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Physicochemical properties of electroactive yeasts surfaces: Seen any effect on extracellular electron transfer and biofilm formation?

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Abstract

This study aimed to understand the biofilm formation of three electroactive yeasts on carbon felt electrode based on XDLVO theory and how physicochemical properties of both electrode and microorganisms surfaces affect current generation. The three yeast (*W. anomalus, C. tropicalis* and *P. fermentans)* strains showed a maximum current density of 34±0.002, 30±0.004 and 28±0.004 mA/m². The physicochemical characterization of the electrode and the yeast stains surfaces was carried out by the sessile drop technique. Moreover, the interfacial free energy of surface adhesion to the carbon was determined. Based on the value of interfacial free energy, the three yeasts should be able to attach to carbon felt. It was established by ESEM and epifluoresence microscopy that the three yeast strains adhered on carbon felt, as predicted theoretically, with adhesion levels of 4,34 %, 3,15 % and 2,85 % found with *W. anomalus, C. tropicalis* and *P. fermentans, respectively*. The correlations between physicochemical properties of the three yeast strains and the current generated were evaluated. Pearson's correlation showed that the current generated correlated significantly and negatively with cell surface hydrophobicity ($r = -0.89$, $P = 0.05$) and significantly and positively with electron acceptor character $\gamma+$ ($r = 0.84$, $P=0.01$). However, there was no significant correlation with electron donor character $\gamma-$.

1. Introduction

In recent years, microbial fuel cells have attracted a lot of attention as new bioelectrochemical devices that use the metabolism of microorganisms in bioanodes for the conversion of chemical energy into electrical energy with minimal or no CO_2 emissions [1,2]. Many researchers studied electrode materials, electroactive microorganisms, reactor configuration and operational conditions of microbial fuel cells (MFCs) [3,4], and pointed out that the interface anode/electroactive biofilm was one of the key elements that affect MFCs performances.

The microbial adhesion to the material is a fundamental step in the development of biofilm formation on the electrode surface [5]. However, the microbial adhesion is directly affected by various parameters including electrode materials, operating conditions, the type of substrate, type of strain including pure or mixed culture and their metabolic pathways, as well as by various physicochemical properties of both electrode material and microbial surfaces. The physicochemical interactions involved in the microorganism adhesion to the electrode surface were: Lewis acid base interactions, electrostatic interactions and Van Der Waals type interactions. These interactions depend on the hydrophobic/hydrophilic effect, electron donor/electron acceptor characteristics, and the charge on both the electrode and the microbial cell surfaces.

According to literature data, the effect of electrode material, their hydrophobicity, surface charge, and surface topography to enhance electro-active biofilms formation and current density have been studied extensively[6–9]. It has been shown that, the positively charged, the rough and hydrophilic anodes could favor electroactive biofilm formation, increase electron transfer and accelerate the startup time [8–10]. Despite knowing that the good comprehension of the microbial adhesion phenomenon cannot be achieved without considering the effect

of the microbial cell surface properties, no work has yet established the effect of the physicochemical properties (both hydrophobicity and electron donor/electron acceptor properties) of electroactive microorganisms on anode surfaces.
On the other hand, the majority of the reported studies performed so far have been done using mixed culture bacteria, in which case correlation of bacterial attachment and biofilm development with the current produced is potentially complicated by changes in microbial populations[8,10]. Consequently, the study of a single species of microorganisms may provide a clearer understanding of the attachment processes and current generation.
Based on calculation of free energy adhesion of the yeast strain to the carbon felt electrode material by the extended DLVO theory, the aim of this present study was to better understand the biofilm formation of three single electroactive yeasts strains on carbon felt while establishing a correlation between yeast strains physicochemical properties and their current generation capacity. Carbon felt was selected for the experiments as a model surface because it is widely used as an anode material in bioelectrochemical systems owing to its low cost, high surface area, good electrical conductivity and biocompatibility[2].

