as acetate, lactate, etc.) as electron donor.^{32,33}

MET consists in the electron transport from the microorganisms to the anode surface promoted by mediators. Mediators act as electron-shuttles and increase the power output generation through a cyclic process in which the mediator alternates between the reduced state, when captures the electron from the microorganism, and the oxidized state, after transferring the electron to the anode surface.²⁴ Mediators are useful to allow the transference of electrons in microorganisms without the capacity of directly transferring electrons to the electrode, and can be synthetic (exogenous) or natural (endogenous).²² Artificial mediators such as neutral red,³⁴ methylene blue³⁵ or thionine,³⁶ can reach high values in terms of electrical generation in a short period, however, synthetic mediators have proved not to be the best option among mediators in a long-term period, since their application can be associated to environmental issues related to water contamination, microorganisms intoxication and extra economical costs.^{23,37} Natural mediators are usually nontoxic and low-cost, and can be self-produced by some microorganisms in the absence of a final electron acceptor or being present in the medium composition. *Pseudomonas aeruginosa* can produce pyocyanin, which can act as an electron shuttle and enhance the power output production.³⁸ Ieropoulos et al.³⁹ demonstrated that *Desulfovibrio desulfuricans* is capable to use sulphate, typical found in wastewaters such as winery wastewater, as mediator. The study

released by Rabaey et al.³⁸ proved that microorganisms can use mediators produced by other different species, this possibility allows the microorganisms survival in conditions that they typical could not adapt.

2.3.MFC materials

The right electrode material choice is crucial to increase the power generation and reduce the internal resistance of the MFC. Electrodes materials should have the following properties: high electrical conductivity to decrease the internal resistances to the electron transference; biocompatible to avoid issues related to microorganism's intoxication; mechanical strong to allow the durability of the system for a long-term period; high surface area and porosity to improve the microbial adhesion to the electrode.¹¹

2.3.1. Anode materials

Carbon materials are the most used in anodes in MFCs, due to their good electrical conductivity, high superficial adhesion, considerable mechanical stability and low-cost. Carbon materials include: graphite rod, graphite felt, graphite brush, carbon cloth, carbon paper and carbon mesh.^{9,11,22,23,25,40} Graphite rod material is very used in MFC anodes, because it is cheaper, however, it produces low power density and has less porosity and superficial surface when compared with others carbon materials. Graphite brush consists in a metal wire (usually titanium) surrounded with graphite fibers, revealing good porosity and higher power generation. Carbon cloths are one of the best carbon material, presenting high mechanical strength, superficial surface area and power production, however their economical cost does not make them viable in a large scale. Carbon paper presents a reasonable superficial surface and electrical conductivity, but does not have sufficient mechanical strength. Metal based materials like or aluminum, gold or cooper are much more conductive than carbon materials, but their application in anodes are inviable, due to their toxicity, corrosion proprieties, high cost and smooth surface that decrease the biofilms adhesion, only noncorrosive stainless steel seems to be a good metal option.^{41,42} Many treatments and modifications had been applied to the anodes surface, ammonia gas treatment increased the power generation by 20% in a carbon cloth anode.^{41,42} The combined heat and acid treatment in anodes improved the power production.^{43,44} Electrodeposition of manganese dioxide (MnO₂) showed to increase the carbon felt anode power generation up to 25%, in fact manganese dioxide application in electrodes can be a good option, because it is cheap, abundance, biocompatible and has eco-friendly proprieties.⁴⁵ Carbon nano tubes (CNTs) and carbon nano fiber (CNF) have become two of electrode materials with the most potential, because of their very good proprieties, however their expensive costs and the possibility of cellular toxicity limit their actual application.^{11,46}

2.3.2. Cathode materials

Cathode materials are generally made by graphite, carbon cloth or carbon paper.¹¹ To increase the reduction reaction with the final electron acceptor, platinum (Pt) has been currently used as catalyst by coating the cathode surface. Despite its good reduction potential, the application of platinum is not seen as a viable solution in an industrial context due to its high economic costs.⁴⁷ Other less expensive materials, such as cobalt tetramethylphenylporphyrin (CoTMPP)⁴⁸, iron phthalocyanine (FePc)⁴⁹ and manganese dioxides (MnO₂)⁵⁰ are being proposed as alternatives to platinum. Binders are used to fix the catalyst onto the cathode. The two most used binders are perfluorosulfonic acid (Nafion) and polytetrafluoroethylene (PTFE).⁴⁸ Using Nafion is possible to obtain 14% more power output than PTFE, due to the Nafion thickness that allows avoiding ohmic losses. However using Nafion represents a highly cost.⁴⁸

2.3.3. PEM materials

PEM allow proton transfer from the anode compartment to the cathode side, generating a positive potential and the oxygen reduction. PEM also avoid problems related to oxygen leakage to the anode chamber, substrate loss and the accumulation of protons.⁵¹ Nafion membranes have been widely used as PEM due to their high selective proton diffusion, however, this material is very expensive making their large-scale application not viable.⁵² For this reason, others cheaper materials are being studied. Glass fiber produces low internal resistance, has a good level of proton transference and low rate of oxygen transference to the anode.⁵³ The increase in the surface area of the PEM result in a decrease in the internal resistances allowing to achieve higher energy densities.⁵⁴

2.4. MFCs configurations

Dual-chamber (DC) and single-chamber (SC) are the two most used configurations in MFC technology.²⁵

DC design consists of an anodic and cathodic chamber separated by a PEM. DC-MFC is the design associated with higher power densities, however, their application in a large scale have high associated economic costs. DC configuration has the limitation of requiring continuous energy input for the cathode aeration to allow the solubilizing of O₂, or the addition of another possible costly final electron acceptor, such as ferricyanide (K₃[Fe(CN)₆]).^{55,14} SC design consist of an anodic chamber separated by a PEM from an open cathode exposed to the air. SC-MFC reduction reaction is not as effective as DC-MFC and for that reason the energy produced is lower, however its application in an industrial scale is more economic viable, as SC-MFC does not need energy requirements for the aeration process by using the O₂ naturally present in the atmospheric air.²³ Figure 2 shows a schematic representation of a typical SC-MFC.

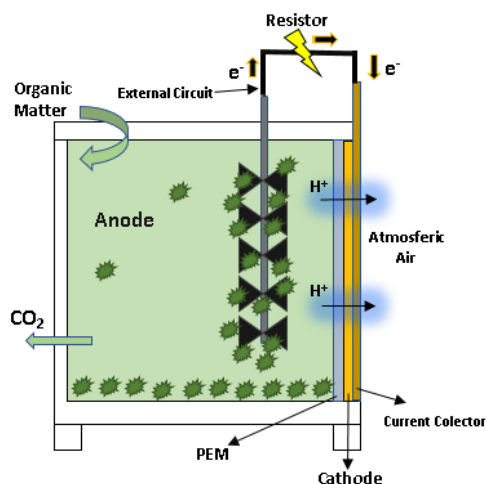


Figure 2: Schematic illustration of a SC-MFC configuration and principle.

2.5. Operational conditions

In addition to the mediators used, the type of materials or the configuration applied other operating conditions show to have high influence on the MFCs performance.

The lower PEM efficiency over the operational activity time of MFC can result in a proton diffusion decrease between the anode and the cathode side.⁵⁶ The accumulation of protons at the anode causes the pH drop to acidic levels. The acid pH, in general (depending on the characteristics of the microorganisms used), is related to a lower metabolic activity which may result in the decrease of the efficiency of the MFC in terms of organic matter removed and energy production.^{21,57} However, the decrease in pH can also be associated with the increase of energy efficiency. Raghavulu et al.⁵⁸ reported a decrease of the H^+ reduction reaction at the anode side, resulting in a higher electron release, energy production and a decrease of metabolites production.

The temperature suitable for optimum growth of the microorganisms involved in the MFC treatment is between the mesophilic temperature range, however, a prior determination of the optimum temperature of the microorganism used as an inoculum should be performed.⁵⁹ The increase in temperature is also related to the increase of the electrical conductivity of the fluid treated. The increase in ionic conductivity translates into a decrease in ohmic losses.⁶⁰

Ohmic losses are also related to the space applied between electrodes and with the anode surface. The existence of a smaller distance between the electrodes and a high porosity anode surface, decreases the internal resistances and provides a smaller loss of electrons.^{40,18}

The nature of the catholyte can have influence in the MFC performance. Ferricyanide was used as final electron acceptor, revealing a more effective reduction reaction and better results associated with the energy production when compared to oxygen.⁵⁷ However, its use implies higher economic costs and toxicity problems related with the probability of ferricyanide crossover the PEM.¹⁴

3. Winery effluent characteristics and composition

To potentiate and determine the ideal operational conditions for the application of MFCs in the winery wastewater treatment it is previously necessary to identify which are the main organic and inorganic components of the winery effluent. The winery effluent characterization represents great similarity in its constitution, however, in quantitative terms it is difficult to be determined or standardized. The reason for the different concentrations of the diverse parameters present in winery effluents is mainly due to the different amounts of water used to clean the infrastructures associated with the wine production.⁶¹ Other factors such as unpredictable climatic conditions; different types and properties of the grapes of the various regions where the wine is produced; the area and soil conditions and especially the wine characteristics sought, have also great influence in the winery wastewater characteristics.^{62,61}

During the high season (harvesting and vinification), more precisely between the months of September and December, the organic load of the winery effluent can increase between three to four times in relation to the organic load of the remaining months of the year.^{63,64} The winery effluent is a type of wastewater characterized by its high organic load, mainly composed of ethanol and sugars (glucose and fructose), which together represent 75% to 90% of the total organic matter present in the winery effluent and organic acids containment which represent about 10% of the total organic load.^{65,66,67} Organic acids have high importance in the organoleptic characteristics of the wine, such as flavor, aroma and texture, besides that, organic acids can perform preservative functions in the quality of the wine, since they allow the lowering of the pH and consequently favor the inhibition and the control of the microorganisms' growth.⁶⁸ The organic acids present in the winery wastewater are divided in two different categories. There are organic acids that are already naturally present in the composition of grapes such as tartaric acid (which is generally not metabolized), malic acid and citric acid, in addition there are organic acids that are metabolized by microorganisms during must fermentation, such as succinate acid, pyruvic acid, lactic acid and acetic acid.⁶⁹ Malic acid is generally found in winery effluents in low concentrations since most wine producers choose to carry out malolactic fermentation after alcoholic fermentation, which consists in the degradation of malic acid into lactic acid with the intervention of lactic acid bacteria, due to taste, aroma and acidity proprieties, allowing to increase the pH by about 0.3.⁷⁰ The application of ascorbic acid together with sulfur dioxide (SO₂) is also present in the wine constitution and consequently in the winery effluent, the objective of these two compounds is to increase the antimicrobial properties and maintain the antioxidant characteristics of the wine,⁷¹ which include the reduction of the oxidation of polyphenols to quinones.⁷² The use of ascorbic acid favors the addition of smaller amounts of SO₂, which intake in the ionic form (HSO₃⁻) is not beneficial to the human health.⁷³ Sulfur dioxide and ascorbic acid are added at concentrations between 50 mg and 200 mg per liter of wine, respectively.⁷¹

Polyphenols are also present in wine and winery effluent composition. Polyphenols are found in the initial composition of the plant and the fruit (such as grapes) and attribute characteristics responsible for color, bitterness and astringency, as well as having human health benefits such as antioxidant and antihypertensive properties.⁷⁴ There is a large difference between the concentration of polyphenols in white wines and in red wines, according to Mattivi et al.⁷⁵ white wines have only a polyphenol concentration between 30 and 120 mg/L, whereas red wines have a rather high polyphenol concentration between 264 mg/L and 3451 mg/L. The difference in polyphenols concentration makes red wines with a higher associated pollutant load. Red wines are subjected to the maceration process, which consists in the extraction of anthocyanins and tannins or other phenolic components from the must, generally during between ten to thirty days to allow maintain the color and taste characteristics typical found in red wines.⁷⁶ Phenolic compounds extraction can also occur during the wine maturation before bottling, which consists in wine storage during several months in tanks with different concentrations of oxygen.⁷⁷ Polyphenols in the biological treatment (including MFC treatment) of the winery wastewater are a disadvantage, since these compounds have antimicrobial properties, which potentiate the inhibition of the proliferation of the microorganisms responsible for the degradation of the organic matter and consequently the COD removal rate decreases.^{78,19} In winery wastewater composition it is also important to mention the presence of microorganisms, Jourjon et al.⁷⁹ identified the presence of acetic, lactic and yeast bacteria between 10^5 and 10^8 CFU/mL.

In Table 1 is possible to analyzed main constituents and parameters based in the manly studies in which is reported the winery wastewater composition.^{6,80,5,65,81,62,67,82,66}

Winery effluent presents several inorganic compounds in its composition. Table 2 presents the analysis performed on eight Spanish wines by Bustamante et al.⁶ Of the various inorganic components analyzed, nitrogen compounds (Nt), phosphorus (P), potassium (K), magnesium, (Mg), iron (Fe), copper (Cu), calcium Manganese and zinc (Zn) are part of the nutritional composition of the grape and therefore are also found in the winery wastewater.⁸³ Metals such as cobalt (Co), chromium (Cr), cadmium (Cd), lead (Pb) and nickel (Ni) were identified in the composition of the winery effluent possibly as consequence of the solubilizing of metallic compounds of the infrastructures where the wine is produced.⁶ It is possible to verify that potassium, sodium and calcium are the inorganic components that are in greater quantity in the winery effluent.

Table 1: Organic composition and characteristics of the winery wastewater from the analysis of nine studies.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	Mean
COD (mg O ₂ / L)	49 105	3 555	3 449	12 750		18 200	-	4 415	14 600	11 246
BOD₅ (mg O ₂ / L)	22 418	1 855	-	-	-	13 810	-		-	12 694
pH	5.5	6.1	4.43	5.6		3.8	-	3.7	5.0	4.9
Electrical conductivity (S/m)	0.19	-	-	2.32		-	-	-	-	1.3
Total solids (mg/L)	18 336	4 000	-	2 050		-	-	-	-	8 1
Suspended solids (mg/L)	5 137	180	-	-	-	995	-	-	-	2 104
Total sugars (g/L)	-	-	1.76	0.805	-	-	0.752	2.8	0.87	1.4
-Glucose (g/L)	-	-	0.9	0.32	-	-	0.32	-	-	0.5
-Fructose (g/L)	-	-	0.86	0.48	-	-	0.43	-	-	0.6
Citric acid (mg/L)	-	-	0.013	-	-	-	55	-	-	27.5
Tartaric acid (mg/L)	-	-	0.48	890	530	-	-	1 445	1260	825.1
Malic acid (mg/L)	-	-	0.0012	6.5	-	-	-	10	7	5.9
Lactic acid (mg/L)	-	-	0.31	180	350	-	-	-	160	172.6
Succinic acid (mg/L)	-	-	-	6		-	-	-		6
Acetic acid (mg/L)	-	-	191	245	10	-	-	-	300	186.5
Polyphenols (mg/L)	140	845	7.3	-	-	-	-	30	-	255.6
Ethanol (g/L)	-	-	-	4.4	3.13	-	1.53	-	-	3
Glycerol (mg/L)	-	-	-	295	14	-	-	-	320	209.7

References: (1), Bustamante et al.⁶ (2005); (2), Vlyssides et al.⁸⁰ (2005); (3), Malandra et al.⁵ (2003); (4), Colin et al.⁶⁵ (2005); (5), Chapman et al.⁸¹ (1995); (6), Zhang et al.⁶² (2008) ;(7), Quayle et al.⁶⁷ (2009); (8), Mosteo et al.⁸² (2006); (9) Bories et al.⁶⁶ (2005).

Table 2: Inorganic composition of the winery wastewater.⁶

Parameter	(mg/L)
Nt	35
P	35
Na	158
K	270
Ca	545
Mg	36
Fe	12
Mn	0.31
Cu	0.79
Zn	0.58
Co	0.17
Cr	0.15
Cd	0.06
Pb	1
Ni	0.12

According to Vlyssides et al.⁸⁰ the winery effluent is produced during the various stages of the wine production. The first stage consists in the reception of grapes which in Mediterranean region it usually occurs from the end of August until the beginning of October.^{3,64,84,85} Then, the production of the must occurs in which step the grapes are crushed by the action of a pneumatic press. During the following 15 to 30 days after the must production, the alcoholic fermentation and the maceration takes place. After, through tubes, the fermented wort is transferred to a decanter, in which the supernatant is removed from the remaining disinterest components, such as yeasts, bacteria and grape residues. Next, the wine that has been decanted may eventually be transferred through pipes to a new tank where the malolactic fermentation may possible occur. After the fermentation processes and the decantation are completed, the wine is matured, this stage usually occurs during the last days of November and allows the wine to stabilize its properties. Then, in the first days of December the wine is filtered to eliminate possible solid residues that may still be contained in the wine. After filtration, between January and February, the wine is transferred through pipes and pumps to the maturation and bottling units. Until the end of July, the wine is packaged and transported to the buyers.

There is a large difference between the quantities of winery effluent produced during the year. The period in which larger quantities of wine residues are produced is directly associated with the months in which grapes harvest and wine production occur. Fernandez et al. reports that 67.5% of the effluents are generated between September and November. At a winery station in Domaine du Mouton, about 63% of the winery effluent was produced between August 24 and October 26.⁸⁵ According to Vlyssides et al.⁸⁰ about 75% of the winery wastewater is produced during the months of August to December, specifically during cleaning of the machines associated with the storage and pressing of the grapes, the decanting and filtration processes, as well as the floors of the various facilities. The cleaning of the boxes and tanks associated with the storage of grapes, must and wine, the pipes and pumps responsible for the transportation of wine and the machinery associated with bottling and packaging also contribute, albeit to a smaller extent, to the production of the winery effluent. In annexes is provided a table based on the study made by Vlyssides et al. which provides information on the production of winery wastewater in liters per one liter of wine produced.

4. Anaerobic treatment vs aerobic treatment vs MFC treatment

Different types of aerobic and anaerobic biological treatment systems are applied with the objective of reducing the pollutant organic load from the effluents of the vineyards, however the great majority of these treatments present high costs associated with the purchase of infrastructures and energy maintenance of the system.⁸

In Portugal, only 17% of the wineries have an environmental license issued by the Portuguese Environmental Agency (APA), under the terms of Decree-Law no. 173/2008, of

August 26 (PCIP Diploma).⁸⁶ In the following table it is possible to observe the emission limit values (ELV) of different parameters, stipulated by Decree-Law no. 236/98 of August 1, 1998 published in “Diário da República”.⁷

Table 3: Portugal legislation of the ELVs in wastewater discharge.⁷

Parameter	ELVs in wastewater discharge
Ph	6 - 9
CBO₅ (mg O ₂ /L)	40
COD (mg O ₂ /L)	150
SST (mg/L)	60
C₆H₅OH (mg/L)	0.5
Fe (mg/L)	2
Mn (mg/L)	2
S (mg/L)	1
SO₃ (mg/L)	1
P (mg/L)	10
NH₄ (mg/L)	10
N (mg/L)	15
NO₃ (mg/L)	50
Pb (mg/L)	1
Cd (mg/L)	0.2
Cr (mg/L)	2
Cu (mg/L)	1
Detergents (mg/L)	2

Anaerobic biological treatments consist in the degradation of the large part of the organic matter present in the effluent through the presence of a consortium of microorganisms in the absence of oxygen. This treatment consists in three steps: hydrolysis; acidogenesis and methanogenesis which results in the production of methane, water and carbon dioxide.⁸⁷ To promote the growth of methanogenic microorganisms, the pH and temperature are maintained, respectively, neutral (between 6 and 8) and mesophilic (between 50 °C and 70 °C).^{88,89} The COD removal rate after the anaerobic treatment of the winery effluent generally ranges from 65% to 95% with a biogas production of 500 L per kg of COD removed (70% of methane).⁹⁰ Aerobic biological treatments consist in the degradation of the organic matter through microorganisms in the presence of oxygen and are generally applied to maximize the efficiency of the organic matter removed after the anaerobic winery effluent treatments.^{90,91}

From an environmental point of view, anaerobic treatments (ANTs) are more advantageous, since they do not require the use of aeration energy such as aerobic systems require (usually 2 mg O₂/L of wastewater treated).^{90,92,93} Almost every carbon dioxide (CO₂) produced during acidogenesis and acetoclastic methanogenesis (conversion of acetic acid to methane and

carbon dioxide) is used with hydrogen in the hydrogenophilic methanogenesis to obtain methane and water.^{89,94} The methane produced is used as biogas and for this reason anaerobic treatment is considered a sustainable way to obtain energy.⁸⁹ Also, ANTs are both economical viable and more efficiency to treat high organic loads.⁹⁵ In contrast, ANTs present some disadvantages, which refer to the costs associated with increasing of the operating temperature in order to reach the optimum mesophilic temperatures for the microorganisms growth; the costs associated with the application of alkaline solutions (between 2500 mg/L and 5000 mg/L) to maintain the pH neutral and to avoid the toxicity caused by the acidity of the intermediate compounds (such as hydrogen sulphide); and to the odors caused by volatile fatty acid emissions during methanogenesis.^{96,91,66} Anaerobic systems have an average associated cost of around € 15 000 per m³ of digester produced,⁹⁰ with an operating costs and generated revenues of 0.01 €/kg of COD and 0.1 €/kg of COD, respectively.⁹⁷

Aerobic treatments (ATs) present advantages related to the high efficiency in terms of organic matter removal and the possibility of operating at ambient temperature without the need for heating.⁹⁵ However, from an economic point of view, aerobic treatment is the system that presents the highest expenses without any type of economical return. ATs requires on average the use of 1 kWh for the aeration supply in the degradation of 1kg of COD, besides the aeration problem, the biomass produced have a post-treatment associated cost around 500-600 euros per ton of dry biomass produced.^{98,9}

Currently, the technology associated with the application of MFCs in the treatment of winery effluents is still in an initial stage and with an economic disadvantage when compared to the conventional treatments. The actual associated costs corresponding to 8 euros / kg of COD, however, it has already been found that the higher share of costs associated with MFC technology is supported by the material used as catalyst to promote the reduction reaction at the cathode side (such as platinum), by about 47% of the overall costs. After the catalyst, is the PEM which represents a highly cost, around 38% of the overall associated costs.⁹⁷ Reducing those expenses and find new possible materials can eventually make this process economically viable, since according to Pham et al.⁹⁹ MFCs can theoretically reach to a maximum of 4 kWh/kg of COD.

MFCs can act as a support and complement to anaerobic treatments, since at an operating temperature of less than 20 °C and reduced organic charges, methanogenesis is not effective enough.⁹⁹ In an hypothetical optimistic scenario, typical aerobic treatments that are used to maximize the COD efficiency of anaerobic systems, could be eventually replaced by MFC technology to reduce the high associated costs of aeration energy input.

The following table was based on the studies referenced in this chapter and summarizes the comparative parameters between the anaerobic treatment, aerobic treatment and MFC treatment.

Table 4: Comparison between anaerobic system, aerobic system and MFC system in the winery wastewater treatment based on the studies referenced in chapter 4.

Parameter	Anaerobic Treatment	Aerobic Treatment	MFC Treatment
Energy requirement	Moderate	High	Low
Organic removal efficiency	65% - 95%	90-99%	10%-93%
Organic Load rate	High	Low	Low
Start up time	2 to 4 months ⁹⁵	2 to 4 weeks ⁹⁵	variable
Nutrient requirements	Low ⁹¹	High ⁹¹	Moderate
Odor problems	High ¹⁰⁰	Low	Low
Methane and hydrogen production	Yes	No	No
Electricity production	No	No	Yes
Sludge production	0.077g/g COD removed ⁹⁸	0.4g/g COD removed ⁹¹	0.07-0.22g/g COD removed ⁹⁸
Actual economical balance costs	Moderate	High	High
Temperature and pH sensitivity	High	Moderate	Moderate

Table 5 shows the different COD removal rates of the existing winery wastewater treatment systems.

