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Bioelectrochemical Characterization of Heavy Metals Resistant yeast: *Hansenula fabianii* Isolated from Tannery Wastewater

Alae Elabed[1,2], Loubna Ezziat[2], Saad Ibnsouda[1,4], Soumya Elabed[4], Benjamin Erable[1]

[1] Laboratoire de Génie Chimique, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France
[2] Laboratoire de Biotechnologie Microbienne, Faculté des Sciences et Techniques, Université Sidi Mohamed Ben Abdellah, Fès, Maroc
[3] Laboratoire de Science et Technologie du Génie des procédés, Université Sidi Mohamed Ben Abdellah, Fès, Maroc
[4] Cité d'Innovation, Université Sidi Mohamed Ben Abdellah- Fès, Maroc
[*]E-mail: soumya.elabed@usmba.ac.ma

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To date, heavy metals present a main drawback for bioelectrochemical systems (BESs) performances. Therefore, our results confirm, for the first time, that *Hansenulafabianii is* an electrochemical active yeast that has a potential tolerance against various heavy metals. The bioreactor inoculated by *Hansenula fabianii* in mediatoless conditions provided a maximum current density of 32 mA/m²; up to 300 mA/m² in the presence of methylene blue. In mediatorless conditions, cyclic voltammetry (CV) on a yeast pellet showed a single oxidative peak at 450mV and a pair peak, the CV of the supernatant confirmed that *Hansenula fabianii* secretedits own mediator. The obtained results proved that *Hansenula fabianii* based biofuel cell could be used for simultaneous current generation and heavy metal bioremediation.

Keywords: Electrochemical active yeast; heavy metals; *Hansenula fabianii*; mediatorless condition.

1. INTRODUCTION

Microbial fuel cells (MFCs) are a form part of an emerging technology that makes it possible to deal with two of the major problems: water and fossil fuels availability. They generate electrical energy through the oxidation of organic and inorganic matter catalyzed by microorganisms (biocatalyst).

Various microbial species and mixed cultures have been widely studied in Microbial Fuel Cells (MFCs) with promising results in terms of wastewater treatment and power output [1,2]. They can be classified into four groups: heterotrophic prokaryote, phototrophic prokaryote, heterotrophic eukaryote, and phototrophic eukaryote [3]. So far, most of the fundamental investigations in the field were performed by the utilization of heterotrophic prokaryotes such as bacteria of the genera *Geobacter*, *Shewanella* and *Pseudomonas*[3,4]. However, relatively less work is available today concerning the

eukaryotic species such as microalgae and yeast as bioelectrocatalyst in fuel cell operations. The easy cultivation, broad substrate spectrum, fast growth and tolerance of the yeast to a wide range of environmental conditions are advantageous for development of yeast-based biofuel cells[3–5]. Also, complicated architecture of yeast cell respiratory chains gives the advantage harnessing the energy from biocatalysts [6] and the membrane of the yeast cells generally have high protein content including natural electron shuttles such as ferredoxin and cytochromes that be used by redox enzymes for electron exchange with an electrode surfaces[7]. However, there is a little information about the yeast cells electrocatalytic activity and electron transfer mechanisms without an addition of exogenous mediator. On the other hand, the treatment of heavy metals by yeast cultures showed higher tolerance and removal rates compared with prokaryotes based bioremediation [8,9].

The removal of heavy metals in MFCs was mostly studied in the cathodic chamber of dual chambered MFCs. The presence of a physical separator is needed to prevent the cross-over of metals from the cathode to the anode, which results in lower power generation, due to the inhibition of electrochemically active anodic microorganisms. However, for practical reasons, such separation in double chamber MFCs may not be feasible for wastewater treatment, as both metals and organic matter co-exist in the same water stream. Indeed, high metal content in tannery wastewater presents a challenge for conventional biological methods of treatment.

Recently, few authors started to evaluate the impact of heavy metals in single chamber MFCs. For instance, Abourached 2014 reported the maximum tolerable concentrations for electroactive microorganisms to be 22.40 mg/L and 26.00 mg/L for Cd and Zn, respectively. The increased Cd concentrations to 33.60 mg/L and Zn to 32.50 mg/L resulted in voltage drops by 71 and 74%, respectively [10]. More recently, Wu (2018) stated that a tolerable concentration of 12.50 mg/L of Cu^{2+} was effectively removed in single chamber MFCs. However, a higher concentration of 15.00 mg/L lead to a decrease in copper removal and inhibited electricity production [11]. It should there be concluded that above tolerable concentrations, heavy metals start to exhibit negative effects on the MFCs performances due to their toxicity [10].