2. Material and methods

2.1. Yeast strains growth

Wickerh amomyces anomalus, Candida tropicalis were previously isolated from soil and wastewater samples heavily contaminated with chemical industrial effluents in Fez and were selected on the basis of their chromium resistance as reported by Bahafid et al. 2013 [11]. It is also the case for *Pichia fermentans* (unpublished data). Yeast strains were seeded on yeast medium agar (1% peptone, 1% yeast extract, 2% glucose) plates and incubated for 2 days at 30 °C.

2.3. Electrochemical characterization

The experiments were carried out at 30 °C and were operated in three electrode bioreactors (600ml), each equipped with a 16 cm² projected surface area working electrodes (WEs) connected electrically via a thin titanium wire, a saturated calomel reference electrode (SCE, +0.24 V vs. SHE) and a 16 cm^2 platinum grid auxiliary electrode. All working electrodes were polarized at -0.1 V/SCE using a multi-channel potentiostat (Biologic VSP2). WEs were exposed to a 1 M of HCl and a 1 M of NaOH solutions (1 h in each solution) for cleaning purposes, and stored in sterile distilled water.

2.4. Cells preparation

A fresh culture of three yeasts was inoculated in yeast medium (YPG) containing (1 % peptone, 1 % yeast extract, 2 % glucose) and incubated at 30°C for 48h. Then, cells were centrifuged at 7000 x g for 15 min. the pellet was rinsed twice with KNO_3 (0.1 M) and resuspended in the same buffer. At 550 nm, the cell density was adjusted to 1 x 10^7 cells.mL^{-1}[12]. Then, the adjusted solutions were filtered on a cellulose acetate membrane filter (0.45 µm). Filters containing microorganisms were air dried for 30–60 min in order to obtain stable lawns for contact angles measurements. Contact angles were measured in triplicate with separately cultured microbes.

2.5. Contact angle measurements (CAM)

2.5.1. Hydrophobicity

Contact angle measurements were performed using a goniometer (GB instruments, France) by the sessile drop method. According to Vogler's approach [13], the value of the water contact angle θ_W determined by contact angle measurement (GBX, France) allows us to evaluate the hydrophobicity of a given surface, qualitatively. A θ_W value that exceeds 65° indicates a hydrophobic surface, conversely when the θ_W value is less than 65° the surfaces are characterized as hydrophilic.

2.5.2. Surface tension components

After measuring the contact angles, the three equation system found by applying the Young-Dupré equation to each probe liquid was used to obtain the Lifshitz-van der Waals (γ^{LW}) and acid-base (γ^{AB}) surface tension components. Two polar liquids (water and formamide) and one apolar liquid (diiodomethane) with known energy characteristics values γ_l^{LW}, γ_l^+, and γ_l^-were used. Thus, the unknown surface tension components of a solid surface (γ_s^{LW}, γ_s^+, and γ_s^-) or microbial surface (γ^{LW}, γ^+, and γ^-) can be calculated.

$$\gamma_L(\cos\theta+1)=2\left[\left(\gamma_s^{LW}\gamma_L^{LW}\right)^{1/2}+\left(\gamma_s^+\gamma_L^-\right)^{1/2}+(\gamma_s^-\gamma_L^+)^{1/2}\right] \quad \textbf{Eq.(1)}$$

In this equation, θ is the measured contact angle and the subscripts (S) and (L) denote solid and liquid phases, respectively. γ^{LW} is the Lifshitz-van der Waals component of the surface free energy, γ^{+} and γ^{-} are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}). The surface free energy is expressed as: $\gamma_S = \gamma_S^{LW} + \gamma_S^{AB}$ where $\gamma_S^{AB} = 2\left(\gamma_S^{-}\gamma_S^{+}\right)^{1/2}$ is the acid-base free energy component.