Table 5: Different treatment systems and associated COD removal rates in the winery wastewater treatment.

Treatment System	COD removal rate	References
Aerobic Treatment		
Fixed Bed Biofilm Reactor (FBBR)	91%	Andreottola et al. ¹⁰¹ (2005)
Membrane Bioreactor (MBR)	>97%	Artiga et al. ⁹³ (2005)
	96%	Bolzonella et al. ⁶⁴ (2010)
Sequencing batch reactor (SBR)	93%	Torrijos et al. ⁸⁵ (1997)
Jet-Loop Bioreactor (JLB)		
Activated-Sludge (AS)	>97%	Fumi et al. ⁶¹ (1995)
	>90%	Petruccioli et al. ¹⁰² (2000)
Aerated lagoons (AL)	91%	Montalvo et al. ¹⁰³ (2010)
Air micro-bubble bioreactor (AMBB)	99%	Oliveira et al. ⁸⁶ (2009)
Anaerobic Treatment		
Upflow anaerobic sludge blanket (UASB)	>90%	Keyser et al. ¹⁰⁴ (2003)
	>80%	Moletta et al. ⁹⁰ (2005)
USBF	98%	Molina et al. ¹⁰⁵ (2007)
Anaerobic sequencing batch reactor (ASBR)	98.8%	Ruíz et al. ¹⁰⁶ (2002)

MFC Treatment		
	90%	Sciarria et al. ¹⁹ (2015)
	11%	Penteado et al. ¹⁵ (2016)
	17%	Penteado et al. ¹² (2015)
	41%	Penteado et al. ¹⁴ (2016)
	10%	Penteado et al. ¹³ (2016)
	87.5%	Rengasamy et al. ¹⁰⁷ (2012)
	93%	Rengasamy et al. ¹⁸ (2016)

5. Potential microorganisms for the inoculation of MFCs in the WW treatment

There are microorganisms with metabolic characteristics that enable their survival in winemaking environments and for that reason could be potential candidates to act as inoculum in the winery effluent treatment through MFC technology. This section contemplates a characterization of the microorganisms identified during the various stages of wine production, wine storage and biological treatments of the winery effluent.

Pseudomonas sp. followed by *Bacillus* sp., *S. cerevisiae* and *Flavobacterium* sp. were the most isolated microorganisms by Petruccioli et al.^{102,108} in the aerobic treatment of winery wastewater by activated sludge bioreactors. Also, *Pseudomonas* sp., *Bacillus* sp. followed by *Candida* sp., *Agrobacterium radiobacter*, *Acinobacter* sp., *Trichosporon capitatum* and *Geotrichum peniculatum* were the main microorganisms isolated during the winery effluent treatment in a jet-loop activated sludge reactor.^{108,109} Keyser et al.¹⁰⁴ identify six strains in the winery effluent as *Enterobacter sakazakii*, *Bacillus licheni-formis*, *Bacillus megaterium*, *Bacillus licheniformis*, *Brevibacillus lateroporus* and *Stphylococcus* sp. *E. sakazakii*, among the genus identified, was the microorganism which produces the higher quantity of volatile fatty acids from the winery effluent degradation, indicating its potential for the winery wastewater treatment. In fact, *E. sakazakii* was tested as inoculum with granular sludge in the USBA performance, removing 90% of the total organic charge present in the winery effluent. Cusick et al.²⁰ analyzed the microbial community present in the biofilm produced during the winery wastewater treatment by using a microbial electrolysis cell (MEC), the predominant bacteria in the biofilm were *Geobacter sulfurreducens* (44%), *Roseivivax* sp. (14%), *Pelobacter propionicus* (8%) and *Rhodopseudomonas palustris* (3%).

Yeasts are facultative anaerobic microorganisms, that can oxidize preferential sugars and others carbon substrates, like for example, alcohols and organic acids into CO₂ and H₂O. When the oxygen is at low concentrations or the substrate is at high inhibiting concentrations, these microorganisms convert sugars into ethanol without the presence of oxygen by the alcoholic fermentative pathway. This anaerobic process is one of the most important in industrial application and it is involved in the processing of diverse alcoholic beverages, such as beer or

wine, and in bioethanol production.¹¹⁰ Due to its good adaptive characteristics to acid environments, high osmotic concentrations and high ethanol tolerance, *S. cerevisiae* yeast is being widely used in beverages and bioethanol industries.¹¹¹ *S. cerevisiae* grows in pH range between 2.5 and 6.2 and a temperature between 30 °C and 35°C.¹¹² *S. cerevisiae* is one of the most alcohol tolerant microorganisms, which is able to metabolize glucose at ethanol concentrations around 20 % (v/v %).¹¹³ Some studies show the presence of this yeast in the winery wastewater treatments using activated sludge. Eusébio et al.¹⁰⁹ isolated *S. cerevisiae* strain during the microbial characterization of activated sludge in the aerobic winery wastewater treatment. Maladra et al.⁵ identified *S. cerevisiae*, among other species, such as *Candida intermedia*, *Hanseniaspora uvarum* and *Pichia membranaefaciens*, as one of the most dominant species in the biofilm aggregation to a rotating biological contactor in the winery wastewater treatment. *Zygosaccharomyces bailii* and *Zygosaccharomyces Rouxii* yeasts are characterized by its low permeability to the weak acids and sulfites used as preservatives in the alimentary and beverage industry.^{114,115} Kalathenos et al.¹¹⁶ identified *Z. bailii* among other yeasts as the one with higher resistance to acetic, malic, tartaric, lactic and citric acids usually present in the wine. *Z. bailii* yeasts can use glucose, ethanol and glycerol typical found in wine as carbon source,¹¹⁷ and can metabolize acetic acid even when glucose is being oxidized.^{118,119} The organic acid tolerance of *Z. bailii* allows its growth in low pH values between the range of 2 and 7.^{118,119} According to Thomas et al.¹¹⁷ this specie can tolerate high sulfite and ethanol concentration present in wine, as can be proved by the identification of *Z. bailii* in wine after nine months of being bottled, which represents well the capacity of this yeast to survive in typical wine conditions.

Acetic acid bacteria are aerobic microorganism that can completely oxidize diverse substrates present in winery effluent such as glucose, glycerol, acetate, and lactate into CO₂ and H₂O.¹²⁰ This microorganism has also the capacity of oxidizing ethanol into acetaldehyde and acetic acid. Joyeux et al.¹²⁰ and Drysdale and Fleet¹²¹ proven that some acetic acid bacteria can survive in acidic pH between 3 and 4 and in high ethanol conditions such as during the fermentation and storage of wine. Joyeux et al. conclude that *Acetobacter aceti* and *Acetobacter pasteurianus* were the most adaptive species during vinification processes of French wines. This study also shows the presence of *A. aceti* and *A. pasteurianus* after nine months of the storage in barrels, which is a good indicative of the capacity of these microorganisms to survive to wine conditions. A more recent study release by Du Toit et al.¹²² demonstrates that *A. pasteurianus*, *Acetobacter liquefaciens* and *Acetobacter hansenii* can survive in winery conditions and that *A. pasteurianus* presents the higher viability in South African wines, respectively, at the middle and at the end of the fermentation process in relation to *G. oxydans*, *A. aceti*, *A. liquefaciens* and *A. hansenii*. The notable adaptive characteristics of acetic acid bacteria and the possibility of using diverse carbon sources present in the winery effluent composition, turn its applicability in the winery wastewater treatment by using MFCs a possible option to be considered. Despite of being

strict aerobic these microorganisms had proven that can eventually use others final electron acceptors despite of oxygen.^{123,17}

Lactic acid bacteria are also present in winery conditions since wine producers use lactic acid bacteria to induce the malolactic fermentation.⁷⁰ Du Plessis et al.¹²⁴ identify *Oenococcus oeni* as the lactic acid bacteria dominated specie responsible for spontaneous malolactic fermentation and present during the must production and wine fermentation. *Lactobacillus hilgardii*, *Lactobacillus brevis*, *Lactobacillus paracasei* and *Lactobacillus vemiciforme* were also responsible for the malolactic fermentation and were identify during different wine stages but in low numbers in relation to *O. oeni*.

6. Analysis of the scientific studies involving the WW treatment with MFCs

This chapter includes an analysis of the studies involving the treatment of the winery effluent by using MFCs. There are only eight studies done on this subject, which reveals the existence of a somewhat unknown field in which few conclusions were made.^{12–15,16,19,20,17,18} Therefore, there is an opportunity in this area of scientific research for the development of the winery wastewater treatment with the application of the MFC technology.

Recently, in 2015, Sciarria et al.¹⁹ report a study involving the treatment of a winery effluent contained in white wine lees (WWL) and red wine lees (RWL). According to Sciarria et al. lees represent 4% of the winery wastewater production, which in an industrial scale means a considerable quantity of organic compounds that needs to be treated before disposal. Microorganisms present in the MFC filled with WWL wastewater indicated to be more suitable to degrade organic matter already present in the effluent and the organic compounds produced during the fermentative processes, in relation to the RWL microorganisms. The most plausible explanation for the different performances and results between these two types of lees is the fact that RWL presents a higher amount of polyphenol concentration in relation to WWL (180 mg/L compared to 15 mg/L). Due that, high polyphenol concentration in RWL was probably responsible for intoxication of the microorganisms involved in the carboxylic acids degradation, and electricity production, WWL biofilm exhibit a much more diversity and quantity in terms of microbial organisms when compared to the RWL biofilm. In this study, winery wastewaters with low polyphenol concentrations were suitable to present more variability of microorganisms, high values of COD removal rate and electricity production in a SC- MFC. This indicates that winery wastewater with high polyphenols concentrations needs to be treated with microorganisms that can possible catabolize or survive in the presence of this compound.

In 2006, Penteado et al.¹⁵ determine the efficiency of the winery wastewater treatment in terms of energy generation and COD removal rate by using three different carbon anode materials in a DC-MFC. The three electrode materials studied were carbon felt, carbon cloth and carbon paper. Due to the high roughness characteristics and superficial area, carbon felt demonstrated to

be the surface with more concentration of microorganisms adhered, in contrast with carbon paper which was the material with lower microorganism concentration in the anode. In this study, the different values of COD removal and power density output in each electrode material type, suggests that despite of being made of the same chemical composition, the superficial surface and the adhesion properties are directly involved in the efficiency of these parameters. The MFC equipped with the carbon felt electrode demonstrates the higher COD consumption rate and power output. In this experiment, carbon cloth and carbon paper demonstrate that were not appropriate electrode options for energy production in this winery effluent by using a DC- MFC.

More recently, in 2016, Penteado et al¹⁴. also studied the behavior of a DC-MFC in the treatment of a winery wastewater in terms of COD removal and electricity production by using ferricyanide as catalyst of the oxygen reduction in the cathode chamber. In this study, sodium phosphate and ammonium sulfate solutions were described as beneficial to the energy production and for that reason were also added to increase the nutrients concentrations of phosphorous and nitrogen, respectively, to 10 mg/L and 100 mg/L. Ferricyanide at low concentrations (0,005M) had showed improvements in terms of electricity generation and COD removal rate when compared to the MFC worked without the addition of ferricyanide. 0,25 M ferricyanide concentrations, confirmed not to be sustainable for this type of treatment under these particular conditions, because the accumulation of high quantities of mediator at the cathode chamber turns possible its leakage to the anode side through the semipermeable membrane, which could affect the microorganism's growth and consequently the organic charge degradation and the efficiency of electrons and protons released. The protons transference to the cathode chamber could be also reduced due to the clogging of the membrane when the ferricyanide diffusion to the anode happen, which could affect the pH at the anode chamber. For these reasons, a very selective and economical viable PEM and an optimal mediator concentration need to be determined and studied, to allow the application of this type of catalysts in optimal concentrations at the cathode chamber for the winery wastewater treatment under specific conditions established, to prevent the possibility of the intoxication of the microorganisms and the contamination of the effluent.

Also in 2016, Penteado et al.¹³ studied the influence of solid retention time (SRT) in the performance of two DC-MFC for the treatment of a winery wastewater inoculated with activated sludge. In this study, the microorganisms with less SRT have probably more capacity to transfer electrons to the anode, which leads to believe in the possibility of the microorganisms responsible for the electrons transference to the anode surface have a higher growth rate when compared to other microorganisms with less capacity. When the SRT increases there are more chances of diverse types of microorganisms (including microorganisms with less ability to transfer electrons to the anode) could grow, which have a negative effect considering the substrate competition behavior unfavorable to the microorganisms that supposedly have more capacity to transfer electrons to the electrode. This study also reveals a high consumption of nitrogen and phosphorous

nutrients at low SRTs, which described the possibility of the microorganisms responsible for the energy production were related with the higher consumption of these nutrients. The term “bio-electrogenetic” and “exoelectrogenetic” used in this study, which supposedly represent the microorganisms with the capacity of directly transferring electrons to the anode surface, is needed to be taken into consideration, because these definitions are not transversal for all type of situations, in fact, this characteristic depends of the operational conditions and wastewater effluent compositions, the same microorganism can be consider “exoelectrogenetic” in some operational conditions and in others difference conditions not. A proper identification of the microorganisms at the different SRTs analyzed could be useful to determine which are the species responsible for the higher power densities.

In 2012, Rengasamy et al.¹⁷ analyzed the performance of 12 DC- MFC in terms of COD removal rate and energy produced during 3 cycles of 72 hours. In this experiment, four different substrates were tested, which were oxidized by 3 different inoculums: *Acetobacter aceti*, *Gluconobacter roseus* and a mixture of these two microorganisms, in different MFCs. The substrates analyzed were glucose, ethanol, acetate (which are present in the composition of the winery effluent) and wine with poor quality. This study reports the relevance of the alcohol dehydrogenase enzyme involving in the synthesis of alcohol and the enzyme quinoxaline protein and quinoprotein glucose dehydrogenase respectively responsible for the degradation of ethanol and glucose, which supposal have the capacity to act as mediators in the electron transference to the anode surface. Analyzing the results of the performance of the different inoculums in the bad wine quality treatment, it was possible to verify that the organic matter removal rates were between 41% and 59%, with *A.aceti* presenting the higher value. The fact of *A. aceti* showed a greater efficiency in the removal of organic matter may be due to the ability of these bacteria to degrade the acetic acid produced by the oxidation of ethanol, unlike *G. roseus* that may possibly accumulate this compound. A mixed culture of both microorganisms was also tested, revealing to be more effective in terms of COD removal rate and energy production in relation to the other two inoculums.

The same authors had analyzed the electrical efficiency and COD removal rate of four DC-MFC equipped with different anode material in the treatment of bad quality wine with a mixed culture containing *A. aceti* and *G. roseus* as inoculum. The anode composition of the four MFC was, nickel foam (NF), nickel foam with polyaniline (NF/PANI), nickel foam with polyaniline and titanium carbide and nickel foam with polyaniline (NF/PANI/TC), titanium carbide and chitosan (NF/PANI/TC/Chit), respectively. The maximum power output was obtained by using the NF/PANI/TC/Chit anode. NF/PANi and NF/PANI/TC anodes revealed almost the same power output densities, which indicates that the TC compound is not relevant for the energy efficiency. NF anode showed the lower power generation. In that experiment, the electricity generation in a closed circuit for 130 hours was analyzed, which results established

that the MFC with NF/PANI/TC/Chit and NF/PANI/TC demonstrated the better results, producing an electrical current at constant power density of, respectively, 13.6 W m^{-3} and 6.2 W m^{-3} , under a constant external resistance of 50, during a period of 80 hours. After 80 hours of the analysis in closed circuit, the power density decreased to values around 0 W m^{-3} , that is probably related with to lower substrate concentration available to be converted into energy at this stage. The possibility of low substrate concentration available at the end of the process can be confirmed with the COD percentage removal rate situated between 87% and 93%, obtained after 130 hours for the four MFCs treatment. These different power densities values obtained for the different anode materials are related with the porosity surface, the conductivity efficiency and lower internal resistance of itch material. The epifluorescence images captured at the end of the operation demonstrated that NF/PANI/TC/Chit anode had fibrillar surface that could promote the attachment of a thick biofilm, which allowed the reduction of internal resistances associated to the distance between the microorganism and the electrode, allowing to increase the coulombic efficiency.

In 2010, Cusick et.²⁰ al made the first economic comparison of the power densities generated in terms of electricity and hydrogen production and the COD removal rate obtained between the operation of MFC and MEC in the treatment of winery and domestic wastewater. In this case, the winery wastewater used to be treated served at the same time as inoculum for the process. After the winery wastewater treatment, a DNA extraction from the bacteria on the anode biofilm was made, which by PCR method determined the presence of 44% of *Geobacter sulfurreducens*, 14% of *Roseivivax* spp. and 7.7% of *Pelobacter propionicus* as the most dominant microorganisms at the end of the process.

Table 6 presents the electrode materials, PEM materials, operational characteristics, and performances related to the studies previously described.

Table 6: Electrode materials, PEM materials, operational characteristics, and performances related to the studies involving the WW treatment using MFCs.

References	Anode Material	Cathode Material	PEM Material	Anode Volume (mL)	Final electron acceptor	Configuration	Maximum COD removed	Maximum power density	Maximum Coloumbic Efficiency	Inoculum
Sciarria et al. ¹⁹ (2015)	Graphite Brush	Carbon Cloth-PTFE with platinum catalyst	-	28	Oxygen	SC-MFC	90%	112.2 mWm ⁻²	15%	Denitrification Wastewater
Penteado et al. ¹⁵ (2016)	Carbon-Felt	Carbon-Felt	Sterion	8	Oxygen	DC-MFC	11%	420 mWm ⁻²	-	Activated sludge
Penteado et al. ¹² (2015)	Carbon-Felt	Carbon-Felt	Sterion	70	Oxygen	DC-MFC	17%	465 mWm ⁻²	14.75%	Activated sludge
Penteado et al. ¹⁴ (2016)	Carbon-Felt	Carbon-Felt	Sterion	70	Ferricyanide	DC-MFC	41%	1783 mWm ⁻²	16%	Activated sludge
Penteado et al. ¹³ (2016)	Carbon-Felt	Carbon-Felt	Sterion	70	Oxygen	DC-MFC	10%	890 mWm ⁻²	42.2%	Activated sludge
Rengasamy et al. ¹⁰⁷ (2012)	Carbon-felt	Graphite	Nafion 117	125	Oxygen	DC-MFC	87.5%	3.8 W/m ³	45%	<i>Acetobacter aceti</i> and <i>Gluconobacter roseus</i>
Rengasamy et al. ¹⁸ (2016)	Carbon-felt	Graphite	Nafion 117	125	Oxygen	DC-MFC	93%	18.8 W/m ³	14.7%	<i>A. aceti</i> and <i>Gl. roseus</i>
Cusick et al. ²⁰ (2010)	Graphite fiber brush	CC-PTFE with platinum catalyst	-	28	Oxygen	SC-MFC	65%	0.26 kwh/kg-COD	18%	Microbial Consortium

7. Materials and methods

7.1. MFC construction and operation

Two Microbial Fuel Cells (MFC1 and MFC2), were inoculated with *Zygosaccharomyces bailii* and *Bacillus cereus*, respectively, and fed with synthetic winery wastewater (SWW) as anolyte. The SWW was prepared under sterile conditions based in the composition described by Malandra et al.⁵ (2003). Both MFCs had a single-chamber configuration, were made of acrylic, had 1 liter of volume, a PEM of Nafion 212 (Quintech, Germany) with 0.0508 mm of thickness, a cooper with gold coating current collector and a rubber gasket to prevent leakage. The anode was a graphite brush with approximately 8 cm length and 2.5 cm of diameter (Mill-Rose Company, USA). The cathode was a plain carbon cloth coated with 1 mg/cm² of platinum black (Quintech, Germany).

Z. bailii was incubated in 200 mL of Yeast Extract Peptone Dextrose medium (1% yeast extract, 2% peptone, 2% dextrose and 95% distilled water) (Liofilchem s.r.l., Italy) for 24 hours at 30 °C. *B. cereus* was incubated in 200 mL of Neutral Broth medium (beef extract 1g/L; yeast extract 2g/L, peptone 5g/L, sodium chloride 5g/L) (ThermoFisher, U.S.A.) for 24 hours at 30 °C. After incubation, both inoculums were centrifuged (3772 g for 15 minutes) (centrifuge 5810 R, Eppendorf, Germany), and the pellet was suspended in 300 ml of SWW and then incubated for 24 hours at 30 °C before inoculation of MFC1 and MFC2.

After inoculation of both MFCs, a previous evaluation of the adaptive behavior of *Z. bailii* and *B. cereus* was made. A sample of each of MFC1 and MFC2 was daily collected for ten and nine days, respectively. For each of the samples taken, the COD removal rate, culturability by plating method, polysaccharides removal rate and open circuit voltage (OCV) were analyzed and compared with the growth curves made for 120 hours in SWW for both microorganisms. To avoid cell death in both cultures, 1/3 of the medium was renewed with fresh SWW on the seventh day of operation of MFC1 and on the ninth day of operation of MFC2. From the analysis of the data obtained it was determined that each batch cycle had a duration of three days (72 hours). Every three days, before starting a new batch cycle, 2/3 of the anode medium was renewed with fresh SWW at sterile conditions.

After the starting of each batch cycle the homogenization of the medium was carried out. At the end of each batch cycle, polarization and power density curves were made and a sample was taken for the analysis of COD, TOC and polysaccharides removal rate and for culturability tests. MFC1 operated for 9 cycles during 26 days after the starting of the first batch cycle. On the 26th day, the quantification of the biofilm adhered to the anode surface in terms of concentration of total solids, total volatile solids and fixed solids was made. MFC2 operated for 8 cycles during 27 days after the starting of the first batch cycle. On the 27th day, the quantification of the biofilm

adhered to the anode surface in terms of concentration of total solids, total volatile solids and fixed solids was accomplished.