To defeat this challenge, this study targeted the isolation of novel electrochemically active yeast from industrial tannery wastewater known for its high Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD) concentrations and also high levels of chromium ranging from 200 to 400mg/L. The goal was isolate a species that is able to tolerate the presence of heavy metals, mainly hexavalent chromium, with the purpose of treating industrial wastewater loaded by heavy metals. The mechanism of yeast electrons transfer and current generation were also studied in bioelectrochemical systems.

2.MATERIALS AND METHODS

2.1. Isolation and identification of yeast strain

The strain AE6 used in this study was isolated following the protocol used by Bahafid 2013 [12] from an industrial tannery wastewater in Fez, Morocco. The yeast was identified by PCR based identification using the following protocols [13,14].

The resulting sequence from genetic analysis system model 3130 was compared to the 5.8S rDNA partial sequences published already in the GenBank, which had been generated using the BLAST tool of the National Center for Biotechnology Information (NCBI).

2.2. Yeast strain and growth conditions

The yeast was grown in YPG (yeast extract, peptone and glucose) medium at temperature of 30°C and on a rotary shaker at 150 tr/min. The cells were then harvested by centrifugation at 6000 rpm, washed several times with deionized water and finally suspended at optical density 600 nm (OD600) = 1.0 in YPG medium..

2.3. Procedures of heavy metals-resistance experiments

Heavy metal resistance of the strain AE6 was assessed on YPG-agar plates. Heavy metals were filtered sterilized and added increasing concentrations (100–4000 mg/L) of Cr (VI), $NiCl_2$, $CoCl_2$, $ZnSO_4$, $CuSO_4$, $Pb(NO_3)_2$ separately to the YPG-agar medium. Minimum inhibitory concentration (MIC) was determined after 48 h of incubation at 30 °C.

2.4. Electrochemical characterization

The experiment was conducted at 30 °C and was operated in three electrode bioreactors, each equipped with a 16 cm^2 carbon felt as working electrodes connected electrically via a thin 0.5 mm diameter platinum wire, a saturated calomel reference electrode (SCE, +0.24 V vs. SHE) and a 16 cm^2 platinum grid auxiliary electrode. All the working electrodes were polarized at-0.1 V/SCE using a multi-channel potentiostat (Biologic VSP2). The polarization was periodically suspended to perform cyclic voltammetries at 1 mV/s in the potential range of +0.6 V/SCE and -0.6 V/SCE.

2.5. Environmental Scanning electron microscope (ESEM)

Microbial biofilms Micrographs were obtained using Environmental scanning electron Microscopy (ESEM, FEI Company).

specimen is chemically fixed in phosphate buffers (400 mM, pH = 7.4) with 4% glutaraldehyde, dehydrated through an acetone series after that immersed in acetone and hexamethyldisilazane (50:50), and in 100% hexamethyldisilazane (HMDS). Then, left to dry at room temperature.

2.6. Epifluorescence microscopy

Firstly, after extraction of the electrodes from the bioreactors, they were washed with sterile physiological water to remove all substances except attached biofilms. Afterwards, electrodes were

stained with 0.03% acridine orange (A6014, Sigma) for 10 minutes, then rinsed with sterile physiological water and finally allowed to dry at room temperature. The biofilms coverage ratio was evaluated by the Carl Zeiss Axio Imager-M2 microscope equipped with an HXP 200 C light source and the Zeiss 09 filter (for epifluorescence analysis).

3. RESULTS AND DISCUSSION

3.1. Molecular identification of the yeast strain

The comparison results of the sequences with those available in the databases (Gen Bank, EMBL, DDJB and PDB) revealed that the yeast strain was closely matched to *Hansenula fabianii* with 100% similarity. *Hansenulafabianii* has commonly been used in wastewater treatment containing a large amount of nitrogen compounds, organic and inorganic substances in the wastewater [15].

3.2. Strain tolerance to heavy metals

Figure 1 illustrates the tolerance potential of the isolated yeast against various heavy metals. The minimum inhibitory concentration (MIC) of various metallic salts viz. $K_2Cr_2O_7$, $NiCl_2$, $CoCl_2$, $ZnSO_4$, $HgCl_2$, $Pb(NO_3)_2$ has been determined.