2.5.3. Calculation of free energy adhesion of the yeast strain to carbon felt by extended DLVO theory

The limitation of the classical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory when studying biofilm formation was highlighted by a number of discrepancies between experimental evidences and DLVO predictions. Thus, extended DLVO (XDLVO) theories were developed with the aim of taking into account the other phenomena implicated in colloidal adhesion and that were primarily neglected by the DLVO approach.
In this theory, the interaction energy (ΔG^{DLVO}) requires the interaction between a microbe (m) and a flat substratum surface (s) immersed in an aqueous environment (l).
The net interaction energy was developed by balancing repulsive or attractive electrostatic energy (G^{EL}) and Lifshitz-van der Waals attractive forces (G^{LW}). The total interaction or adhesion energy as a function of the separation distance (d) between a bacterium (sphere) and a substratum (flat plane) surface can be formulated as follows:

$$\Delta G^{DLVO}(d) = \Delta G^{LW}(d) + \Delta G^{EL}(d) \quad \textbf{Eq.(2)}$$

An extension of the DLVO theory (XDLVO) was proposed [14,15], in which the acid-base interactions (ΔG_{AB}) were added to formulate the extended DLVO theory. In this approach the total interaction energy between microbial cells (m) and substratum (s) through water (w) is described as a balance between attractive Lifshitz-van der Waals forces, repulsive or attractive electrostatic forces and acid-base interaction forces, being expressed as follows:

$$\Delta G^{XDLVO}(d) = \Delta G^{LW}(d) + \Delta G^{EL}(d) + \Delta G^{AB}(d) \quad \textbf{Eq.(3)}$$

In this equation:

$$\Delta G^{LW} = \left(\left(\gamma_M^{LW}\right)^{1/2} - \left(\gamma_S^{LW}\right)^{1/2}\right)^2 - \left(\left(\gamma_M^{LW}\right)^{1/2} - \left(\gamma_L^{LW}\right)^{1/2}\right)^2 - \left(\left(\gamma_S^{LW}\right)^{1/2} - \left(\gamma_L^{LW}\right)^{1/2}\right)^2 \quad \textbf{Eq.(3.a)}$$

$$\text{and } \Delta G^{AB} = 2\left[\left(\gamma_L^{+}\right)^{1/2}\left(\left(\gamma_M^{-}\right)^{1/2} + \left(\gamma_S^{-}\right)^{1/2} - \left(\gamma_L^{-}\right)^{1/2}\right) + \left(\gamma_L^{-}\right)^{1/2}\left(\left(\left(\gamma_M^{+}\right)^{1/2} + \left(\gamma_S^{+}\right)^{1/2} - \left(\gamma_L^{+}\right)^{1/2}\right) - \left(\gamma_L^{-}\gamma_S^{+}\right)^{1/2}\right) - \left(\gamma_L^{+}\gamma_S^{-}\right)^{1/2}\right]$$

Eq.(3.b)

The XDLVO prediction of the free energy of adhesion between yeast strains and carbon felt was investigated. The total free energy of interaction defined by the extended DLVO theory is the sum of the Lifshitz-van der Waals, electrostatic and acid-basic interactions as calculated from Eq.(3). The utilization of a suspension liquid with high ionic strength (KNO_3 0.1M) allows the negligence of electrostatic interaction free energy ΔG^{EL}as done before [16,17].

2.6. Environmental Scanning Electron Microscopy and epifluorescence analysis

The electrode was extracted from the bioreactors and washed with sterile physiological water to remove all materials except the attached biofilms and were imaged by using environmental scanning electron microscopy (ESEM Quanta 200) and Carl Zeiss Axio Imager-M2 microscope equipped for epifluorescence with an HXP 200 C light source and the Zeiss 09 filter (excitor HP450e 490, reflector FT 10, barrier filter LP520). For epifuerorence images, the specimens was treated with 0.03% acridine orange (A6014, Sigma) for 10 min, rinsed with sterile physiological water and then left to dry at room temperature. The biofilms were imaged with a digital camera (Zeiss AxioCamMRm) every 0.5 µm along the Z-axis and the set of images was processed with the Zen® software.

2.7. Statistical analysis

Statistical analysis was performed to evaluate correlations between the physicochemical properties of yeast strains and their potential of current generation. Pearson's correlation was calculated using Statistica for Windows version 7.0 (StatSoft Inc., USA).