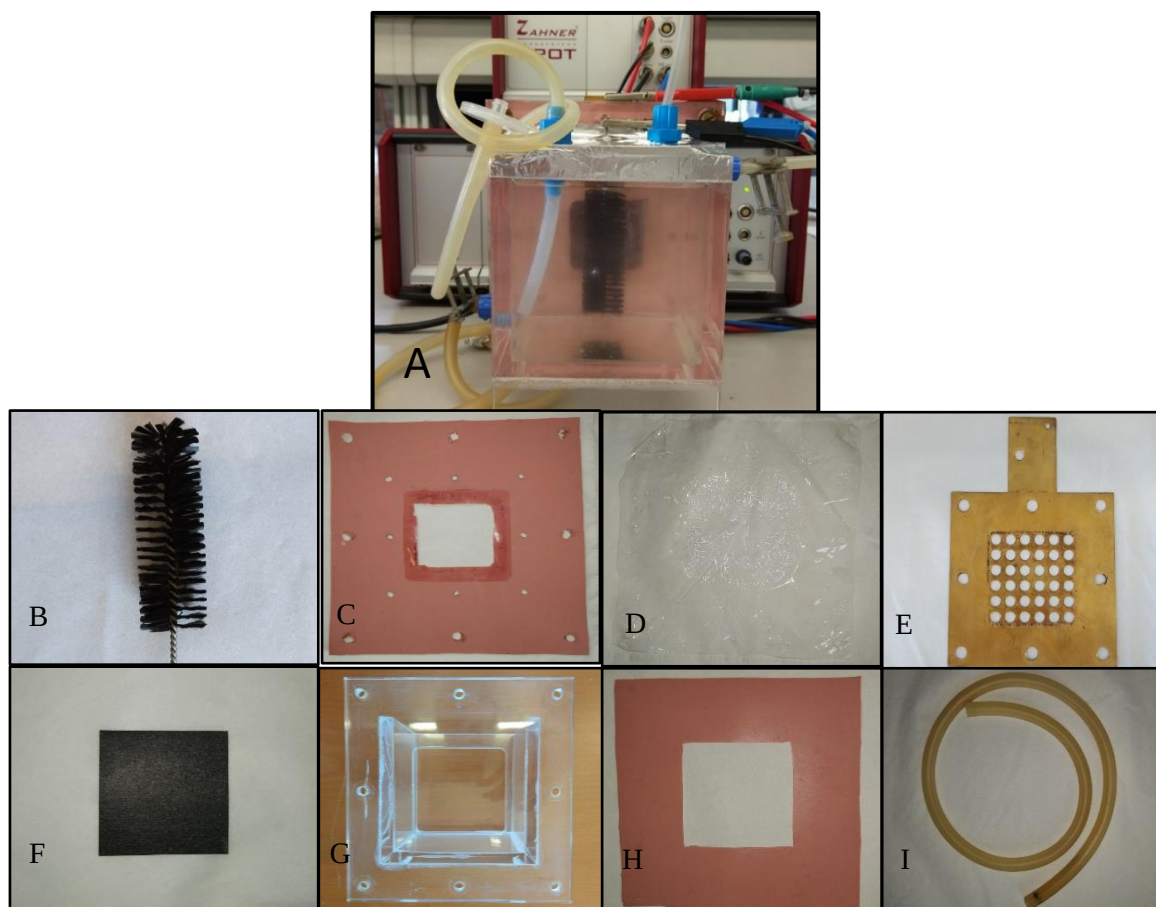


Figure 3: A) SC-MFC connected to an external resistor (Zahner - Electric GmbH & Co.); B) Graphite brush anode; C) Internal rubber gasket; D) Nafion 212; E) Copper with gold coating current collector; F) Carbon plain cathode coated with 1 mg/cm² of platinum black; G) Acrylic anode chamber; H) External rubber gasket; I) Tube for medium renewal.

7.2. Preliminary evaluation tests for the inoculum determination

Acetobacter aceti, *Gluconobacter oxydans*, *Saccharomyces cerevisiae*, *Z. bailii*, *Zygosaccharomyces rouxii*, *B. cereus*⁶⁰ and *Escherichia coli* were evaluated as potential candidates to act as inoculum in MFCs for the WW treatment.

A. aceti and *G. oxydans* were kindly provided by Professor Maria José Saavedra (Universidade de Trás-os-Montes e Alto Douro). *Z. bailii* and *Z. rouxii* were kindly provided by Professor Manuela Côrte-Real (Universidade do Minho).

A. aceti, *G. oxydans*, *S. cerevisiae*, *Z. bailii*, *Z. rouxii*, *B. cereus* and *E. coli* were inoculated as isolated cultures in 60 mL SWW at room temperature for 96 hours. Colony forming units (CFUs) counting by drop plating method in six-fold dilutions was made to determine the microorganism's culturability in terms of CFUs/mL. The microorganisms were cultivated in Plate Counting Agar (PCA) (0.5% peptone, 0.25% yeast extract, 0.15 % glucose, 1.5 % agar) (VWR Prolab Chemicals) at 30 °C and at different intervals of time (0 hours, 2 hours, 24 hours, 48 hours and 72 hours after inoculation). The CFUs counting was performed according to the different

growth cycles observed for each microorganism. For *B. cereus*, the CFU/mL counting was done after 16 hours, for *E. coli* after 24 hours for *A. aceti*, *G. oxydans*, *S. cerevisiae* and *Z. bailii* after 48 hours and for *Z. rouxii* after 72 hours of cultivation. The analysis of the COD removal rate was performed for each microorganism at 24 hours and 72 hours after inoculation in SWW medium. The CFU counting was performed in duplicate.

7.3. Growth curves

A growth curve for *Z. bailii* and *B. cereus* was performed before both MFCs inoculation by CFUs counting by using the drop plating method in four-fold dilutions. *Z. bailii* and *B. cereus* were inoculated in 250 mL of SWW at 30°C with agitation during 120 hours. For 29 time intervals. *Z. bailii* samples were cultivated in Yeast Extract Peptone Dextrose - Agar (YPD - agar). For 29 time intervals *B. cereus* samples were cultivated in Neutral Broth-Agar (NB-agar). Each sample was analyzed in duplicate.

7.4. Synthetic winery wastewater

SWW was prepared from the chemical composition described by Malandra et al.⁵ (2003) SWW was adjusted to pH 4 with a solution of 1M NaOH. The composition of the SWW is represented in the following table:

Table 7: SWW chemical composition and characteristics .⁵

Components	mg/L	Company
Glucose (C ₆ H ₁₂ O ₆)	1800	Sharlab
Fructose (C ₆ H ₁₂ O ₆)	1800	VWR Prolab Chemicals
Citric acid (C ₆ H ₈ O ₇)	1	AppliChem
Tartaric acid (C ₄ H ₆ O ₆)	2	Merck
Malic acid (C ₄ H ₆ O ₅)	2	ThermoFisher
Lactic acid (C ₃ H ₆ O ₃)	2	ThermoFisher
Acetic Acid (C ₂ H ₄ O ₂)	250	VWR Prolab Chemicals
Propanol (C ₃ H ₈ O)	1.24	VWR Prolab Chemicals
Butanol (C ₄ H ₁₀ O)	1	VWR Prolab Chemicals
Amyl alcohol (C ₅ H ₁₁ OH)	3.8	VWR Prolab Chemicals
Ethanol (C ₂ H ₆ O)	10	AGA SA
Ethylacetate (CH ₃ COOCH ₂ CH ₃)	4	VWR Prolab Chemicals
Propionic acid (C ₃ H ₆ O ₂)	8	Merck
Valeric acid (C ₅ H ₁₀ O ₂)	1	Merck
Hexanoic acid (C ₆ H ₁₂ O ₂)	0.5	Merck
Octanoic acid (C ₈ H ₁₆ O ₂)	0.7	VWR Prolab Chemicals

YPD (Yeast Nitrogen Base)	1700	VWR Prolab Chemicals
Ammonium sulfate ((NH ₄) ₂ SO ₄)	5000	VWR Prolab Chemicals

7.5. Experiment analytical parameters

7.5.1. Polarization, power density curves and open circuit voltage

Polarization and power density curves were performed at the end of each batch cycle for both MFCs. The polarization curves were carried out using the galvanostatic method, where the current intensity is controlled and imposed and the resulting electric potential difference are measured by an electrochemical device (Zahner - Electric GmbH & Co.). The tests were performed by adjusting diverse current intensities values and measuring the corresponding different electric potential difference. The power was calculated using the following equation:

$$P = U * \frac{I}{A} \quad (4)$$

Where P is the power measured in Watts per square meter (W/m²), U is the current voltage measured in volts (V), I is the current intensity applied in amperes (A), A is the PEM area in square meters (m²).

OCV (V) was daily measure by the same electrochemical device.

7.5.2. COD quantification

COD removal rate was calculated to determine the percentage of organic matter that was oxidized during three days of MFC operation. Standard method number 5220 C¹²⁵ was used to determine the COD concentration (mg O₂ /L) of each sample taken at the end of each cycle.

The following equation represents the COD removal rate efficiency:

$$COD\ removed\ rate\ (\%) = \frac{\Delta COD}{COD_{SWW}} * 100 \quad (5)$$

ΔCOD (mg O₂/L) corresponds to the difference between the SWW COD concentration (mg O₂/L) calculated before the MFC inoculation and the COD_{SWW} concentration (mg O₂/L) calculated at the end of each cycle. To avoid errors associated to the distribution of suspended solids, each of the samples analyzed was centrifuged (3772 g for 15 minutes) (centrifuge 5810 R, Eppendorf, Germany) and the pellet was removed. The COD digestion was made in a thermoreactor (Spectroquant® TR 420, Merck Millipore, USA). Each sample was analyzed in triplicate.

7.5.3. TOC quantification

TOC removal rate was calculated to determine the percentage of the total organic carbon that was consumed during three days of the MFC operation. Standard method number 5310 A¹²⁶ was used to determine the TOC concentration (mg/L) of each sample taken at the end of each cycle.

The following equation represents the TOC removal rate efficiency:

$$TOD\ removal\ rate\ (\%) = \frac{\Delta TOD}{TOD_{SWW}} * 100 \quad (6)$$

ΔTOD (mg /L) corresponds to the difference between the SWW TOC concentration (mg /L) calculated before the MFC inoculation and the TOC_{SWW} concentration (mg /L) calculated at the end of each cycle. To avoid errors associated to the distribution of suspended solids, each of the samples analyzed was centrifuged (3772 g for 15 minutes) (centrifuge 5810 R, Eppendorf, Germany) and the pellet was removed. The TOD measurement was made in a total organic carbon analyser (TOC-L®, Shimmadzu Corporation, Japan).

7.5.4. Culturability tests

The CFUs counting by drop plating method in six-fold dilutions was made at inoculation, at the beginning of the 1st cycle and at end of each cycle for both MFCs. MFC1 samples were cultivated in YPD - agar medium and incubated for 48 hours at 30°C before counting. MFC2 samples were cultivated in NB-agar medium and incubated during 24 hours at room temperature. Each sample was analyzed in triplicate.

7.5.5. Polysaccharides quantification

Colorimetric method from Dubois et al.¹²⁷ was used to determine the polysaccharides concentration (mg/L) of the samples taken at the end of each cycle.

The following equation represents the efficiency of the polysaccharides removal rate:

$$Polysaccharides\ removal\ rate\ (\%) = \frac{\Delta Polysaccharides}{Polysaccharides_{SWW}} * 100 \quad (7)$$

$\Delta Polysaccharides$ (mg /L) corresponds to the difference between the SWW Polysaccharides concentration (mg /L) calculated before the MFC inoculation and the $Polysaccharides_{SWW}$ concentration (O₂ mg /L) calculated at the end of each cycle. To avoid errors associated with the distribution of suspended solids, each of the samples analyzed was centrifuged (3772 g for 15 minutes) (centrifuge 5810 R, Eppendorf, Germany) and the pellet was removed. Each sample was analyzed in duplicate.

7.5.6. Biofilm quantification

At the end of both MFC operation, the biofilm adhered on the anode surface was scraped and diluted in 20 mL of buffer (2 Mm Na₃PO₄, 2 mM NaH₂PO₄, 9 mM NaCl and 1 mM KCl, at pH 7) for extraction of extracellular polymeric substances.¹²⁸ The concentration of total solids (TS) (g/L), total volatile solids (TVS) (g/L) and fixed solids (FS) (g/L) of the biofilm extracted where quantified by the Standard Method number 2540 (A to D).¹²⁹ Culturability tests were also made to determine the microorganism's viability in terms of CFUs/mg of biofilm. Colorimetric method from Dubois et al.¹²⁷ was used to determine the polysaccharides concentration (mg/mg of biofilm).

By the following equations were obtained TS, TVS and FS:

$$TVS(g/L) = TS - FS \quad (8)$$

$$TS(g/L) = \frac{m_b(g) - m_a(g)}{V(mL)} * 1000 \quad (9)$$

$$FS(g/L) = \frac{m_c(g) - m_a(g)}{V(mL)} * 1000 \quad (10)$$

7.5.7. pH and conductivity

pH was measured for the frozen samples taken at the end of each cycle with a pH/mV/°C meter (pHenomenal® pH 1100L; VWR Collection, U.S.A.). Electrical conductivity of the SWW was measure once with a conductimeter (GLP 31, Crison, Spain).

7.6. Statistical analysis

Pearson correlation coefficient was used to identify the relationship between the experiment analytical parameters analyzed. Linear regression analysis with enter selection of variables was made to estimate equation for the correlated parameters. Analysis of variance (ANOVA) was performed to determine the significant differences between the microorganism's culturability during the preliminary evaluation tests for the MFC inoculum determination with Scheffe Post-Hoc test comparison. All statistical tests were performed by using a significance level of 0.05. Statistical analysis of similarity data was performed using *IBM Statistical Package for the Social Sciences Statistics 24* (SPSS) software.

8. Results and discussion

8.1. Preliminary evaluation tests for the MFC inoculum determination

A. aceti, *B. cereus*, *G. oxydans*, *S. cerevisiae*, *Z. bailii* and *Z. rouxii* were assessed as potential candidates to act as inoculum in the winery effluent treatment through MFC technology. These microorganisms were chosen based in the bibliographic research described in Chapter 5. These microorganisms were already described as tolerant to ethanol and resilient to acid pH environments and for those reasons most of them were identified during diverse stages of wine production, wine bottling and winery wastewater treatments. *E. coli* was also chosen due its great versatility to survive in different conditions and environments and for being considered a typical model organism easily to handle in laboratory.¹³⁰ Among the seven microorganisms, the two that presented the better results associated with the CFU/mL concentration and COD removal rate of SWW were selected and properly characterized, with the objective of being further tested in a SC-MFC. Figure 4 shows the different log CFU/mL concentrations observed at different time intervals (0 hours, 2 hours, 24 hours, 48 hours, 72 hours and 96 hours) for the seven microorganisms selected. In the annexes, it is possible to visualize an individualized analysis of the results obtained for each microorganism related to the cell growth in concentrations of log CFU/mL and OD at 600 nm during the 96 hours.

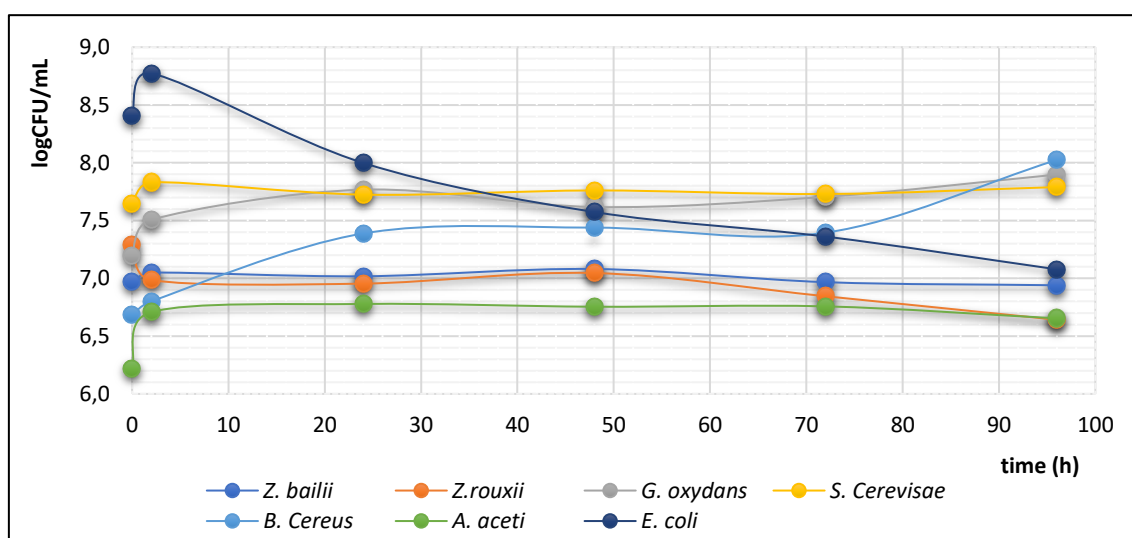


Figure 4: Microorganisms Culturability in SWW for 96 hours period.

Figure 4 shows that *B. cereus*, *G. oxydans* and *S. Cerevisae* presented the higher CFU/mL in SWW during 96 hours of growth. Through ANOVA analysis, significant differences were found between the seven microorganisms for the 96 hours of incubation ($p < 0.05$). Posteriorly, the Post-Hoc test allows to verify that *B. cereus*, *G. oxydans* and *S. cerevisiae* did not present significant differences between each other ($p > 0.05$), being significantly different from the remaining four microorganisms selected ($p < 0.05$). In annexes, Table A4 include the significant differences between the diverse microorganisms from Figure 1.

By a more detailed observation of Figure 4 other conclusions can be made. Five of the seven microorganisms showed an increase in CFU/mL during the first two hours of incubation. Only *Z. bailii* and *Z. rouxii* did not showed increase of CFU/mL during the first two hours of the experiment. *B. cereus* despite of having presented the second lower concentration of CFU/mL at the initial time, it was the microorganism with the highest CFU/mL over the remaining 94 hours of the experiment in the range of 10^8 CFU/mL. *G. oxydans* and *S. cerevisiae* exhibited the second and the third higher culturability, respectively, in the range of 10^7 CFU/mL after 96 hours. *A. aceti* maintained its cell concentration in the order of 10^6 CFU/mL until the 72 hours of experiment. Between the 72 hours and 96 hours, *A. aceti* revealed a slight decrease in the CFU/mL. *A. aceti* is one of the most resistance acetic acid bacteria to wine conditions, being capable to survive during wine fermentation and wine bottling in a concentration from a range between 10^2 to 10^3 CFU/mL.¹²⁰ In contrast, *G. oxydans* demonstrated to be more sensitive to high ethanol concentration, being only capable to contaminate fresh musts at the beginning of the fermentation.¹²² In the present study *G. oxydans* exhibited the opposite behavior by showing a higher CFU/mL compared to *A. aceti*. *Z. bailli* expressed very uniform and stable values by maintaining its cell concentration levels at the transition range between 10^6 CFU/mL and 10^7 CFU/mL during the 96 hours analyzed, however, a more evident cell growth would be expected. *E. coli*, despite being the microorganism with initial higher CFU/mL concentration and having shown a significant increase in CFU/mL at the first two hours of inoculation, afterwards manifested a drastic decrease in the concentration of CFU/mL until the end of the experiment. In fact, *E. coli* transited from 10^8 CFU/mL to 10^7 CFU/mL. *Z. rouxii* displayed a decrease in CFU/mL, from the order of 10^7 CFU/mL at inoculation to 10^6 CFU/mL at the end of the experiment, expressing a certain inadaptability to SWW over time.

Jourjon et al.⁷⁹ performed a study involving the microbiological characterization of winery effluents. Jourjon et al.⁷⁹ revealed that in the harvesting and wine production period acetic acid bacteria and yeasts were respectively present in the range between 1.62×10^7 to 1.8×10^8 CFU/mL in WW. The same author verified that fecal contamination is almost inexistence in WW, finding the presence of a maximum of 114 CFU of *E. coli* per mL of WW. Making a comparison between the microbiological characterization by Jourjon et al.⁷⁹ and the present study it is possible to verify that yeasts and acetic acid showed similar concentrations. The tendency exhibited by *E. coli* can be justified by its inadaptability to the WW. Malandra et al.⁵ also presented some similar results, yeasts were isolated from a range of 4.0×10^6 and 7.2×10^7 CFU/mL, respectively after one and 42 days of inoculation in the same SWW composition.

The following figure represent the results of COD removal rate from the SWW during 24 hours and 72 hours of experiment for each microorganism evaluated.

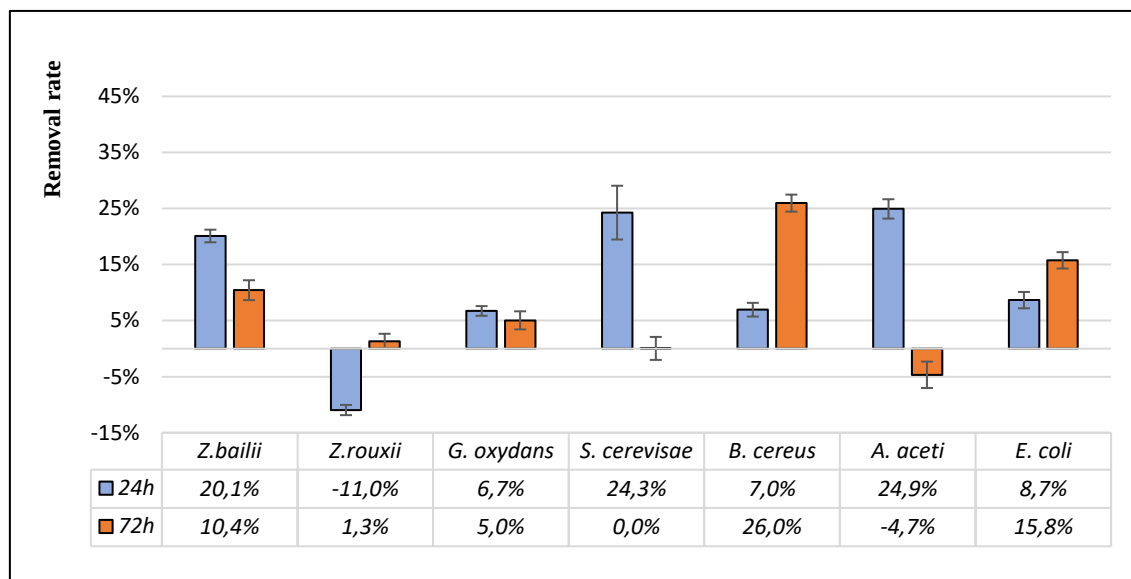


Figure 5: COD removal rate of SWW for 24 hours and for 72 hours of experiment for each microorganism selected.

B. cereus was the microorganism that showed the highest COD removal rate in the SWW after 72 hours of inoculation (26 ± 1.3 %). Comparing the results of the COD removal rate between 24 hours and 72 hours, it is possible to verify that *B. cereus* was also the microorganism which COD removal rate more increased between 24 hours and 72 hours (near 19 %). During the three days analyzed, *B. cereus* presented a daily average COD removal rate around 9 %. *E. coli* presented the second highest value of the COD removal rate for 72 hours (15.8 ± 1.5 %), however the previous results associated to the concentration of CFU/mL make the choice of *E.coli* for the inoculation of the MFC not feasible. During the experiment *E. coli* was capable of reducing the COD concentration by oxidizing sugars by anaerobic fermentation producing metabolites such as lactate, ethanol or acetate.¹³¹ The fast production of this type of metabolites induced the increase of ethanol concentration and the pH decrease to values that possibly inhibit *E. coli* growth. According to Alterthum and Ingram¹³² *E.coli* is not capable to growth at pH lower than 4 and ethanol concentration higher than 8.8 % (v / v %). *Z.bailii* was the third microorganism with higher COD removal rate during 72 hours of experiment (10.4 ± 1.8 %). *Z.bailii* and *S. cerevisiae* showed the best COD removal rate results in the first 24 hours of inoculation (20.1 ± 1.1 % and 24.3 ± 4.8 %), however, during the following 48 hours the COD removal rate decreased to 10.4 ± 1.8 % and to 0 ± 2 %, respectively. This is probably due to the accumulation of organic components, such as ethanol and glycerol, resulting of the anaerobic metabolism.¹¹⁰ The COD removal rate diminishing between 24 hours and 72 hours was more prominent in *S. cerevisiae* than in *Z.bailii*, this can possibly be explained due the fact that ethanol production, for the same sugar quantity, is approximately the double in *S. cerevisiae* compared to *Z.bailii*.¹¹⁴ *G. oxydans* despite presented

a higher CFU/mL after 96 hours of the experiment, did not reveal a satisfactory COD removal rate, evidencing only $6.7 \pm 0.9 \%$ and $5.0 \pm 1.6 \%$, respectively at 24 hours and 72 hours. *Z. rouxii* in the first 24 hours of experiment evidenced the lower COD removal rate ($-11.0 \pm -0.9 \%$) probably due to the high synthesis of alcohols and organic acids (malic acid and succinic acid) via fermentative pathway.^{133, 134} The high production of alcohols and organic acids by *Z. rouxii* during sugar consumption make its application unfeasible in a microbial consortium for the WW treatment in MFC technology. *B. cereus* and *Z. bailii* showed the better results associated with the conjugation of CFU/mL concentration and COD removal rate and for that reason were both chosen to be tested in a SC-MFC.