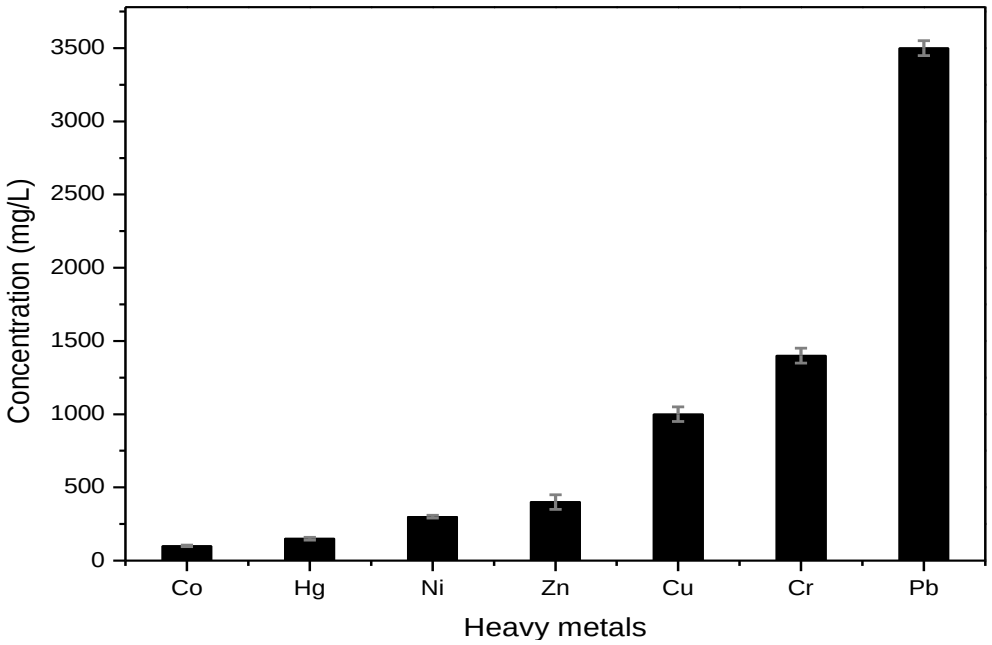


Figure 1. Resistance of *Hansenula fabianii* strain to different heavy metals

The level of survival against Cr (VI) toxicity was up to 1400 (mg/L). The MIC of various other heavy metal salts viz, Hg^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, were 150, 100, 300, 400, 1000, 3500 mg/L,

respectively. It is known that microorganisms isolated from environments contaminated with heavy metals often exhibit greater tolerance to heavy metal pollutants[12,14,16]. The mechanisms of microbial resistance against toxic metals differ for each isolate, which depends on various parameters such as; metal flow channels, metal resistance plasmids, adsorption uptake, DNA methylation and the biotransformation of metals by specific enzymes or indirectly through cellular metabolites. This difference generates several heavy metal resistance profiles. Furthermore, the comparison of metal resistance levels with values reported in the literature is still complicated due to different used media and culture conditions. In general; yeast strains have much higher metal resistance than that found with bacteria[17].