3. Results and discussion

3.1. Chronoamperometries analysis of yeast strains

The extracellular electron transfer capability of the three yeast strains was investigated by chronoamperometric analysis. The experiments were carried out in three electrode bioreactors and the working electrode "carbon felt" was polarized at -0.1 V/SCE. The bioreactors inoculated with *W. anomalus, C. tropicalis* and *P. fermentans*

showed a gradual increase of current, reaching a maximum of 34±0.002, 30±0.004 and 28±0.004 mA/m², respectively. However, no electroactivity was recorded for the sterile medium, indicating that the generated current originated from in vivo produced electrons. To our knowledge, this is the first work to report W. *anomalus*, C. *tropicalis* and P.*fermentans*as electrochemically active yeasts. However, several studies reported yeast species as biocatalysts in fuel cells such as *Saccharomyces cerevisiae*[18–20], *Hansenula polymorpha* [21], *Arxula adeninivorans*[22,23] and *Candida melibiosica*[24,25].

3.2. Physicochemical characterization of carbon felt and yeast strains surfaces

3.2.1. Carbon felt electrode characterization

In the last decades, carbon felt have been extensively investigated and envisaged as an electrode material in MFCs. In addition to being conductive, porous with high specific surface area, excellent microbial adhesion to carbon felt has also been demonstrated in several studies [13–19]. These characteristics make carbon felt highly promising as an electrode materialfor achieving high current densities. The contact angle water measurements and the electron acceptor–donor properties are shown in the table.1. The used carbon felt surface is relatively hydrophobic (θw= 96.2°)and exhibits a strong electron acceptor–donor proprieties ranging from y^-=35.5 ± 0.6 mJ.m^{-2} and y^+=37.3 ± 1.23 mJ.m^{-2}, respectively. These results were in agreement with those found with wu et al. (2013), in which similar values of hydrophobicity were reported [26].

Table1: Contact angle values using water (θw), formamide (θ_F) and diiodométhane (θ_D), Lifshitz-vander Waals (γ^{LW}), Electron-Donor (γ-) and Electron-Acceptor (γ+) of carbon felt electrode material

	Contact angles (°)			Surface tension: components and parameters (mJ.m^{-2})		
	θ_w	θ_F	θ_D	γ^{LW}	γ^+	γ^-
Carbon felt	96.2±2.72	118.8±1.70	0±0.00	50.8±0.28	37.3±0.08	35.4±1.19

3.2.2. Surface properties of yeast strains

Table 2 lists the physicochemical surface properties of the three yeast strains as measured by contact angle measurements. According to Vogler [13], hydrophobic surfaces exhibit a water contact angle greater than 65°, whereas hydrophilic ones exhibit a water contact angle which is less than 65°. Taking into account the values of the water contact angles (Table 2), it can be concluded that all yeast strains tested are hydrophilic, with values of water contact angles ranging from 63.9 to 42.7°. These findings agree with previous results reporting the hydrophilic caracter of yeast cells[27]. The hydrophobicity of yeast strains has been correlated with the high surface protein content [28,29].

Table.2: Contact angle values using water (θw), formamide (θ_F) and diiodométhane (θ_D), Lifshitz-vander Waals (γ^{LW}), Electron-Donor (γ^-) and Electron-Acceptor (γ^+) Parameters of yeast strains.

Yeast strains	Contact angles (°)			Surface tension: components and parameters (mJ.m^{-2})		
	θ_D	θ_w	θ_F	γ^{LW}	γ^+	γ^-
Wickerhamomyces anomalus	42.7±0.95	36.4± 1.28	63.7±0.72	38.2±0.28	2.6±0.06	79.5±1.24
Candida tropicalis	63.9±1.12	37.7± 0.59	37.9±2.56	31.5±0.47	2.5± 0.09	43.5±1.83
Pichia fermentans	45.8±0.15	44.3± 0.60	78.1± 1.22	31.8±0.06	2.4±0.03	90.1± 0.49

All of the yeast strains studied demonstrated very similar Lifshitz-van der Waals (γLW) values. The three yeast strains exhibited weak electron acceptor (γ^+) properties and strong electron donor (γ^-) surface properties. γ^- was found to be much greater than γ^+, i.e., the yeast strains surfaces appeared to behave predominantly as electron donors/Lewis bases (Table 2).