Besides the preliminary tests, other factors were considered for the selection: *Z. bailii* has a metabolic system capable to regulate the intracellular acetic acid and propionic acid concentration in the presence of ethanol even when glucose is being oxidize, this mechanism is associated to a low permeable membrane and a mediated transport system which allow the capacity of *Z. bailii* to resists to high acetic acid concentrations such as during winemaking conditions. In contrast, other yeasts, such as *S. cerevisiae*, does not have a mediated system to control the acid entrance, and for that reason acetic acid enters in cell through diffusion when the extracellular pH is at low levels, which can easily intoxicate the microorganism.¹¹⁸ Kalathenos et al.¹¹⁶ identified *Z. bailii* among other yeasts as the one with highest resistance to acetic, malic, tartaric, lactic and citric acids usually present in the wine. *Z. bailii* yeasts are facultative anaerobes and can use glucose, ethanol and glycerol typical present in wine as carbon source.¹¹⁷ The organic acid tolerance of *Z. bailii* allows its growth in low pH values between the range of 2 and 7.^{118,119} According to Thomas et al.¹¹⁷ this species can tolerate high sulfite (> 3 ppm) and ethanol concentrations ($18\% (v / v \%)$) typically present in wine, as can be proved by the identification of *Z. bailii* in wine after 9 months of being bottled. This yeast growth at an optimal temperature between $22-26^{\circ}\text{C}$, which is a good indicative to its application in MFCs context, particularly in south Europe countries during harvesting period, since possible would not need energy required to the temperature increase for optimal cell growth. Browne and Dowds¹³⁵ demonstrated the capacity of *B. cereus* to resist to stress factors such as 12% ($v / v \%$) ethanol concentrations or acid pH. *B. cereus* adaptably behavior to wine conditions was identified in high occurrence in a jet-loop reactor system after 180 and 360 days of WW treatment.^{108,109} *B. cereus* have demonstrated good adhesion proprieties to stainless steel that can be useful for the biofilm formation in the anode surface.⁶⁰

8.2. Analysis of MFC1 and MFC2 performances

To determine the duration of each batch cycle for both MFCs, culturability, COD removal rate, polysaccharides removal rate and OCV measurement were made and compared with their respectively growth curves. After the analysis and comparison of the results obtained, it was

determined that each cycle had the duration of 72 hours. The data obtained can be visualized in annexes.

8.2.1. Culturability

Culturability tests were made to determine the microbial CFU/mL and to determine the possibility of contamination in both MFCs. Figure 6 shows the log CFU/mL observed for both microorganisms at the beginning of the 1st cycle and at the end of each cycle for MFC1 and MFC2, respectively. Growth curve from the inoculation of both MFCs can be visualized in annexes.

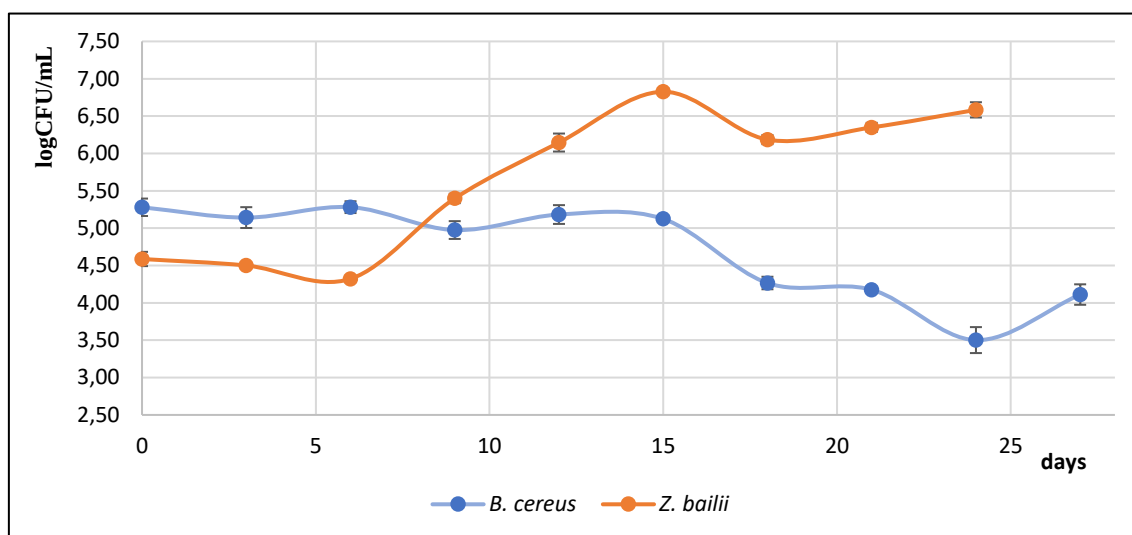


Figure 6: Graphical representation of log CFU/mL concentrations for both MFCs.

Through the observation of Figure 6 it is possible to verify that in MFC1 the concentration of CFU/mL over time showed a very increasing tendency after the end of the 2nd cycle, which probably corresponds to the log phase. The concentration of *Z. bailii* in MFC1 was maximal at the end of the 5th cycle of operation (6.7×10^6 CFU/mL). *Z. bailii* transited from 10^4 CFU/mL of inoculation to 10^6 CFU/mL at the end of the last cycle. *B. cereus* showed a different behavior, in the first five cycles demonstrated a very uniform cellular viability of 10^5 CFU/mL, *B. cereus* probably have reached the steady state during the first days of inoculation before renewal of 2/3 of SWW. After the 5th cycle the CFU/mL concentration decreased to the range of 10^4 CFU/mL, which can possible correspond to the beginning of the death phase.

8.2.3. Polysaccharides removal rate

Based on the colorimetric method development by DuBois et al.¹²⁷ the polysaccharides concentration determination was made. By quantifying the polysaccharides concentration of the SWW (3714 mg/L) and the polysaccharides concentration at the end of each cycle it was possible to calculate the polysaccharides removal rate in SWW for the different batch cycles. Table 8 shows the polysaccharides removal rate observed at the end of each cycle for MFC1 and MFC2, respectively.

Table 8: Polysaccharides removal rate observed at the end of each cycle for both MFCs.

Cycle	MFC1 Polysaccharides removal rate	MFC2 Polysaccharides removal rate
1	52 ± 4%	94 ± 3%
2	77 ± 4%	96 ± 2%
3	92 ± 4 %	90 ± 2%
4	94 ± 5%	95 ± 3%
5	92 ± 5%	92 ± 2%
6	98 ± 4%	96 ± 2%
7	94 ± 4%	93 ± 2%
8	94 ± 4%	95 ± 2%
9	-	98 ± 2%

By the analisis of Table 8 it is possible to verify that in MFC1 90% of the total of the sugars contained in the SWW were removed by *Z. bailii* from the end of the 3rd cycle. In MFC2 it was found that at the end of all cycles, 90% or more of all the sugars contained in the SWW were removed by *B. cereus*.

The following figure makes a graphical relationship between the polysaccharides concentration of SWW and the culturability of *Z. bailii* during the MFC1 operational activity.

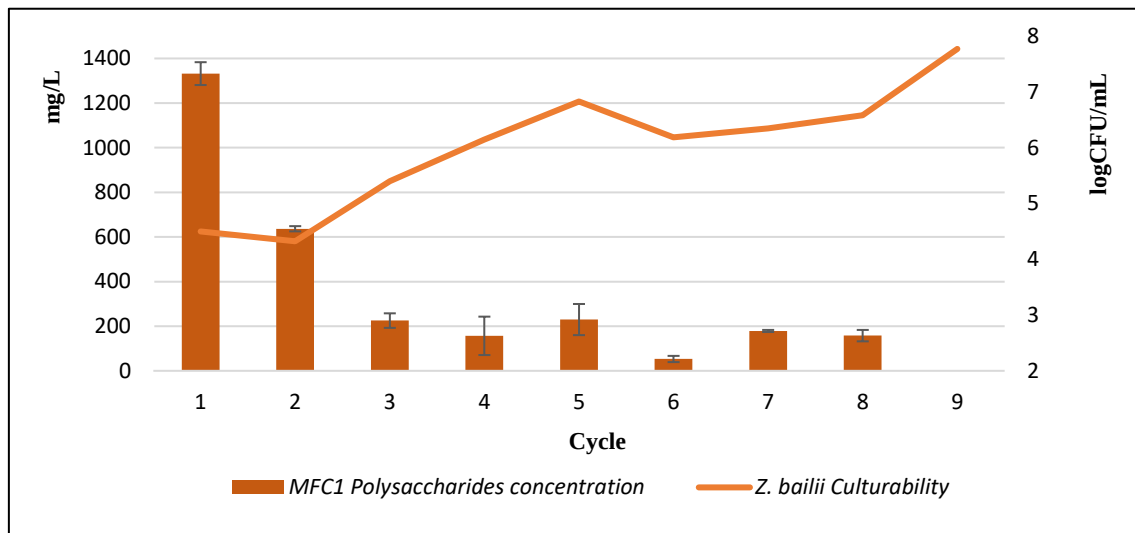


Figure 7: Relation between the Polysaccharides concentration of SWW and the Culturability of *Z. bailii* during the MFC1 operational activity.

The increase in the CFU/mL evidenced from the beginning of the third cycle, is directly related to the increase in the consumption of polysaccharides such as glucose and fructose present in SWW. Possibly *Z. bailii*, during the first two cycles, was still in lag phase. The following figure gives a graphical relationship between the polysaccharides concentration of SWW and the culturability of *B. cereus* during the MFC2 operational activity.

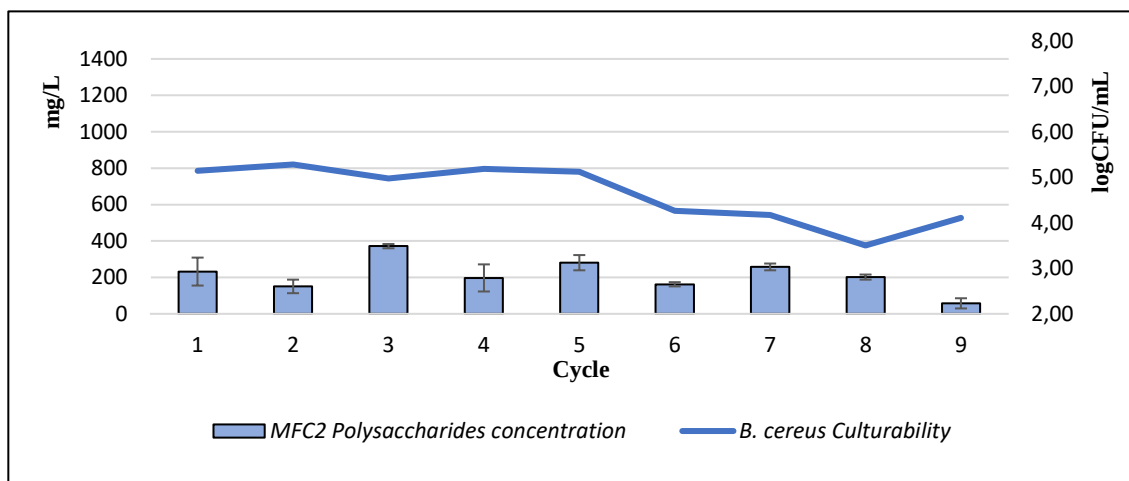


Figure 8: Relationship between the Polysaccharides concentration of SWW and the Culturability of *B. cereus* during the MFC2 operational activity.

The decrease of *B. cereus* cell growth from the beginning of the 6th cycle did not result in an increase of the polysaccharides concentration in the SWW. Probably, because *B. cereus* even at lower CFU/mL is capable of degrading higher amounts of sugars (> 90%) contained in the SWW.

8.3.3. COD removal rate

COD measurement infers the theoretical concentration of the oxygen requires for the oxidation of the total organic components of a given sample under controlled conditions. By the quantification of the COD concentration of the SWW (6010 mg O₂/L) and the COD concentration at the end of each cycle it was possible to calculate the COD removal rate during the MFC performance. Figure 9 and Table 9 show the COD removal rate observed at the end of each batch cycle for MFC1 and MFC2.

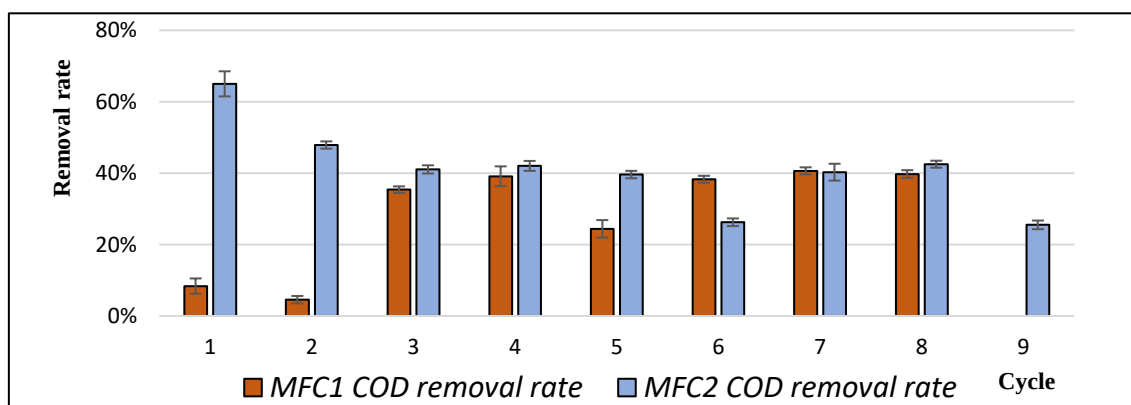


Figure 9: Graphical representation of the COD removal rate observed at the end of each cycle for both MFCs.

Table 9: COD removal rate observed at the end of each batch cycle for both MFCs.

Cycle	MFC1 COD removal rate	MFC2 COD removal rate
1	8 ± 2%	65 ± 4%
2	5 ± 1%	48 ± 1%
3	35 ± 1 %	41 ± 1%
4	39 ± 3%	42 ± 1%
5	24 ± 2%	40 ± 1%
6	38 ± 1%	26 ± 1%
7	41 ± 1%	40 ± 1%
8	40 ± 1%	43 ± 2%
9	-	26 ± 1%

Z. bailii presented an average COD removal rate of 29% at the end of each cycle. *Z. bailii* exhibited a maximum COD removal rate at the end of the 7th cycle (41 ± 1%). Since the total polysaccharides contribute to approximately 48% from the total COD, the low COD removal rate in the first two cycles may be associated with the low polysaccharides removal rate evidenced for the same cycles.

B. cereus revealed an average COD removal rate of 41% at the end of each cycle. These bacteria showed a maximum COD removal rate at the end of the 1st cycle (65 ± 4%). In the remaining cycles, the COD removed rate was always between 40% and 48%, except for the 6th and 9th cycles in which 26 ± 1% and 26 ± 1% of the removed COD was obtained.

The following figure provides the relationship between the COD concentration of SWW and the culturability of *Z. bailii* during the MFC1 operational activity.

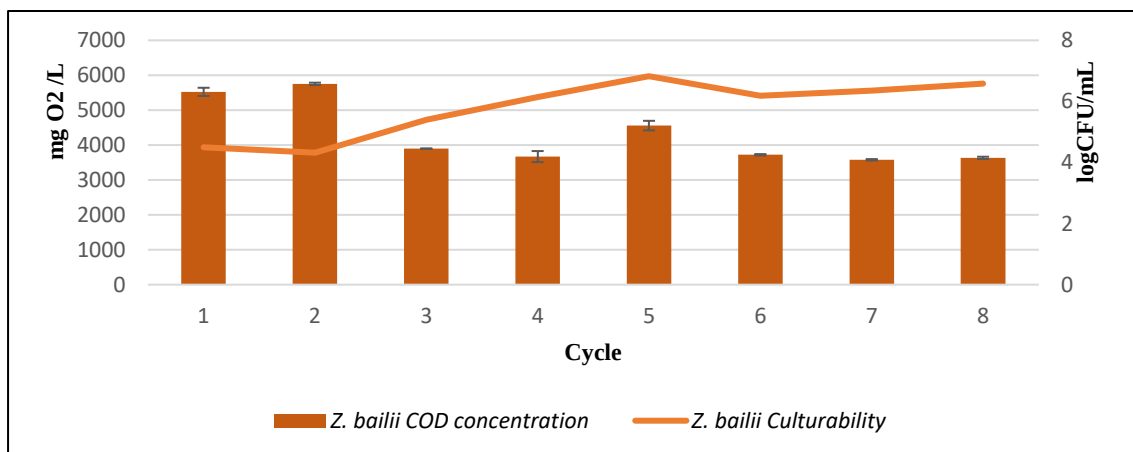


Figure 10: Relationship between the COD concentration and the Culturability of *Z. bailii* during the MFC1 operational activity.

Through Figure 10 it is possible to verify that the decrease in COD concentration is related to the increase of CFU/mL during the MFC1 activity period. However, between the end of the 4th cycle and the end of the 5th cycle, COD concentration increased around 1000 mg O₂/L,

which may be a consequence of the high production of fermentative compounds resulting from the higher concentration of CFU/mL between the 4th and 5th cycles.

There is a significant correlation between polysaccharides concentration and COD concentration ($p < 0.05$), during MFC1 operational activity. A significant correlation between COD concentration and culturability concentration in MFC1 was found ($p < 0.05$).

The following figure makes a relationship between the COD concentration of SWW and the culturability of *B. cereus* during the MFC2 operational activity.

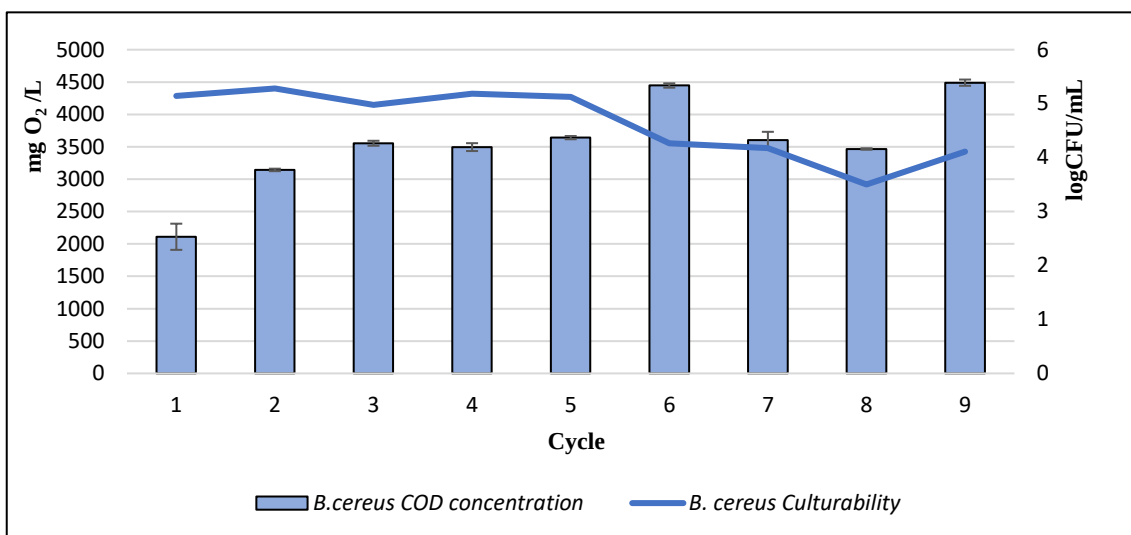


Figure 11: Relationship between the COD concentration and the culturability of *B. cereus* during the MFC2 operational activity.

The uniformity of the CFU/mL over the first five cycles is not reflected in the COD concentration for the first two cycles. The COD concentration increases by about 1500 mg O₂/L between the end of 1st and 3rd cycle, to values close to 3500 mg O₂/L. The increase in COD concentration in the first three cycles may be associated to the progressive accumulation of organic compounds resulting from anaerobic metabolism, which is possibly not significant at the end of the 1st cycle. After the decrease of the CFU/mL between end of the 5th cycle and the end of the 9th cycle, it was also expected that would be an increasing in the COD concentration, but this increase was only evident at the end of the 6th and 9th cycles (up to approximately 4500 mg O₂/L). At the end of the 7th and 8th cycles, the COD concentration remained similar to that observed at the end of the 3rd and 5th cycles (3500 mg O₂/L), where the concentration of CFU/mL was higher. The increase in organic load at the end of the 6th and 9th cycles should be associated with the decrease of the polysaccharides removal rate, but this did not occur, the polysaccharides removal rate remained like the observed in the first five cycles (> 90%) in which the COD removal rate was higher than 40%.

There is no significant correlation between polysaccharides concentration and COD concentration in MFC2 operational activity, ($p > 0.05$). Significant correlation between COD concentration and culturability concentration in MFC2 was not found ($p > 0.05$).

8.3.4. TOC removal rate

TOC quantification is a viable alternative to COD since it allows a direct and more precise measurement of the total amount of the organic carbon present in a sample. TOC, unlike COD, has the advantage of not producing toxic waste during the analysis and therefore has no associated costs with toxic waste disposal.¹³⁶ By the quantification of the TOC concentration of the SWW (2478 mg/L) and the TOC concentration at the end of each cycle it was possible to calculate the TOC removal rate during the various cycles in both MFCs performances. Figure 12 shows the relationship between the TOC removal rate and COD removal rate in both MFCs.

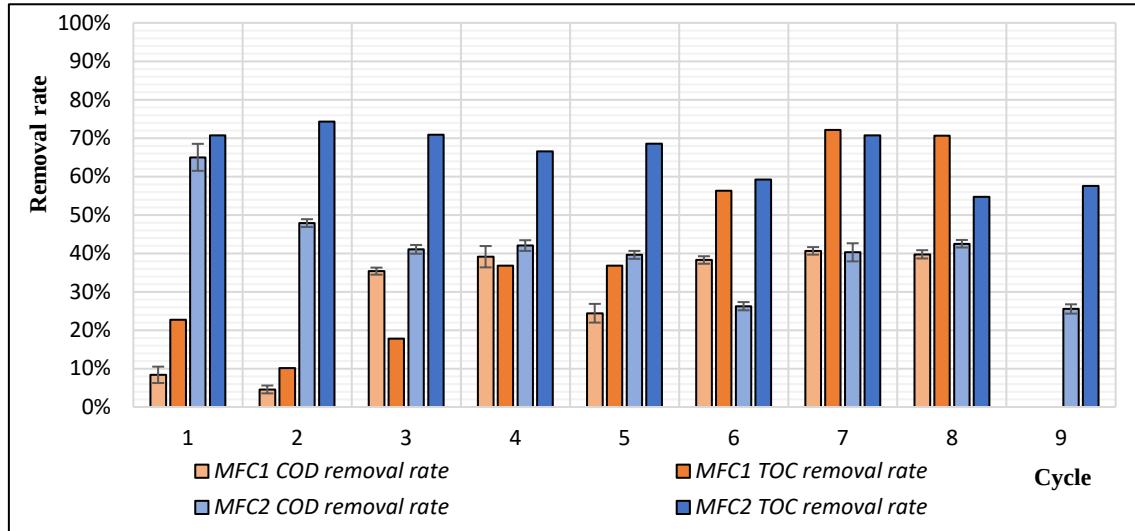


Figure 12: Relationship between the TOC removal rate and COD removal rate in both MFCs.

The following table presents the TOC removal rate obtained at the end of each cycle for both MFCs.

Table 10: TOD removal rate observed at the end of each batch cycle for both MFCs.

Cycle	MFC1 TOC removal rate	MFC2 TOC removal rate
1	23%	71%
2	10%	74%
3	18%	71%
4	37%	67%
5	37%	69%
6	56%	59%
7	72%	71%
8	71%	55%
9	-	58%

Comparing TOC removal rate results with the culturability results in the diverse cycles during MFC1 activity, it can be observed that the progressive increase in the CFU/mL of *Z. bailii*

is in agreement with the increase of the TOC removal rate. COD and TOC removal rates variations during the operational activity of MFC1 are mostly in accordance between them. The increase of TOC removal rate verified at the end of the 7th cycle and at the end of the 8th cycle is not very proportional with the increase of the COD removal rate verified at the same cycles. Comparative analysis between the results of Figure 12 and the culturability expressed in CFU/mL for the diverse cycles during MFC2 activity show that the decrease in the concentration of CFU/mL from the end of the 6th cycle is mostly associated with reduction of the TOC removal rate. At the end of the first five cycles, an approximate average of 70% of TOC removal rate was obtained. The uniformity of the TOC removal rates obtained at the end of the first five cycle are significantly in agreement with the uniformity of the CFU/mL concentration for the same cycles analyzed.

Significant correlation between polysaccharides concentration and TOC concentration, during the operation of MFC1, was not found ($p > 0.05$). There was a significant correlation between COD concentration and TOC concentration ($p < 0.05$), during the operation of MFC1. There was a significant correlation between culturability and TOC concentration ($p < 0.05$), during the operation of MFC1. No significant relation was found between COD concentration and TOC concentration ($p > 0.05$) and between culturability and TOC concentration ($p > 0.05$) in MFC2.

8.3.5. pH

Figure 13 shows the pH values obtained at the end of each cycle for both MFCs.

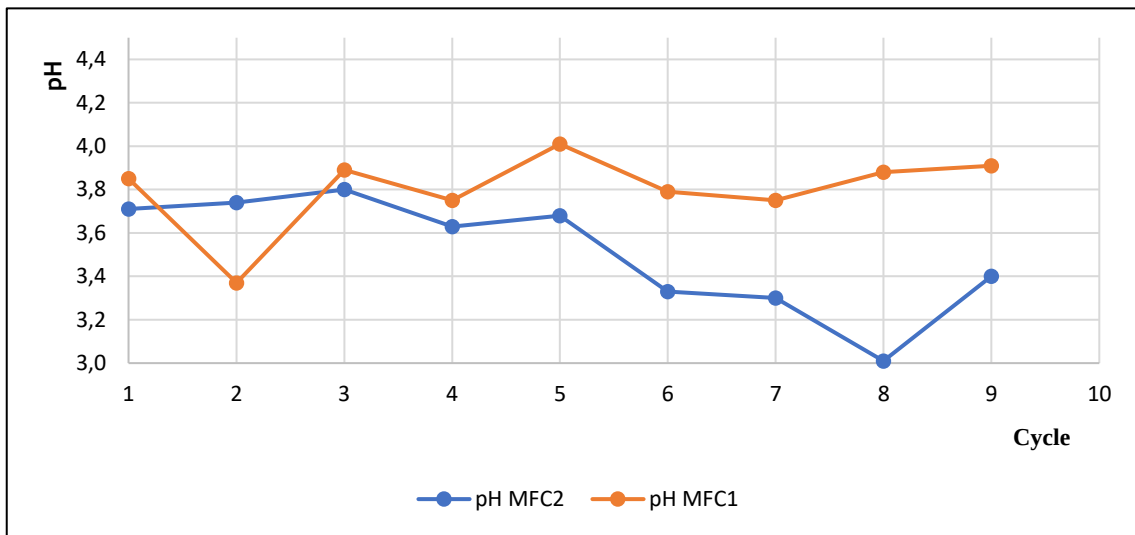


Figure 13: pH values obtained at the end of each cycle for both MFCs.

The pH in MFC1 (with the exception of the 2nd cycle) was maintained in the range between 3.7 and 4.0. This stability reveals that the transfer of protons through the membrane was provided efficiently. On the other hand, in MFC2 the higher power densities obtained contributed to a higher proton production during the MFC2 activity. Between the end of the 5th cycle and the end of the 9th cycle it was found that PEM was not sufficiently efficient in the transport of protons

to the cathode side. The PEM inefficiency from the end of the 5th cycle will have resulted in an accumulation of protons in the anode chamber and consequently a decrease in pH.⁵⁶ The pH decrease from the 5th cycle may be the cause of the CFU/mL concentration decrease for the same time interval. As already described, the pH decrease in the anode side can negatively affect the microbial cell growth and consequently the energy production.^{21,57}

SWW conductivity was performed once and it was 10.63mS/ cm.

8.3.6. Open circuit voltage

Open circuit voltage was daily measure after inoculation as can be visualized in Figure 14.

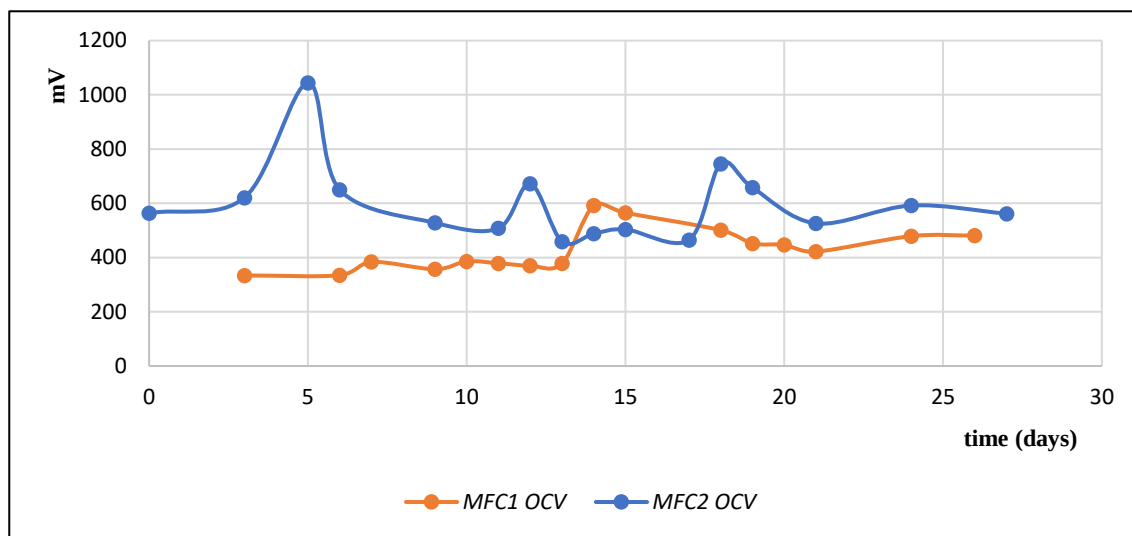


Figure 14: Open circuit voltage during both MFCs operational activity.

OCV of MFC1 was increasing during the operational time from 334 mV at inoculation to 481 mV at the end of the experiment. OCV was uniform until the 13th day of operation, between the 13th day and the 14th day, which corresponds to the 5th cycle, the OCV in MFC1 increases from 378 mV to the maximum OCV obtained of 592 mV. The maximum OCV is related with the maximum CFU/mL obtained in the 5th cycle of MFC1 operational activity, since more microorganisms are capable to transfer electrons. The OCV increasing is also related with the beginning of the log phase of *Z. bailii*. After the 15th day, the OCV decreases and stabilized in the range between 400 mV and 500 mV until the end of the experiment. MFC2 demonstrated an unpredictable behavior in relation to OCV, showing voltage peaks at the 5th (1044 mV) 12th (672 mV) and 18th (746 mV) of operation, which can be associated to days with high room temperatures, in fact *B. cereus* have an optimal growth temperature higher than *Z. bailii*.^{137,117}

There was no significant correlation between polysaccharides concentration and OCV, during the operation of both MFCs, ($p > 0.05$). There is no significant correlation between COD concentration and OCV, during the operation of both MFCs, ($p > 0.05$). TOC demonstrate no significant correlation between OCV during the operation of both MFCs ($p > 0.05$). There was a

significant correlation between culturability and OCV, during the operation of MFC1, ($p < 0.05$). Significant correlation between culturability and OCV, during the operation of MFC2 was not found, ($p < 0.05$).

8.3.7. Polarization and power density curves

Figure 15 presents the polarization and power density curves made at the end of 1st cycle for both MFCs.

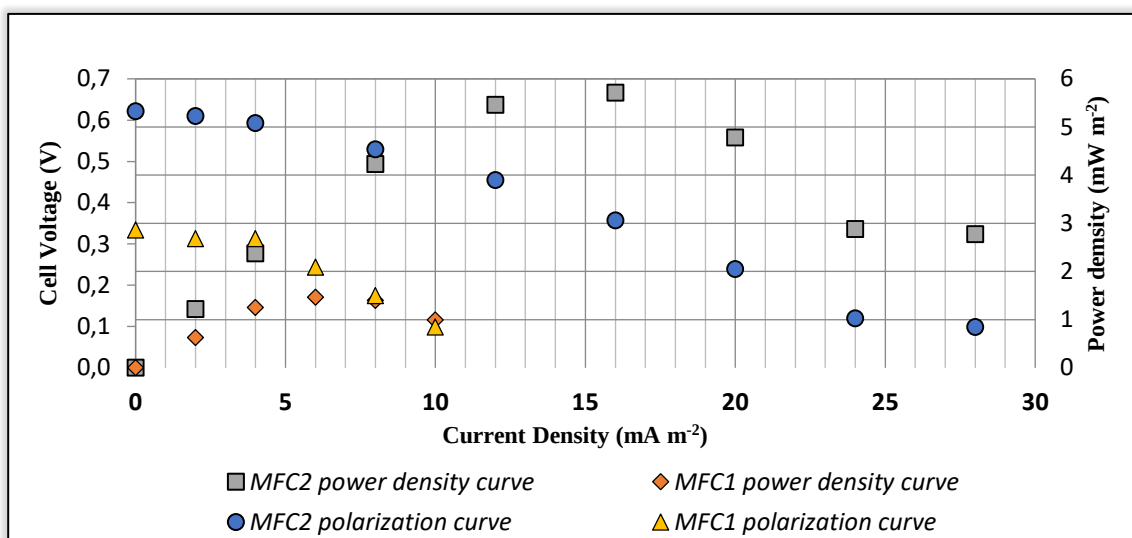


Figure 15: Polarization and power density curves at the end of 1st cycle for both MFCs.

MFC2 reached a maximum power output of 5.71 mW/m² and a maximum current density of 16 mA/m². MFC1 reached a maximum power output of 1.46 mW/m² and a maximum current density of 6 mA/m². MFC2 produces more power output than MFC1, possible due to the very higher COD consumption verified at the end of the 1st cycle, which is related to a higher electron release. Figure 16 shows the polarization and power density curves made at the end of 2nd cycle for both MFCs.

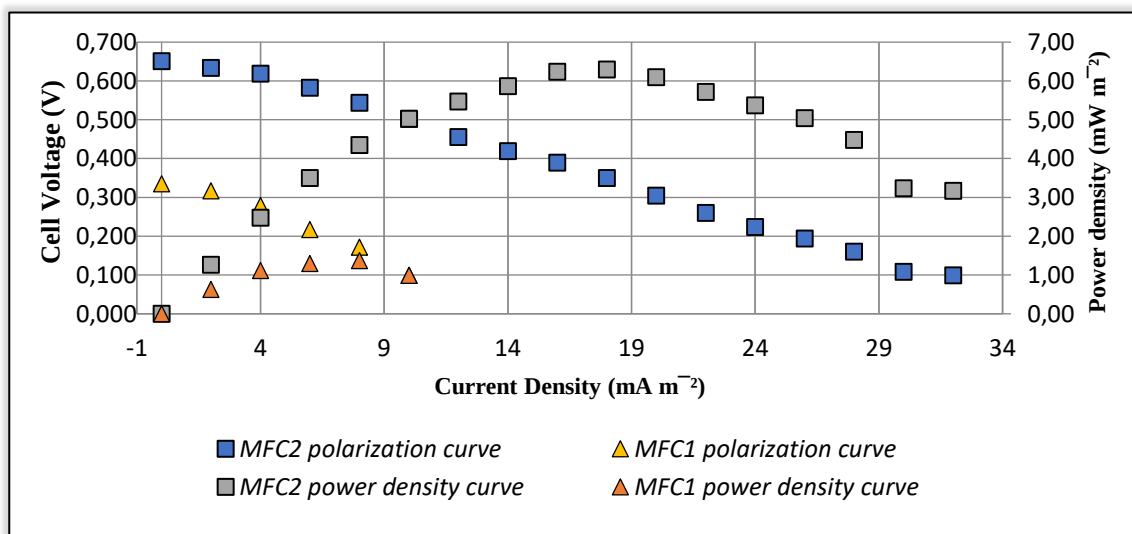


Figure 16: Polarization and power density curves at the end of 2nd cycle for both MFCs.

MFC2 reached a maximum power output of 6.30 mW/m² and a maximum current density of 18 mA/m². MFC1 reached a maximum power output of 1.38 mW/m² and a maximum current density of 8 mA/m². At the end of the 2nd cycle both MFCs showed very similar values to those obtained at the end of the 1st cycle, probably due the similarity between the analytical parameters results evidenced in the two first cycles.

Next figure shows the polarization and power density curves made at the end of 3rd cycle for both MFCs.

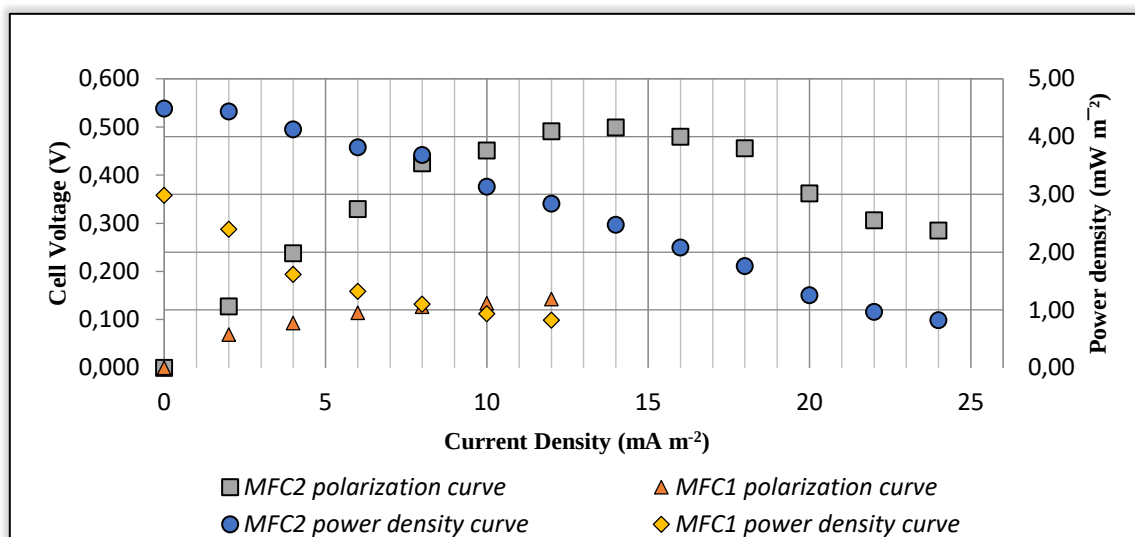


Figure 17: Polarization and power density curves at the end of 3rd cycle for both MFCs.

MFC2 reached a maximum power output of 4.16 mW/m² and a maximum current density of 14 mA/m². MFC1 reached a maximum power output of 1.19 mW/m² and a maximum current density of 12 mA/m². MFC2 exhibit a significant decrease of the maximum power density achieve compared to the 2nd cycle. The decrease of the energy production between the 2nd and 3rd cycle in MFC2 is in accordance with the slightly decrease revealed for the polysaccharides, COD and TOC removal rates analyzed at the same time interval. The maximum power output decrease revealed for MFC1 is not related with the increase of the polysaccharides, COD and TOC removal rate, and with the increase of CFU/mL of *Z. bailii* between the 2nd and the 3rd cycle.

Figure 18 presents polarization and power density curves made at the end of 4th cycle for both MFCs.

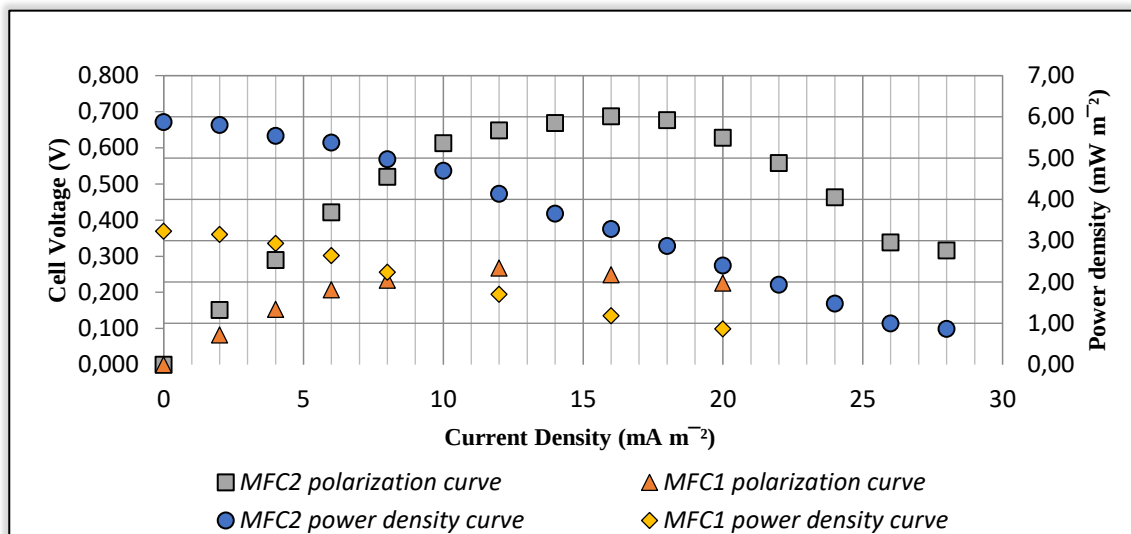


Figure 18: Polarization and power density curves at the end of 4th cycle for both MFCs.

MFC2 reached a maximum power output of 6.02 mW/m² and a maximum current density of 16 mA/m² the end of 4th cycle. MFC1 reached a maximum power output of 2.34 mW/m² and a maximum current density of 12 mA/m². MFC2 exhibit an increase of the maximum power density achieve in relation with the 3rd cycle. This small increase can not be related with any analytical parameter, since for the same time interval the TOC and polysaccharides removal rate slightly decrease and the COD removal rate and UFC/mL were maintaining at very similar values to those obtained at the end of the 1st cycle. Possible at the end of this cycle the biofilm aggregation was becoming to be more prominent, allowing the more efficient transference of electrons to the anode surface.¹⁶ The increase of the maximum power output of MFC1 is directly associated with the increase of the parameters analyzed at the end of the 4th cycle. Figure 19 shows polarization and power density curves made at the end of 5th cycle for both MFCs.

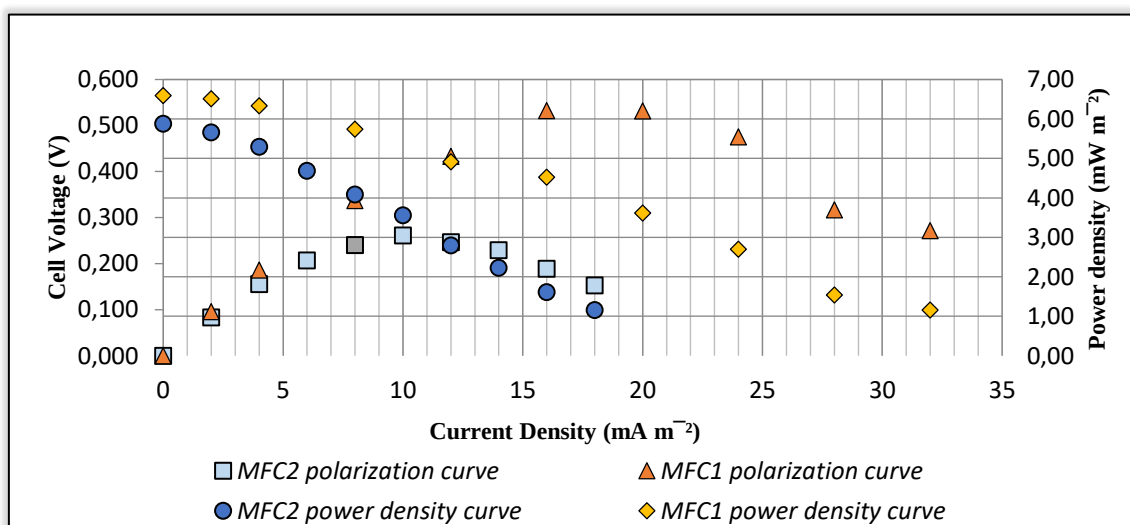


Figure 19: Polarization and power density curves at the end of 5th cycle for both MFCs.

MFC2 reached a maximum power density of 3.05 mW/m² and a maximum current density of 10 mA/m² at the end of 5th cycle. MFC1 energy production decrease in terms of maximum power production can not be associated with the any of the parameters analyzed, because the parameters results obtained at the end of the 5th cycle are very similar to those obtained at the end of the 4th cycle. MFC1 reached a maximum power output of 6.21 mW/m² and a maximum current density of 16 mA/m² at the end of 5th cycle. This was the higher power generation obtained for MFC1 during the experiment. The highly increase is directly associated with high CFU/mL of *Z. bailii* verified during the operation of MFC1.

Figure 20 shows polarization and power density curves made at the end of 6th cycle for both MFCs.

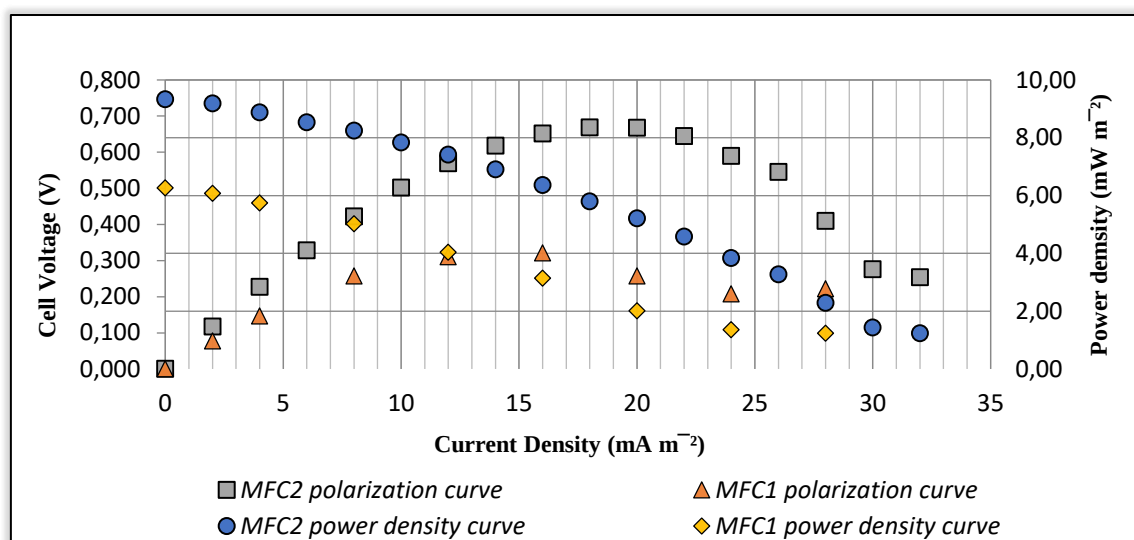


Figure 20: Polarization and power density curves at the end of 6th cycle for both MFCs.