3.2.3. Calculations of free energy adhesion of the yeast strains to the carbon felt anode

The XDLVO predictions of the free energy of adhesion per unit area for the yeasts and substrata are presented in the table 3, as calculated from Eqs. (3a) and (3b). In the present case, the XDLVO predictions show that the adhesion is favorable for all the yeast strains–carbon felt anode combinations since the total free energy of adhesion was negative (ΔG_{XDLVO}<0). The negative values of ΔG_{XDLVO} of *W. anomalus, C. tropicalis* and *P. fermentans* were -9.41, -7.86 and -1.66 mJ m^{-2}, respectively.

Additionally, the values of the ΔG^{LW} component (Table 3) were found to be negative for all of the three strains studied, which means that all long range forces (Van Der Waals interactions) and short range forces may contribute to yeast strains adhesion to carbon felt. It should be noted that the negative value of the ΔG^{AB} component was higher than the ΔG^{LW} component (Table 3) for *W. anomalus* and *C. tropicalis*. This means that the theoretical adhesion to the carbon felt electrode should be governed mainly by short range forces (acid–base interactions). In contrast, the adhesion phenomena of *P. fermentans on* the carbonfelt are governed only by the Van Der Waals interactions.

3.2.4. Experiments of adhesion of yeast strains to the carbon felt anode

The results obtained here suggest that yeast strains are able to adhere to the carbon felt material (Figure. 1). The yeast strains were found to be randomly dispersed and attached to the carbon felt as single cells or pairs of cells (Figure. 1). On the other hand, the three yeasts appeared to be strongly clumped forming cell aggregates to carbon felt surfaces (Fig. 1B).

The ability to adhere on carbon felt is more pronounced for *W. anomalus,* (Fig. 1B) than *C. tropicalis*(Fig. 1B) and *P. fermentans* (Fig. 1B). Based on the epiflueoresence images, it appears that the percentages of the surface covered by *W. anomalus, C. tropicalis* and *P. fermentans* strains are 4,34 %, 3,15 % and 2,85 % respectively.

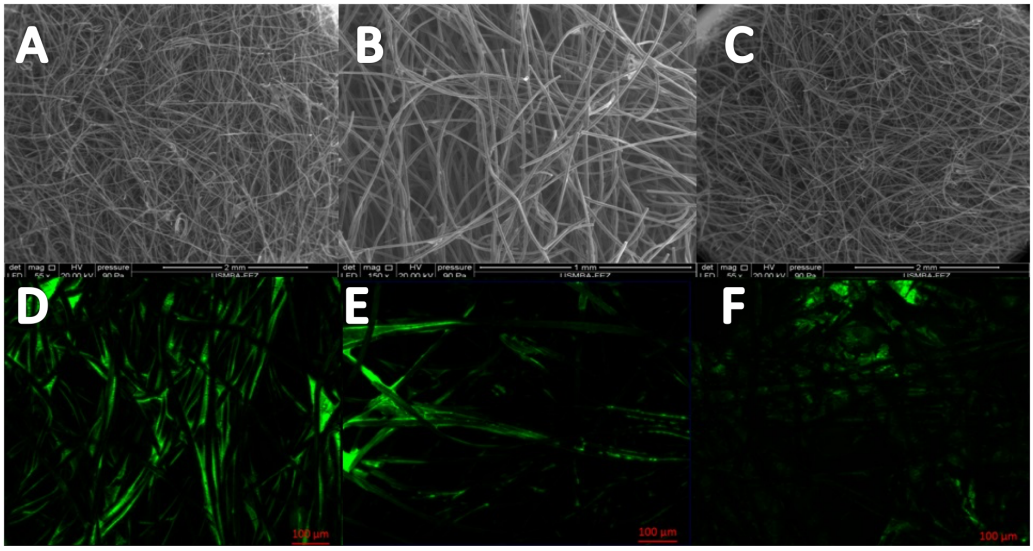

Figure.1: ESEM images of yeast strains bioanodes formed on carbon felt (A: *Wickerhamomyces anomalus*; B: *Candida tropicalis* and C: *Pichia fermentans*) and epifluorescence microscopy images of yeast strains bioanodes formed on carbon felt (D: *Wickerhamomyces anomalus*; E: *Candida tropicalis* and F: *Pichia fermentans*)