MFC2 reached a maximum power output of 8.35 mW/m² and a maximum current density of 10 mA/m² at the end of 6th cycle. This was the higher energy production obtained during the operational activity for both MFCs. However, the power output of MFC2 is not in agreement with the decreasing of CFU/mL at the same time interval. The hypothesis of another microorganism could possible interfere in the substrate consumption and energy production from the end of the 6th cycle should be taken in consideration. MFC1 reached a maximum power output of 4.02 mW/m² and a maximum current density of 16 mA/m² at the end of 6th cycle. The slightly decrease in CFU/mL could possible explain the decrease of power output obtained. However, the COD removal rate increasing verified between the 5th and the 6th cycles should have been translated in an increase of the energy generated.

Following figure presents polarization and power density curves made at the end of 7th cycle for MFC2. Polarization and power density curves were not performed at the end of the 7th cycle for MFC1.

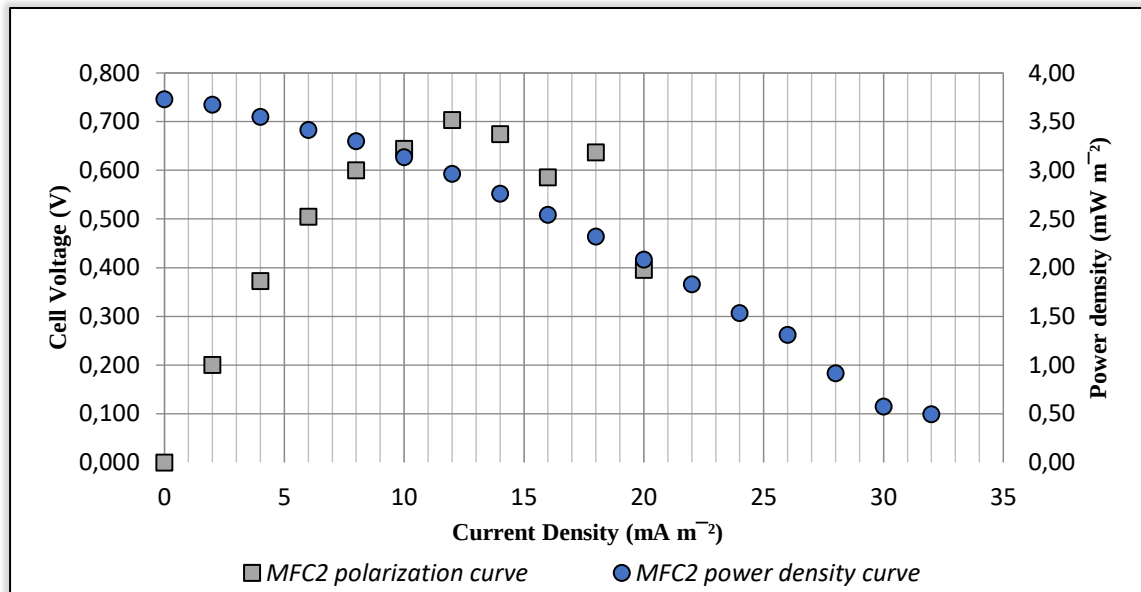


Figure 21: Polarization and power density curves at the end of 7th cycle for MFC2.

MFC2 reached a maximum power output of 3.52 mW/m² and a maximum current density of 12 mA/m² at the end of 7th cycle. The decrease in CFU/mL of *B. cereus* and pH can be related with the power density decrease. However, the power output decreasing of MFC2 is not in agreement with the increasing of the COD and the TOC removal rate for the same time interval.

Figure 22 presents polarization and power density curves made at the end of 8th cycle for both MFCs.

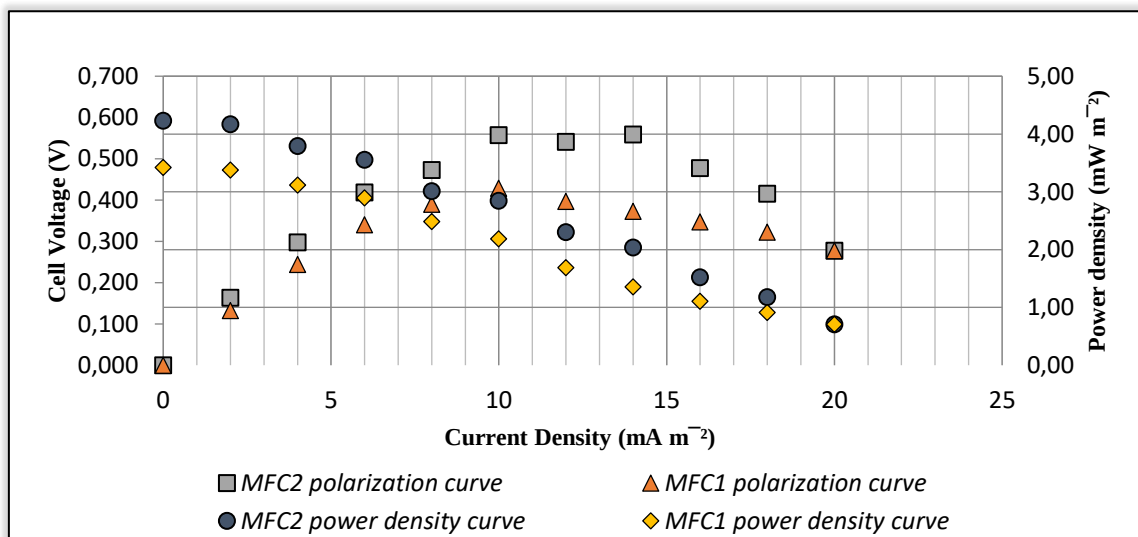


Figure 22: Polarization and power density curves at the end of 8th cycle for both MFCs.

MFC2 reached a maximum power output of 3.98 mW/m² and a maximum current density of 10 mA/m² at the end of 8th cycle. MFC1 reached a maximum power output of 3.06 mW/m² and a maximum current density of 10 mA/m² at the end of 8th cycle. This cycle presents the most approximated results in terms of energy produced between both MFCs. In MFC2, the power generated at the end of this cycle is almost similar than the energy obtained at the end of the

previous cycle, probably because the COD removal rate and the CFU/mL results at the end of this cycle are almost the same than those obtained in the end of the 7th cycle. MFC1 reached a maximum power output of 3.06 mW/m² and a maximum current density of 10 mA/m² at the end of 8th cycle. Between the 6th and the 8th cycle, MFC1 exhibit a decrease in the energy production, which contrary the increasing of the COD and TOC removal rates and the CFU/mL verified between the end of the 6th cycle and the end of the 8th cycle.

Figure 23 shows polarization and power density curves made at the end of 9th cycle for both MFC2.

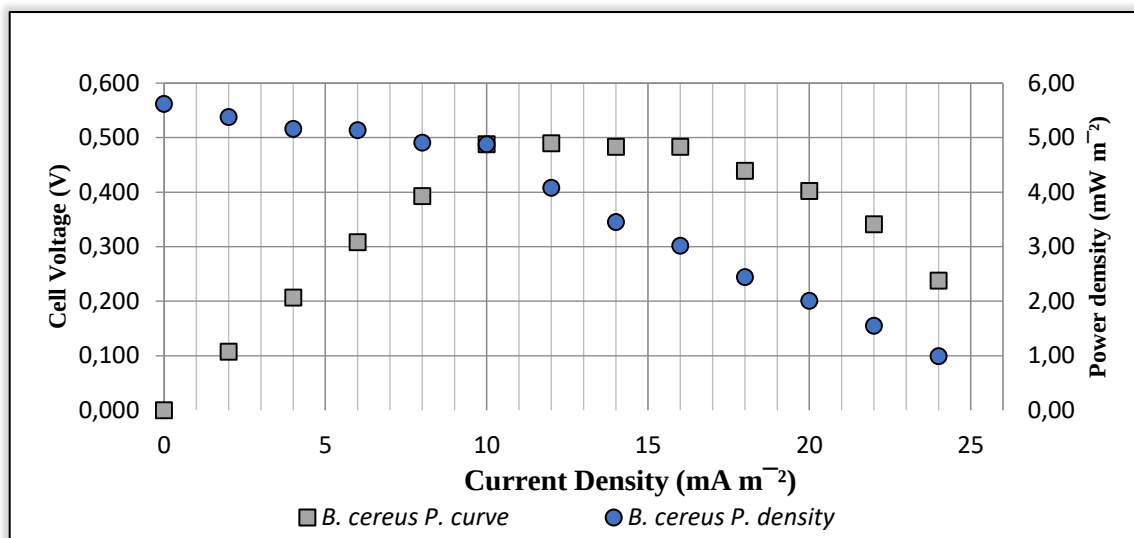


Figure 23: Polarization and power density curves at the end of 9th cycle for MFC2.

MFC2 reached a maximum power output of 4.90 mW/m² and a maximum current density of 12 mA/m² at the end of 9^h cycle. The increase in the energy production verified at the end of this cycle can be related with the increase of the pH for the same time interval. At the end of this cycle protons could be more effectively transferred through PEM compared to the 8th cycle, allowing the increase of the electron transference to the cathode side.⁵⁷

Table 11 compiles the external current, current density and voltage values associated to the maximum power density obtained at the end of each cycle for both MFCs. *B. cereus* and *Z. bailii*, although not considered exoelectric microorganisms, were able to generate energy by treating SWW in a SC-MFC. The energy transfer possible have been intermediated by compounds present in SWW, such as YPD. Sayed et al.¹³⁸ demonstrated that the use of YPD in MFC inoculated with *S. cerevisiae* can be used as a natural mediator by increasing the power output from 12.9 mW/cm² to 32.6 mW/cm². By the analysis of the different polarization curves and the power density, it is possible to observe that most of the energetic densities obtained by MFC inoculated with *B. cereus* were higher than the energy densities generated by the MFC inoculated with *Z. Bailii*. As can be seen in Table 11, MFC1 showed a maximum power density of 6.21 mW/m² and a maximum current density of 16 mA m⁻² at the end of the 5th cycle. In turn, MFC2 showed a maximum power density of 8.35 mW/m² and a maximum current density of 18 mA m⁻²

² at the end of the 6th cycle. The higher energy production observed during MFC2 activity in relation to MFC1 can be related to the higher consumption of the organic matter present in the SWW. However, a direct relation between the power output and the experiment parameters analysed (culturability, COD, TOC and polysaccharides removal rate) is not feasible to do for every cycles in both MFCs. In fact, the maximum power output obtained in MFC1 is related to the higher CFU/mL concentration of *Z. bailii* during the MFC1 activity but not with COD and TOC removal rates, since in MFC1, COD and TOC removal rates are higher at the end of the 8th cycle, which does not coincide with a supposed higher power output obtained for the same cycle. Possible high energy densities are also associated with an increase of COD and TOC concentration since, despite of the higher electrons release, metabolic end products are also being synthesized in higher amounts by microorganisms. Also, in MFC2 the higher power output was observed during the decrease of CFU/mL concentration and decrease of COD and TOC removal rates, these results allow to consider the possibility of contamination from the beginning of the 6th cycle, which could eventually explain the decrease of the cell growth of *B. cereus* by substrate competition and the simultaneously increase of the electricity generated at the end of the 6th cycle.

Table 11: External current, current density and voltage associated to the maximum power density obtained at the end of each cycle for both MFCs.

Cycle	MFC1				MFC2			
	External current (mA)	Current density (mA m ⁻²)	Voltage (V)	Power density (mW/m ²)	External current (mA)	Current density (mA m ⁻²)	Voltage (V)	Power density (mW/m ²)
1	0.015	6	0.244	1.46	0.04	16	0.357	5.71
2	0.002	8	0.172	1.38	0.045	18	0.350	6.3
3	0.03	12	0.099	1.19	0.035	14	0.297	4.16
4	0.03	12	0.195	2.34	0.04	16	0.376	6.02
5	0.04	16	0.388	6.21	0.025	10	0.305	3.05
6	0.04	16	0.251	4.02	0.045	18	0.464	8.35
7	No data	No data	No data	No data	0,03	12	0.293	3.52
8	0.025	10	0,306	3.06	0.025	10	0.398	3.98
9	-	-	-	-	0.03	12	0.408	4.9

Significant correlation between polysaccharides concentration and power density, during the operation of both MFCs was not found ($P > 0.05$). There was no significant correlation between COD concentration and power density, during the operation of both MFCs, ($p > 0.05$).

There was a significant correlation between culturability concentration and power density ($p < 0.05$), during the operation of MFC1. TOC demonstrate no significant correlation between power density during the operation of both MFCs, ($p > 0.05$). Highly significant correlation between OCV concentration and power density during the MFC1 operational activity was found ($p < 0.01$). No significant correlation between OCV concentration and power density during the MFC2 operational activity was found ($p > 0.01$)

The results of the maximum COD removal rates and power densities described in Table 6 are mostly different from the results obtained in this experiment. Vilas Boas et al.¹⁶ presented approximated results, since although different inoculums and anolytes were used, the materials utilized in the MFC assembly were similar. The divergences of the different results observed Table 6 may be related to the fact that the winery effluent presents distinct characteristics regarding to its composition, which makes the efficiency of the WW treatment variable. The different operational conditions (temperature, pH, inoculum, final electron acceptor, configuration, etc.), as well as the different materials used for the construction of MFC also have a high influence on the efficiency of organic matter removal and energy production. Other factor to be considered is that SWW used in this experiment does not present polyphenols in its composition. The low COD removal rate observed in some of the studies mentioned in Table 6 are also associated with the presence of phenolic compounds in high concentrations, which can inhibit the cellular growth of the microorganisms involved in the degradation of the organic matter and consequently the energy production.¹⁹

8.3.8. Biofilm quantification

Quantification of the biofilm adhered to the anode can be very useful to determine which is the microorganism with the best adhesion proprieties to the anode surface. Higher cell adhesion to the electrode surface is associated with the decrease of the internal resistances and the increase of the power output.¹³⁹ In this experiment, it is necessary to take into account that the biofilm quantification of MFC1 was performed on the 27th day after the MFC1 inoculation , two days after the last polarization and power density curves performance for the same MFC1. The biofilm quantification of MFC2 was performed on the 28th day after MFC2 inoculation, on the same day of the last polarization and power density curves performed.

Table 12 presents the characterization of the biofilm attached to the anode surface in terms of total solids, total volatile solids, fixed solids, culturability and polysaccharides concentration released at the end of the experiment.

Table 12: Characterization of the biofilm attached to the anode surface in terms of total solids, total volatile solids, fixed solids, culturability and polysaccharides concentration at the end of the experiment.

Parameter	MFC1 Biofilm	MFC2 Biofilm
Total solids (g/ L)	6.80	6.94
Total volatile solids (g/ L)	5.51	5.35
Fixed solids (g/ L)	1.29	1.59
Culturability (CFU/ mg)	3.6×10^6	4.3×10^4
Polysaccharides (mg/ mg)	90	55

TS concentration obtained for both biofilms are very similar between them. MFC2 showed a slightly higher quantity of TS when compared to MFC1 but lower organic contentment (TVS). MF1 presents a higher CFU/mg when compared to MFC2. Biofilm culturability results are in accordance with the culturability for the two microorganisms during the MFCs activity. Both microorganisms showed a very similar adhesion capacity to the anode surface. However, due to the relationship between the last measurement of the energy density obtained before quantifying the biofilm in both MFCs, it is possible to verify that MFC2, despite having a lower cellular viability in the biofilm, showed a higher energy density than MFC1. *B. cereus* even presenting a lower cell viability in the biofilm can possible originate higher energetic densities than *Z. bailii*. Through the analysis of the culturability results for each microorganism during both MFCs activity it is expected that in the possibility of the experiment had been continued, *Z. bailii* would possibly present a higher TS concentration in relation to the biofilm that would be formed by *B. cereus*.

9. Statistical relations between the experiment analytical parameters in both MFCs

Pearson correlations were made to determine if the different relations between COD (mgO₂/L), TOC (mg/L), polysaccharides (PL) (mg/L), culturability (C) (logCFU/mL), OCV(mV) and P (mW/m²) analyzed during the experiment were statistically significant ($p < 0.05$). The parameters significant correlated were estimated by equations using regression analysis. Table 13 presents the regression analysis between the correlated analytical parameters for the SWW treatment by using MFC1. No correlation was found between the experiment analytical parameters analyzed for MFC2 ($p > 0.05$) and for that reason no regression analysis was performed for MFC2.

Table 13: Correlation and regression analysis between COD, TOC, PL, C, OCV and P for the SWW treatment by using a SC-MFC with *Z. bailii* as inoculum.

Relation	Correlation coefficient	Regression equation	r ²
PL: COD	r = 0.826* p = 0.012	PL = 0.395 * COD - 1322.32	68.2%
PL:C	r = - 0.781* p = 0.022	PL = - 350.148 * TOC - 2398.58	60.9%
COD: TOC	r = 0.727* p = 0.041	COD = 1100 * TOC + 2668.251	52.9%
COD:C	r = - 0.793* p = 0.019	COD = - 744.473 * C + 8602.503	62.9%
C: TOC	r = - 0.756* p = 0.03	C = -0.001* TOC + 7.59	57.2%
C: OCV	r = 0.846** p = 0.008	C = 0.009 * OCV + 1.88	71.6%
C: P	r = 0.787* p = 0.036	C = 0.43 * P + 4.501	61.9%
OCV: P	r = 0.954** p = 0.001	OCV = 48.94 * P + 282.692	90.9%

r: Pearson correlation coefficient; p: significance level; r²: percentage of the variation explained by regression;

*, **: significant correlated at (p < 0.05), significant at (p < 0.01).

10. Conclusions and suggestions for future work

B. cereus and *Z. bailii* showed the best results associated with the conjugation of CFU/mL concentration and COD removal rate in the SWW during 96 hours of incubation and for that reason were chosen to be tested in a SC-MFC. Both microorganisms demonstrated to be able to degrade the organic matter present in the winery effluent and simultaneously produce electricity. *B. cereus* and *Z. bailii* proved to be possible options to act as inoculum in the winery effluent treatment by using MFC technology. By the analysis of the performance of the two MFCs, it is possible to conclude that for 24 days of the operational activity, MFC2 presented better results regarding the maximum COD removal rate ($65 \pm 4\%$) and to the maximum power density obtained (8.35 mW/m^2) in relation to MFC1, which demonstrated a maximum COD removal rate of $41 \pm 1\%$ and a maximum power density of 6.21 mW/m^2 . However, from the end of the 5th cycle, *B. cereus* cell culturability and the COD removal rate in MFC2 decreased, possibly due to the pH decreasing at the anode chamber for the same time interval, which can possible represent the initial death phase of bacterial growth of *B. cereus*. The opposite behavior was exhibited by *Z. bailii* which demonstrated a gradual increase in both CFU/mL concentration and COD removal rate during the MFC1 operational activity. This situation allows to propose that *Z. bailii* might be at an exponential phase of bacterial growth and for that reason, *Z. bailii* could be the best option among these two microorganisms analyzed, for long-term period winery wastewater treatment such as in large-scale applications. *Z. bailii* and *B. cereus* revealed almost the same adherence capacity to the anode surface during both MFCs operational activity by presenting a TS quantification of 6.8 g/L and 6.94 g/L, respectively.

The main issue in the application of *Z. bailii* as inoculum in MFC1 is the production of ethanol as metabolite resulting from the oxidation of polysaccharides through the fermentative pathway, which hinder the COD removal rate efficiency. In this context, the creation of a microbial consortia with the insertion of a microorganism capable of degrading the ethanol produced by *Z. bailii* could be beneficial to the MFC performance and for the further development of this project. In a first analysis, acetic acid bacteria could be a plausible choice, as already was mentioned, these microorganisms have the capacity to degrade ethanol to acetic acid. Additionally, the genus *Acetobacter* is capable to use acetic acid as carbon source.^{120,121} *Z. bailii* could possibly adapt to this hypothetically scenario. This yeast has a mechanism capable of regulating the concentration of intracellular acetic acid, which allows its survival in environments with high acidity.¹¹⁸ Despite of existing studies^{17,18} demonstrating the enzyme-mediated transference of electrons from acetic acid bacteria, the fact of acetic acid bacteria being strictly aerobic could create doubts regarding to the applicability of these microorganism in a possible microbial consortium. *Pelobacter propionicus* can represent an appropriate choice for the application of a consortium involving *Z. bailii* in the winery wastewater treatment by MFCs. *P.*

propionicus is an anaerobic bacterium capable of degrading ethanol, lactate, propanol and butanol, typical present in winery wastewater, to acetic acid.¹⁴⁰ *P. propionicus* was found as one of the most representative microorganisms in a MFC fed with lactic acid and in a MFC fed with ethanol after more than one year of operational activity.^{32,33} The capacity of *P. propionicus* to convert ethanol and lactate to acetic acid can be useful to lower the concentration of ethanol synthesized by *Z. bailii* and at the same time provide a carbon source which could be easily metabolized by an eventually exoelectrogenic microorganism. As is known, microorganisms capable of transferring electrons directly to the anode have the drawback of being metabolically limited by being only capable of degrading metabolic products such as, for example, lactate or acetate.³³ The introduction of *G. sulfurreducens* in the same consortium of *P. propionicus* and *Z. bailii* could potentiate the energy production in the treatment of the winery effluent by MFCs technology. *G. sulfurreducens* has the advantage of being able to transfer electrons directly to the anode by mainly using acetate as a carbon source.³¹ Kiely et al.³³ testified the dominance of *G. sulfurreducens* in MFCs containing acetate as substrate. The possibility of *P. propionicus* and *G. sulfurreducens* cohabiting in the same environment was proved by Kiely et al.³³, who reported their simultaneous presence in a MFC fed with acetate as substrate, and by Cusick et al.²⁰ in a biofilm produced during the winery wastewater treatment using a microbial electrolysis cell (MEC). In this theoretical hypothesis, the metabolic and synergic relationship between the three microorganisms described could probably allow a greater reduction of the organic load and a more efficient transfer of electrons and energy production.

The application of MFC to the treatment of the winery effluent seems to be a possible option to act as a complementarity treatment to the actual anaerobic system. In an optimistic perspective, the technology associated with MFCs could be used as an alternative to the aerobic system which is usually applied to maximize the efficiency of the anaerobic winery wastewater treatments when the organic concentration is at low levels and the anaerobic treatment is not being effective. MFCs can allow the generation of electricity and simultaneously the degradation of organic matter, maintaining the system self-sustainable without high external energy requirements, this attractive attribute could provide an incentive for the eventual application of this technology in smaller vineyards without spending large amounts of money to treat winery wastewaters. For this purpose, it is necessary to investigate and determine the best materials, operational conditions and microorganisms suitable for the application of MFCs in the treatment of the winery wastewater in order to obtain the best organic matter removal rates and energy densities favorable to the environmental and economic development and investment of MFC technology.

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12. Annexes

Table A1 is based on the study carried out by Vlyssides et al.⁸⁰ which provides information on the production of winery wastewater in liters per one liter of wine produced.

Table A1: Production of winery wastewater in liters per one liter of wine produced.

Different wine processes and infrastructures	Liters of wastewater produced per liter of alcohol content in wine (L/L)
Reception grapes	
Machinery Washing	0.77
Floor washing	0.57
Must production	
Fermentation vessels pre-washing	0.24
Machinery washing	1.54
Floor washing	0.57
Must losses	0.08
Fermentation	
Decanting	
Fermentation vessels post-washing	0.24
Storage tanks pre-washing	0.24
Washing of transportation pumps	0.77
Floor washing	0.57
Fermenting must losses	0.08
Maturation and stabilization	
Filtration	
Post-washing of maturation tanks	0.24
Pre-washing of storage tanks	0.24
Machinery washing	1.54
Floor washing	0.57
Wine losses	0.08
Disposal from storage tanks into the vessels of transportation trucks	
Post-washing of storage tanks	0.24
Machinery washing	0.77
Floor washing	0.57
Wine losses	0.08

Figure A1 represents the microorganisms OD at 600 nm in SWW for 96 hours period.

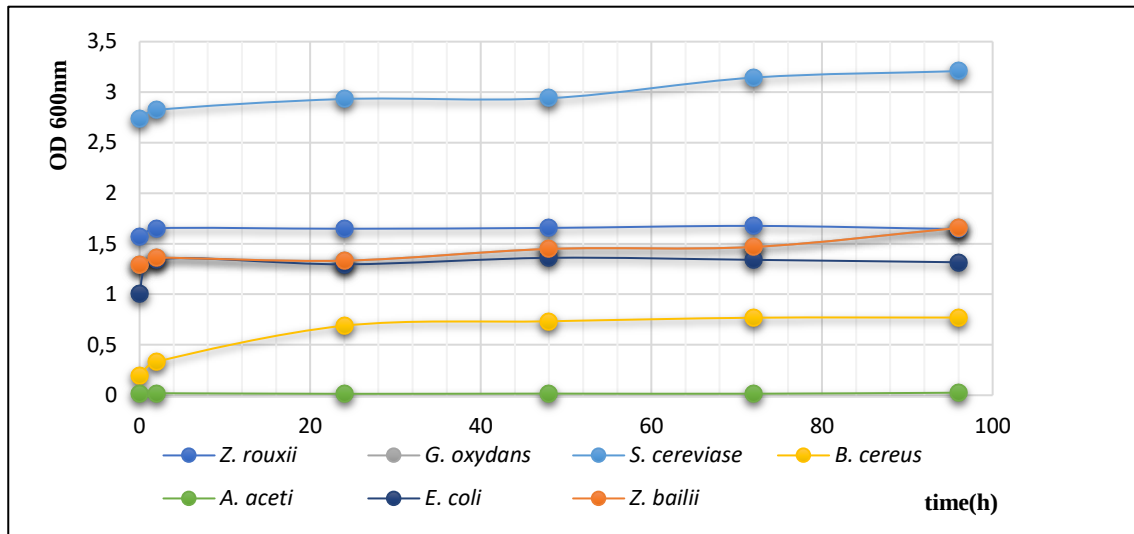


Figure A1: Microorganisms OD at 600nm in SWW for 96 hours period.

Table A2 shows the microorganisms OD at 600 nm in SWW for 96 hours period.

Table A2: Microorganisms OD at 600 nm in SWW for 96 hours period.

	0 hours	2 hours	24 hours	48 hours	72 hours	96 hours
<i>Z. rouxii</i>	1.564	1.652	1.648	1.656	1.676	1.644
<i>Z. bailii</i>	1.296	1.360	1.332	1.448	1.468	1.656
<i>G. oxydans</i>	0.118	0.198	0.192	0.214	0.237	0.288
<i>S. cerevisiae</i>	2.740	2.824	2.932	2.940	3.144	3.208
<i>B. cereus</i>	0.190	0.332	0.688	0.732	0.767	0.769
<i>A. aceti</i>	0.018	0.020	0.014	0.016	0.015	0.025
<i>E. coli</i>	1.004	1.344	1.296	1.360	1.340	1.316

Table A3 presents the microorganisms culturability in terms of CFU/mL in SWW for 96 hours period.

Table A3: Microorganisms culturability in terms of CFU/mL in SWW for 96 hours period.

	0 hours	2 hours	24 hours	48 hours	72 hours	96 hours
<i>Z. bailii</i>	9.40*10 ⁶	9.00*10 ⁶	1.30*10 ⁷	1.21*10 ⁷	9.85*10 ⁶	8.75*10 ⁶
<i>Z. rouxii</i>	1.95*10 ⁷	1.90*10 ⁷	1.12*10 ⁷	1.12*10 ⁷	7.00*10 ⁶	4.40*10 ⁶
<i>G. oxydans</i>	1.60*10 ⁷	3.35*10 ⁷	5.85*10 ⁷	4.15*10 ⁷	7.00*10 ⁶	4.40*10 ⁶
<i>S. cerevisiae</i>	4.40*10 ⁷	6.80*10 ⁷	5.30*10 ⁷	5.80*10 ⁷	5.40*10 ⁷	6.25*10 ⁷
<i>B. cereus</i>	1.45*10 ⁶	6.50*10 ⁶	2.45*10 ⁷	2.75*10 ⁷	2.50*10 ⁷	1.10*10 ⁸
<i>A. aceti</i>	1.65*10 ⁶	5.20*10 ⁶	6.00*10 ⁶	5.70*10 ⁶	5.95*10 ⁶	4.65*10 ⁶
<i>E. coli</i>	2.55*10 ⁸	5.95*10 ⁸	1.00*10 ⁸	3.75*10 ⁷	2.30*10 ⁷	1.20*10 ⁷

Table A4 include the significant differences between the microorganism's culturability during the preliminary evaluation tests for the MFC inoculum determination with Scheffe Post-Hoc test comparison using SPSS.

Table A4: significant differences between the microorganism's culturability during the preliminary evaluation tests for the MFC inoculum determination with Scheffe Post-Hoc test comparison using SPSS.

Microorganism (I)	Microorganism (J)	Mean Difference (I-J)	Std. Error	Sig.	Lower Bond	Upper Bound
<i>B. cereus</i>	<i>S. cerevisae</i>	0.234946	0.102716	0.558296	-0.259755	0.729647
	<i>G. oxydans</i>	0.127017	0.102716	0.941837	-0.367684	0.621718
	<i>Z. bailii</i>	1.084660	0.102716	0.000564	0.589959	0.621718
	<i>A. aceti</i> *	1.370861	0.102716	0.000123	0.876160	1.865562
	<i>Z. rouxii</i> *	1.382958	0.102716	0.000116	0.888257	1.877660
	<i>E. coli</i> *	0.946941	0.102716	0.001332	0.452240	1.441642
<i>S. cerevisae</i>	<i>B. cereus</i>	-0.234946	0.102716	0.558296	-0.729647	0.259755
	<i>G. oxydans</i>	-0.107929	0.102716	0.972070	-0.602630	0.386772
	<i>Z. bailii</i> *	0.849714	0.102716	0.002594	0.355013	1.344415
	<i>A. aceti</i> *	1.135915	0.102716	0.000419	0.641214	1.630616
	<i>Z. rouxii</i> *	1.148012	0.102716	0.000392	0.653311	1.642714
	<i>E. coli</i> *	0.711995	0.102716	0.007372	0.217294	1.206696
<i>G. oxydans</i>	<i>B. cereus</i>	-0.127017	0.102716	0.941837	-0.621718	0.367684
	<i>S. cerevisae</i>	0.107929	0.102716	0.972070	-0.386772	0.602630
	<i>Z. bailii</i> *	0.957643	0.102716	0.001241	0.462942	1.452344
	<i>A. aceti</i> *	1.243844	0.102716	0.000232	0.749143	1.738545
	<i>Z. rouxii</i> *	1.255942	0.102716	0.000218	0.761240	1.750643
	<i>E. coli</i> *	0.819924	0.102716	0.003218	0.325223	1.314625
<i>Z. bailii</i>	<i>B. cereus</i> *	-1.084660	0.102716	0.000564	-1.579361	-0.589959
	<i>S. cerevisae</i> *	-0.849714	0.102716	0.002594	-1.344415	-0.355013
	<i>G. oxydans</i> *	-0.957643	0.102716	0.001241	-1.452344	-0.462942
	<i>A. aceti</i>	0.286201	0.102716	0.368309	-0.208500	0.780902
	<i>Z. rouxii</i>	0.298299	0.102716	0.330757	-0.196403	0.793000
	<i>E. coli</i>	-0.137719	0.102716	0.918400	-0.632420	0.356982
<i>A. aceti</i>	<i>B. cereus</i> *	-1.370861	0.102716	0.000123	-1.865562	-0.876160
	<i>S. cerevisae</i> *	-1.135915	0.102716	0.000419	-1.630616	-0.641214
	<i>G. oxydans</i> *	-1.243844	0.102716	0.000232	-1.738545	-0.749143
	<i>Z. bailii</i>	-0.286201	0.102716	0.368309	-0.780902	0.208500
	<i>Z. rouxii</i>	0.012097	0.102716	1.000000	-0.482604	0.506799
	<i>E. coli</i>	-0.423920	0.102716	0.099167	-0.918621	0.070781
<i>Z. rouxii</i>	<i>B. cereus</i> *	-1.382958	0.102716	0.000116	-1.877660	-0.888257
	<i>S. cerevisae</i> *	-1.148012	0.102716	0.000392	-1.642714	-0.653311
	<i>G. oxydans</i> *	-1.255942	0.102716	0.000218	-1.750643	-0.761240
	<i>Z. bailii</i>	-0.298299	0.102716	0.330757	-0.793000	0.196403
	<i>A. aceti</i>	-0.012097	0.102716	1.000000	-0.506799	0.482604
	<i>E. coli</i>	-0.436017	0.102716	0.088111	-0.930719	0.058684

<i>E. coli</i>	<i>B. cereus</i> *	-0.946941	0.102716	0.001332	-1.441642	-0.452240
	<i>S. cerevisae</i> *	-0.711995	0.102716	0.007372	-1.206696	-0.217294
	<i>G. oxydans</i> *	-0.819924	0.102716	0.003218	-1.314625	-0.325223
	<i>Z. bailii</i>	0.137719	0.102716	0.918400	-0.356982	0.632420
	<i>A. aceti</i>	0.423920	0.102716	0.099167	-0.070781	0.918621
	<i>A. aceti</i>	0.436017	0.102716	0.088111	-0.058684	0.930719

*: significantly different ($p < 0.05$).

Figure A2 shows the relationship between CFU/mL and OD600nm of *G. oxydans* in SWW for 96 hours period.

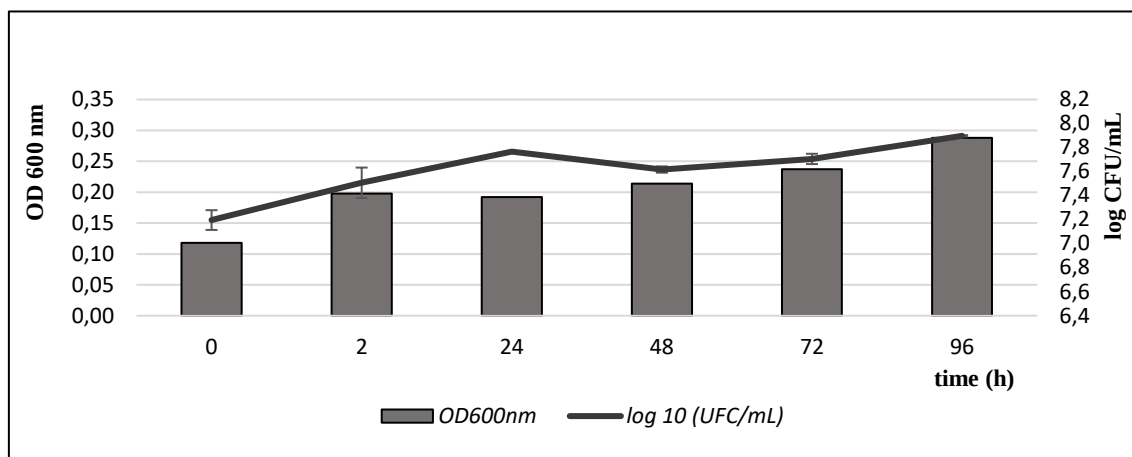


Figure A2: Relationship between CFU/mL and OD600nm of *G. oxydans* in SWW for 96 hours period.

Figure A3 presents a relationship between CFU/mL and OD600nm of *B. cereus* in SWW for 96 hours period.

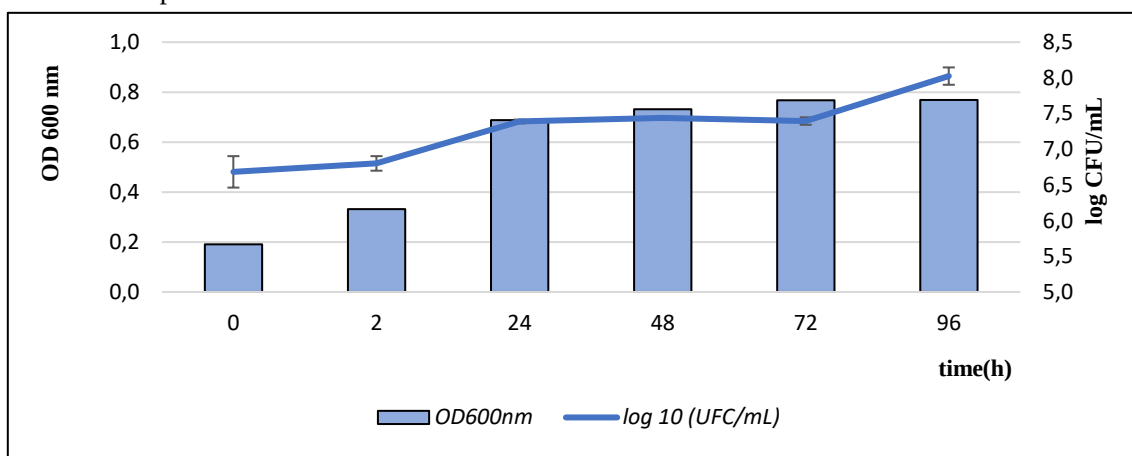


Figure A3: Relationship between CFU/mL and OD600nm of *B. cereus* in SWW for 96 hours period.

Figure A4: Relationship between CFU/mL and OD600nm of *S. cerevisiae* in SWW for 96 hours period.

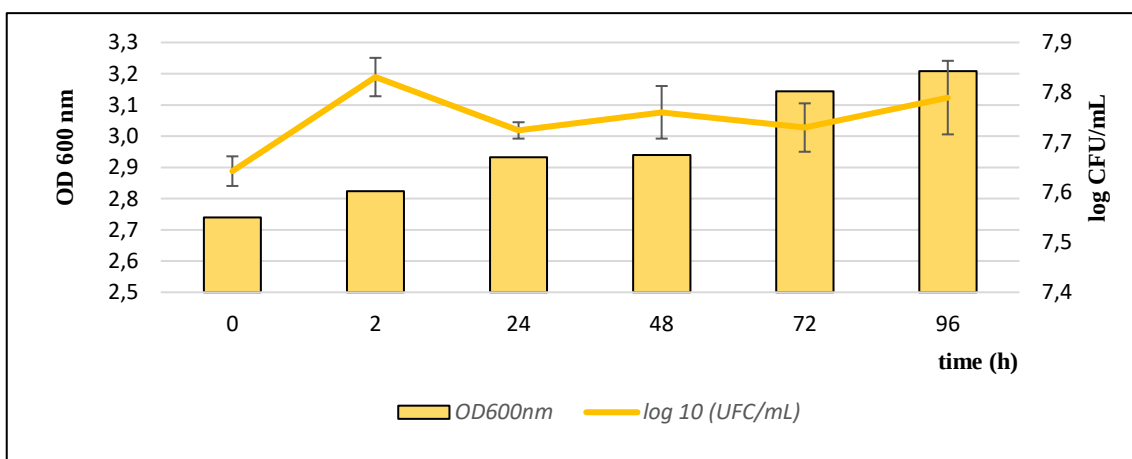


Figure A4: Relationship between CFU/mL and OD600nm of *S. cerevisiae* in SWW for 96 hours period.

Figure A5 presents the relationship between CFU/mL and OD600nm of *Z. bailii* in SWW for 96 hours period.

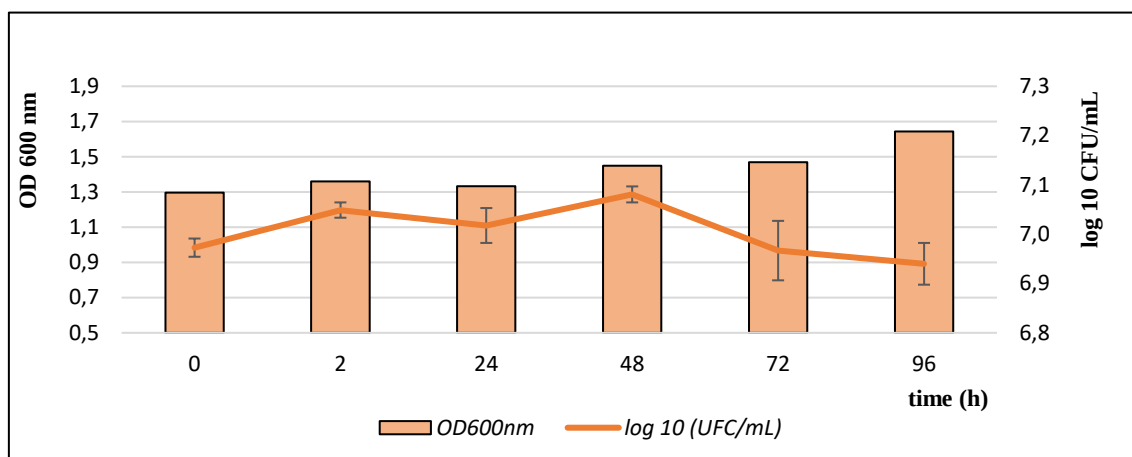


Figure A5: Relationship between CFU/mL and OD600nm of *Z. bailii* in SWW for 96 hours period.

Figure A6 represents the relationship between CFU/mL and OD600nm of *A. aceti* in SWW for 96 hours period.

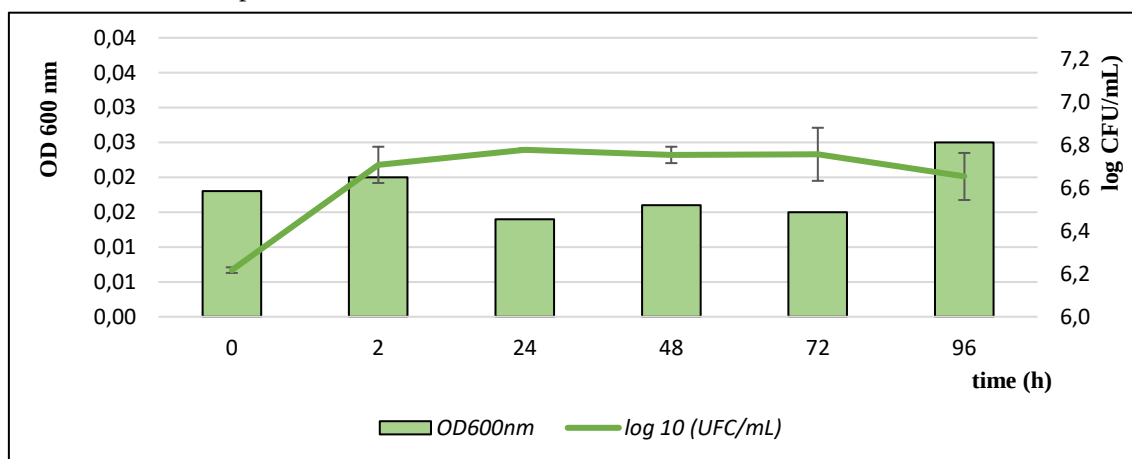


Figure A6: Relationship between CFU/mL and OD600nm of *A. aceti* in SWW for 96 hours period.

Figure A7 shows the relationship between CFU/mL and OD600nm of *Z. rouxii* in SWW for 96 hours period.

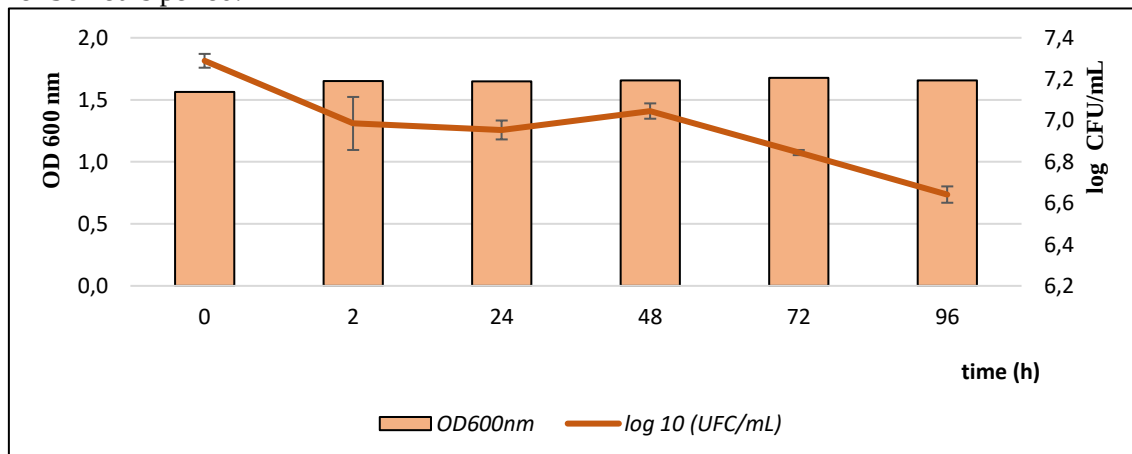


Figure A7: Relationship between CFU/mL and OD600nm of *Z. rouxii* in SWW for 96 hours period.

Figure A8 shows the relationship between CFU/mL and OD600nm of *E. coli* in SWW for 96 hours period.

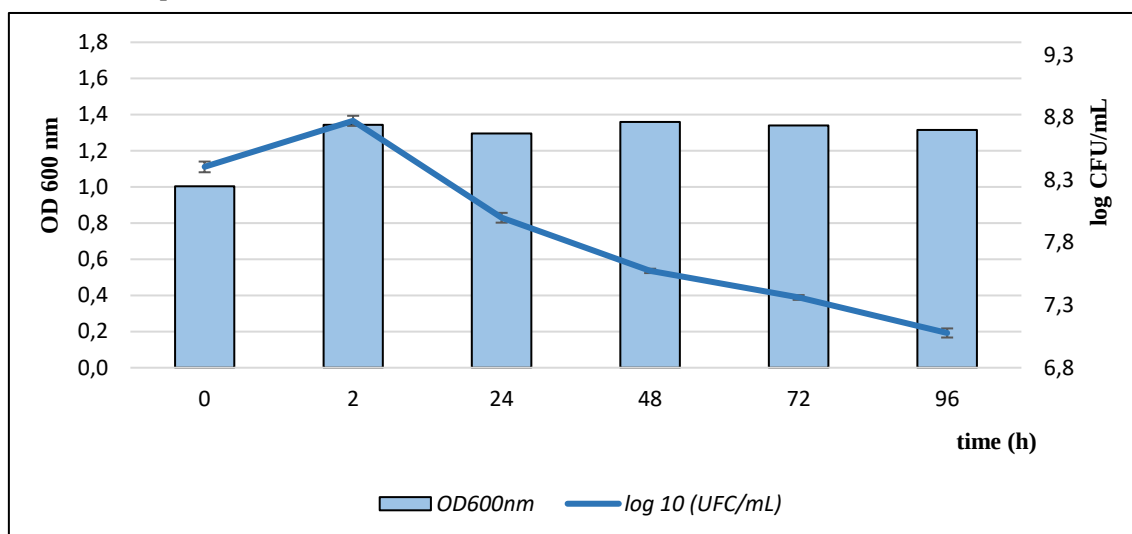


Figure A8: Relationship between CFU/mL and OD600nm of *E. coli* in SWW for 96 hours period.