3.2.5. Comparison between XDLVO and adhesion experiment results

The prediction of yeasts adhesion to anode surfaces by XDLVO has not been investigated. As indicated in (Table 3), a negative value of the total free energy of the yeast strains to the carbon felt anode was found ($G_{XDLVO}<0$), therefore, the adhesion is favorable for all yeast strains involved in interaction with carbon felt. It can thus be concluded that the XDLVO predictions are similar to what was observed by ESEM and epiflueresence microscopy (Figure 1).

Table.3: Lifshitz–Van Der Waals ΔG^{LW} ($mJ.m^{-2}$), acid–base ΔG^{AB} ($mJ.m^{-2}$) and total free energy of interaction ΔG^{XDLVO} ($mJ.m^{-2}$), for the adhesion of yeasts strains.

Yeast strains	ΔG^{LW}	ΔG^{AB}	ΔG^{XDLVO}
Wickerhamomyces anomalus	-7.42	-1.99	-9.41
Candida tropicalis	-4.76	-3.09	-7.86
Pichia fermentans	-4.63	2.97	-1.66

The first stage in the formation of biofilms is microbial adhesion; the latter depends principally on the physicochemical surface characteristics of micro-organisms and substrata. Thus, a change of the surface properties of each of these compounds may result in a change in their bioadhesive behaviour. According to the physicochemical approach, the hydrophobic cells tend to attach to hydrophobic substrata and the hydrophilic

cells tend to attach to hydrophilic substrata. This approach is insufficient to explain our results since the three hydrophilic yeast strains were able to adhere to hydrophobic carbon felt material. Until now, several works reported such contradictions for this hydrophobic/hydrophilic aspect [30–32] because the adhesion phenomenon cannot be treated only by taking into account the sole hydrophobicity effect, but the AB and LW interactions of microbial cells and surface colonization have to be investigated and considered as well [33], since the electron donor/electron acceptor (acid-base) properties play an important role in interfacial phenomena between microorganisms and substrata[34–36]. The adhesion of the three yeasts on carbon felt can be explained by the AB and LW interactions presented in (Table. 3). As indicated before, the adhesion of *P. fermentans* on the carbon felt electrode material is governed by the Van Der Waals interactions. In contrast, the adhesion of *W. anomalus* and *C. tropicalis* are gouverned by t both Van Der Waals and acid–base interactions.

3.2.6. Relation between cell CSH and electron donor/electron acceptor properties of yeast strains and their potential current generation

Pearson's correlation, physicochemical characteristics (CSH and electron donor/electron acceptor properties) of yeast strains and the current generated were used to evaluate the existence of correlations. A negative significant correlation was found between yeast CSH and their current generation capacity (r = - 0.89, P= 0.05) (figure 2A).