Figure A9 represents the COD calibration curve made to determine the COD concentration through the absorbance values of the SWW samples analyzed during the experiment.

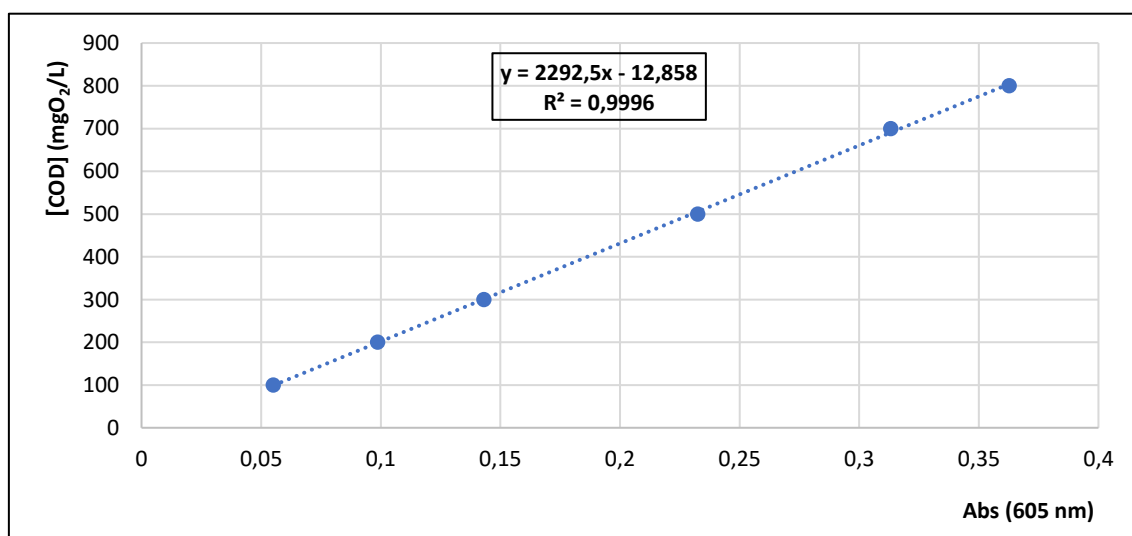


Figure A9: COD calibration curve. absorbance at 490 nm versus COD concentration in milligrams of oxygen per liter of SWW.

Figure A10 represents the Polysaccharides calibration curve made to determine the polysaccharide concentration through the absorbance values of the SWW samples analyzed during the experiment.

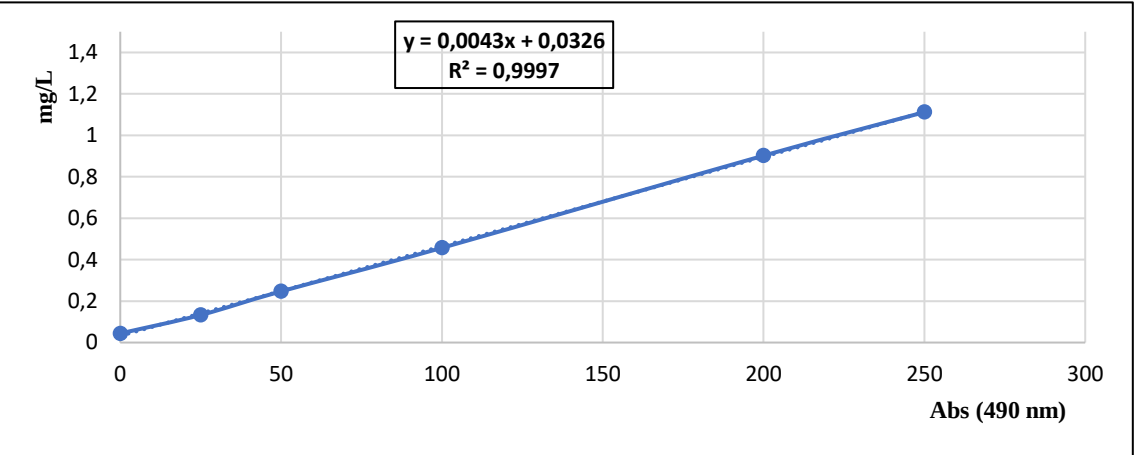


Figure A10: Colorimetric method calibration curve. from DuBois et al.¹²⁷ absorbance at 490 nm versus COD concentration in milligrams of oxygen per liter of SWW

Next Figure A11 shows the growth curve of *Z. bailii* since the inoculation of MFC1.

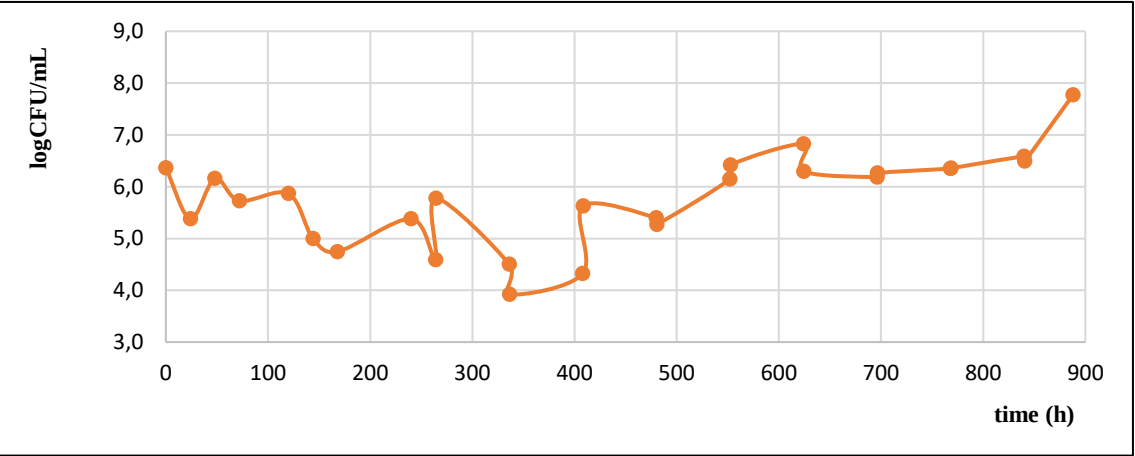


Figure A11: Growth curve of *Z. bailii* since the inoculation of MFC1.

Following Figure A12 represents the growth curve of *B. cereus* since the inoculation of MFC2.

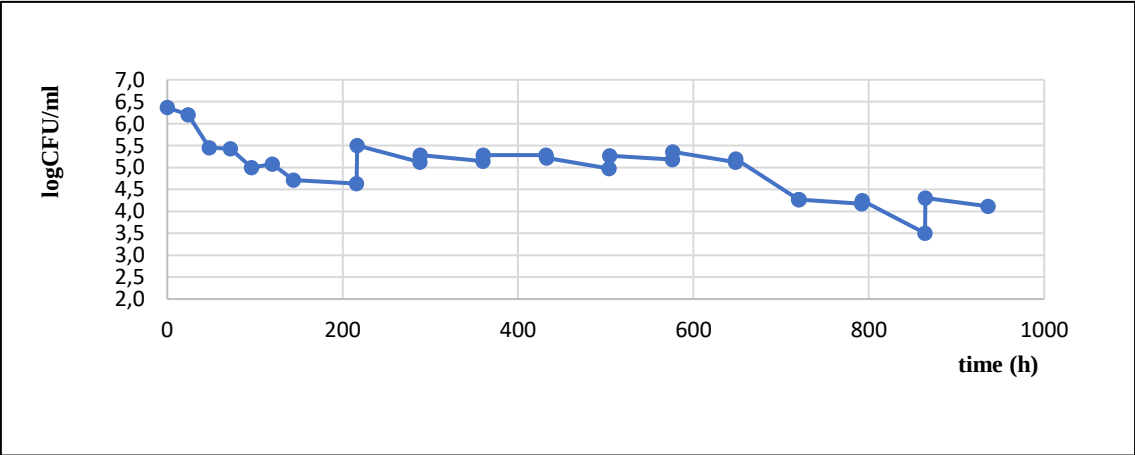


Figure A12: Growth curve of *B. cereus* since the inoculation of MFC2.

Figure A13 gives the previously evaluation of COD removal rate in MFC1.

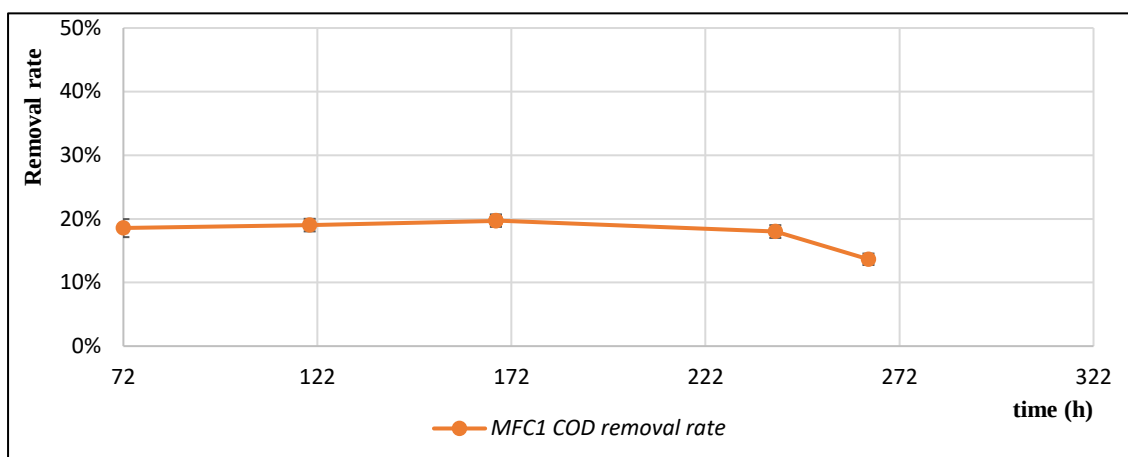


Figure A13: Previously evaluation of COD removal rate in MFC1.

Figure A14 represents the previously evaluation of COD removal rate in MFC2

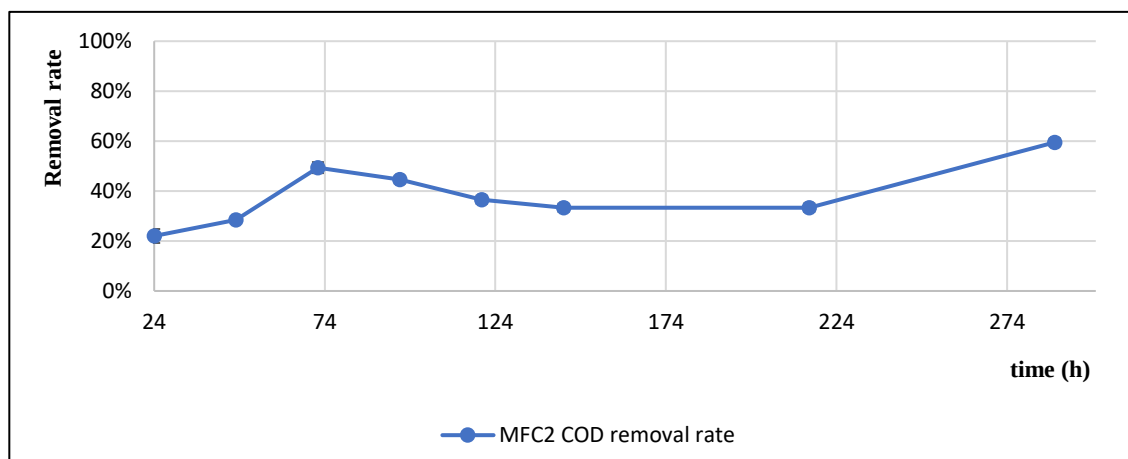


Figure A14: Previously evaluation of COD removal rate in MFC1.

Figure A15 presents the previously evaluation of polysaccharides removal rate in MFC1.

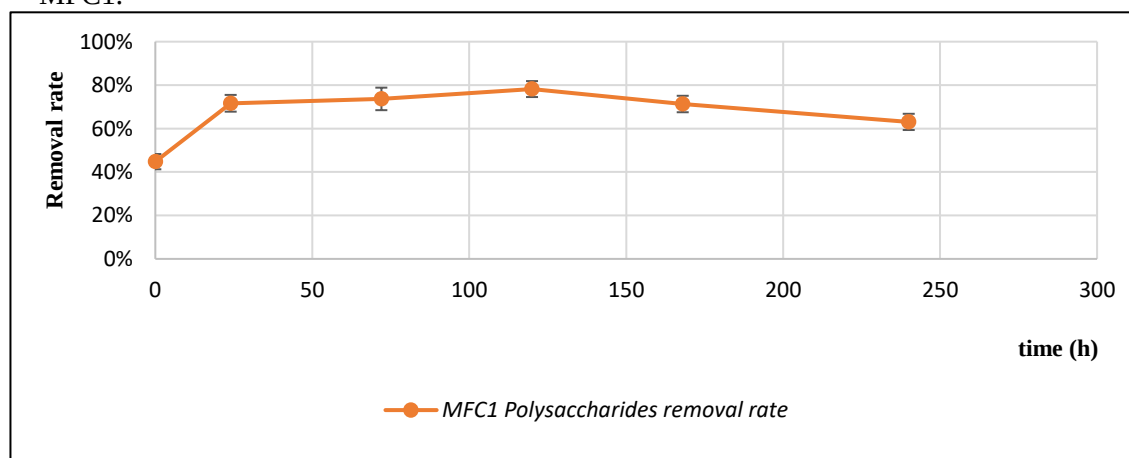


Figure A15: Previously evaluation of polysaccharides removal rate in MFC1.

Figure A16 represents the previously evaluation of polysaccharides removal rate in MFC2.

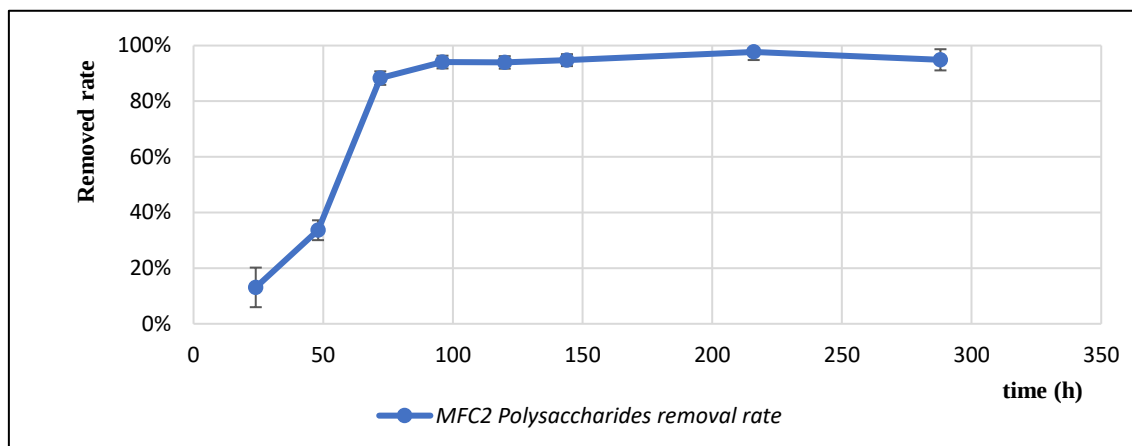


Figure A16: Previously evaluation of polysaccharides removal rate in MFC2.

Figure A17 presents the previously evaluation of culturability of *Z. bailii* in MFC1.

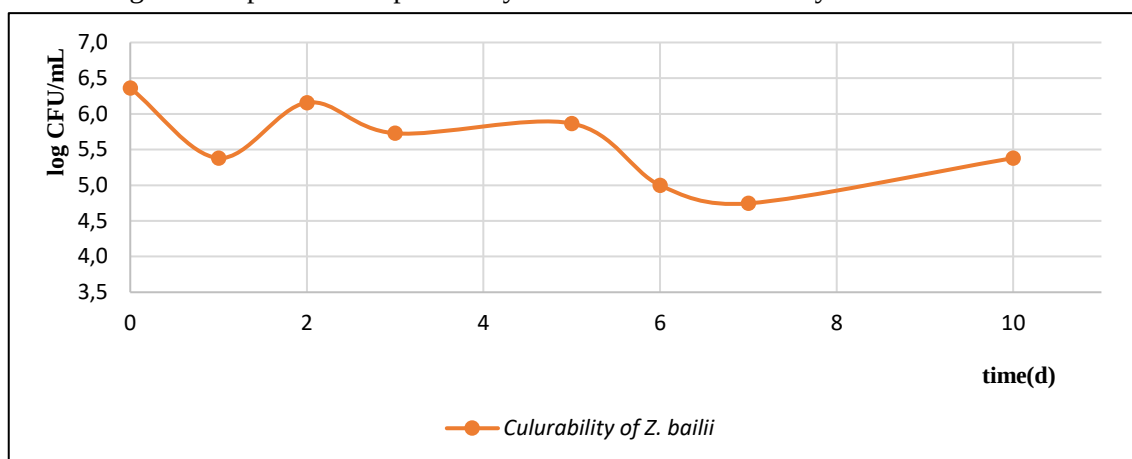


Figure A17: Previously evaluation of *Z. bailii* culturability in MFC1.

Figure A18 presents the previously evaluation of *B. cereus* culturability in MFC2.

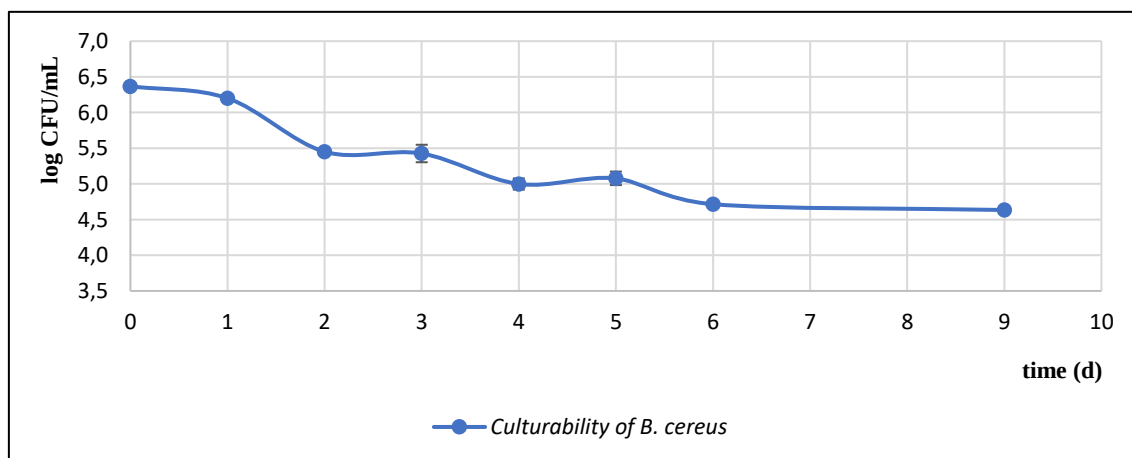


Figure A18: Previously evaluation of *B. cereus* culturability in MFC2.

Figure A19 presents the previously evaluation of OCV in MFC1.

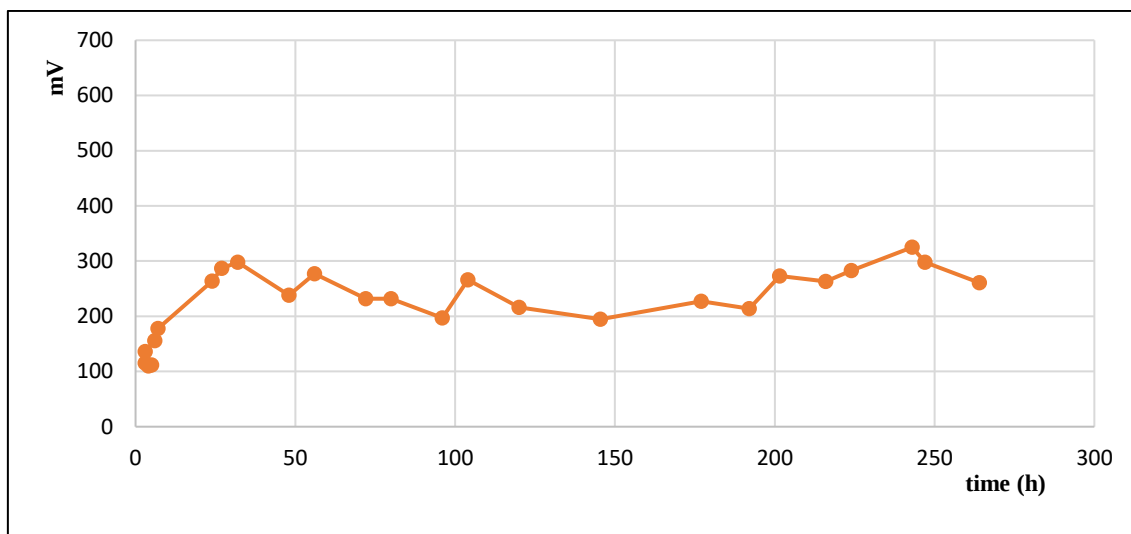


Figure A19: Previously evaluation of OCV in MFC1.

Figure A20 presents the previously evaluation of OCV in MFC2.

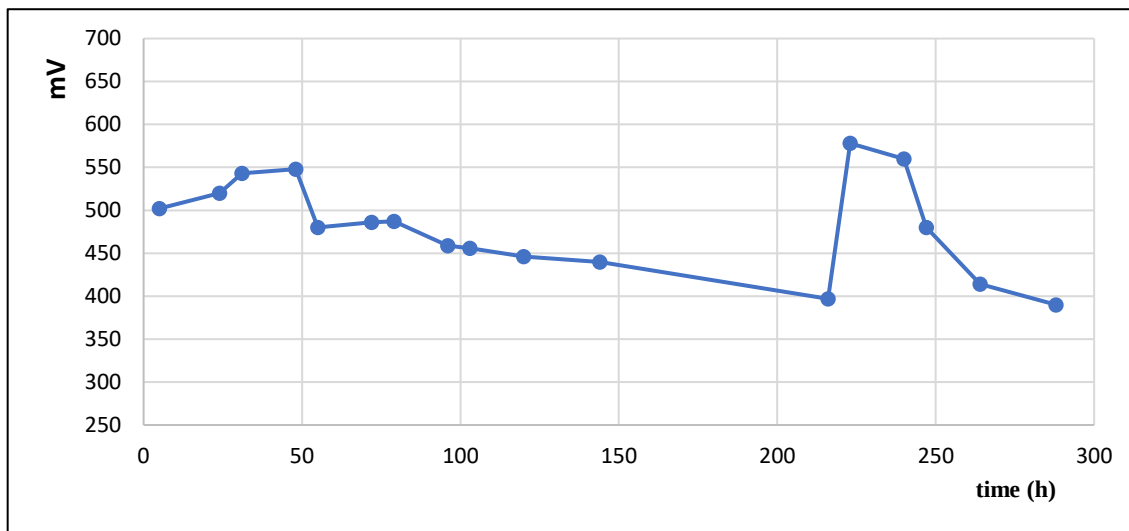


Figure A20: Previously evaluation of OCV in MFC2.