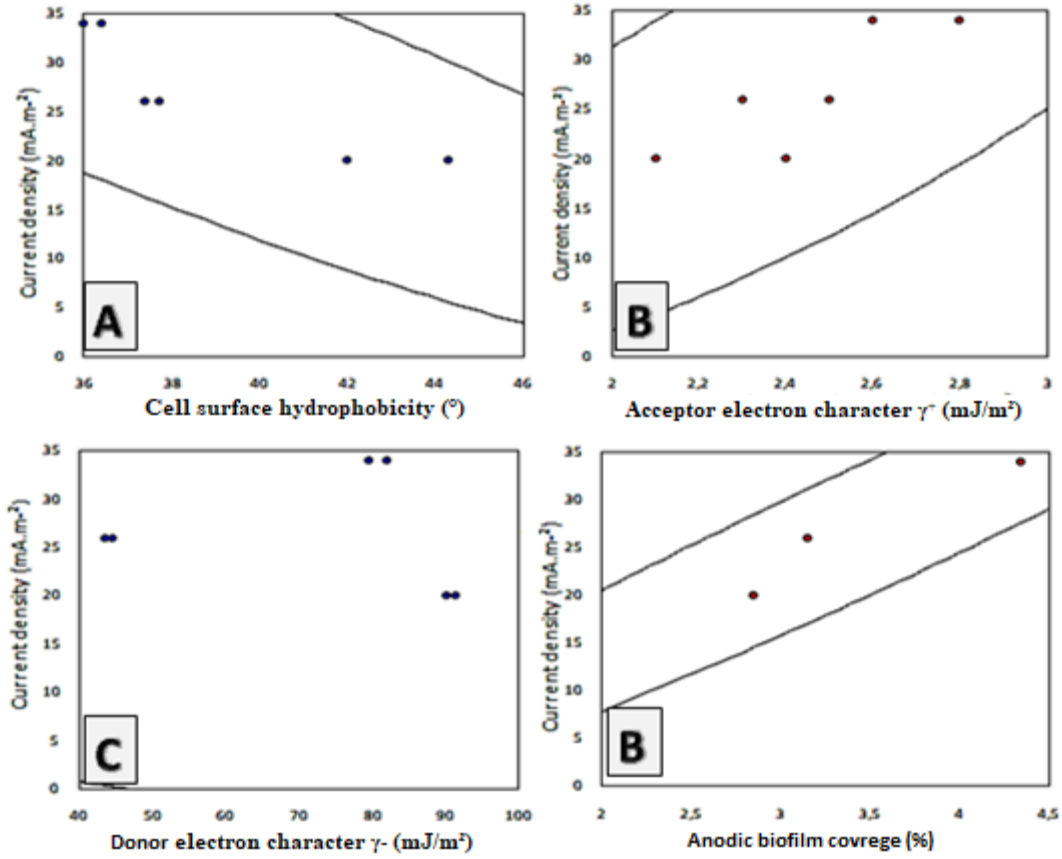

Figure.2: Correlation between surface physicochemical properties yeast strains and anodic biofilm coverage and their potential current generation. (A) Cell surface hydrophobicity (CSH), (B) Acceptor electron character γ^+ and (C) donor electron character γ- and (D) anodic biofilm coverage.

These results clearly demonstrate that the more hydrophilic yeast strains have a better current generation potential. Figure.2 also shows a high positive correlation between yeasts electron acceptor character γ+ and their current generation potential (r = 0.84, P=0.01) (Figure 2B). Yeast strains with high donor electron character present weak current generation potential, while no correlation was found between yeasts current generation and donor electron character γ- (r = - 0.26) (Figure 2C). These findings suggest that surface yeast charge can significantly influence their biofilm formation and current output. As shown in figure 2D, a positive significant correlation(r=0.96) was obtained between anodic biofilm coverage by the yeast strains and their current generation capacity.

Conclusion

The findings presented in this study, explored on the one hand the theoretical prediction attachment of the three yeasts on carbon felt by the XDLVO theory. This theoretical prediction was confirmed by the visualization of the experimental adhesion using environmental electronic and epiflueresence microscopy. On the other hand, the relationship between cell surface physicochemical properties and the ability of these strains to generate current was calculated. The current generation capacity of the three yeast strains presented a significant positive correlation with their electron acceptor character ($r = 0.84$, $P=0.01$) and a percentage of anodic biofilm coverage ($r=0.96$), while a negative significant correlation with hydrophobicity value was shown.

To our knowledge, this is the first report about the effect of cell surface hydrophobicity, acid-basic component on current output in bioelectrochemical systems using contact angle measurements. This paper confirms the importance of physiochemical characteristics of microbial strains as selection criteria of microorganisms for their potential use as inoculums in BESs.

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