3.3. Effect of chromium on the growth of yeast

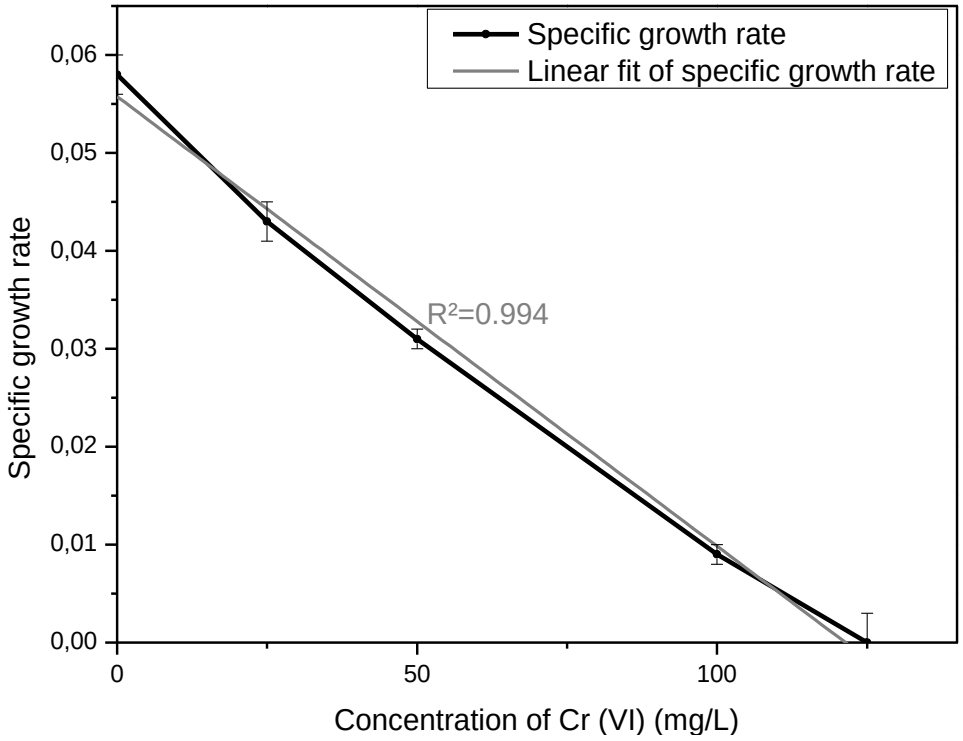


Figure 2. The effect of the initial Cr(VI) concentration on the specific growth rate of *Hansenula fabianii*

Batch cultures of Hansenula fabianii were grown in YPG media containing different Chromium (VI) concentrations ranging from 0 to 125 mg/L.

The relationship between the biomass yield and the initial concentration of Cr (VI) is shown in Fig.2. The growth of cells was heavily influenced by Cr (VI) at a concentration of 100 mg/L, while Cr (VI) at 25 mg/L had only a slight effect on the growth. Such behavior suggests that Cr (VI) may affect negatively the metabolic activity of Hansenula fabianii. These findings are in good agreement with earlier published studies. previous works by Liliana Morales-Barrera (2008). and Juvera-Espinosa (2006) have shown that Cr (VI) has an inhibitory effect on the growth of some strains in particular;

Hypocrea tawa [14] and *Candida sp.* [16] respectively, with a significant decrease in the specific growth rate values. The concentrations of hexavalent chromium that inhibited activated sludge range from the lower limit of 1 mg / L in an earlier study [18] to 87 mg / L Cr (VI) [19]and up to 40–201 mg / L of Cr (VI) [20]**.**

3.4. Bioelectrochemecal analysis

3.4.1. Chronoamperometry

In order to investigate the extracellular electron transfer capability of the yeast strain *Hansenula fabianii*, a chronoamperometric analysis was performed under anaerobic conditions in the presence and in the absence of methylene blue. Methylene blue was already demonstrated as an exogenous electron transfer dye mediator that shuttle electrons between yeast cells and the electrode[6]. The experiment was carried out in a 3-electrodes electrochemical bioreactor and the working electrode was polarized at -0.1 V/SCE in YPG liquid medium inoculated by 5% (v/v) of *Hansenula fabianii*.

Figure 3 depicts the evolution of current density according to time. The current generated by *Hansenula fabianii* in mediatorless conditions gradually increased, reaching 32 mA/m² after 25 days following the inoculation. In order to confirm that generated current originates from in vivo electrons, a sterile medium was studied as control. No current exchange was recorded on the -0.1 V/SCE polarized electrode in the sterile medium which indicated that *Hansenula fabianii* yeast plays an undeniable role in the current generated. In short, this continuous production of anodic current demonstrates that *Hansenula fabianii* is electrochemically active yeast. Several authors already reported yeast species as anodic electrobiocatalysts in fuel cells such as *Saccharomyces cerevisiae* [6,21,22], *Hansenula polymorpha* [23], *Arxula adeninivorans* [24,25] and *Candida melibiosica* [26,27]. But only *A. adeninivorans* and *C. melibiosica* yeast species have been reported as electroactive microorganisms and can be used as an electrobiocatalyst in a yeast-based fuel cell even in the absence of an artificial exogenous mediator.

The influence of methylene blue as an electron shuttle was studied separately, in order to increase the rate of electron extraction,. The plots of current density vs. time in the presence of methylene blue are presented in Fig.3B. The current density began to recover rapidly and reached a higher level of 500 mA/m² than in the absence of methylene blue. The current decreased over time, reaching a stable current of 32 mA/m² after 5 days of inoculation. The current density obtained in mediator conditions was 100 times higher than that in mediatorless conditions. Once the entire reduced mediator pool has been consumed after day 5, the basal current density is again of the same order of magnitude as previously determined in Figure 3.A, i.e. 30-40 A/m². *Hansenula fabianii* therefore does not seem to be able to ensure the reduction of methylene blue as other yeasts do very well, such as *Saccharomyces cerevisiae, Hansenula polymorpha, Arxula adeninivoransor Candida melibiosica.*

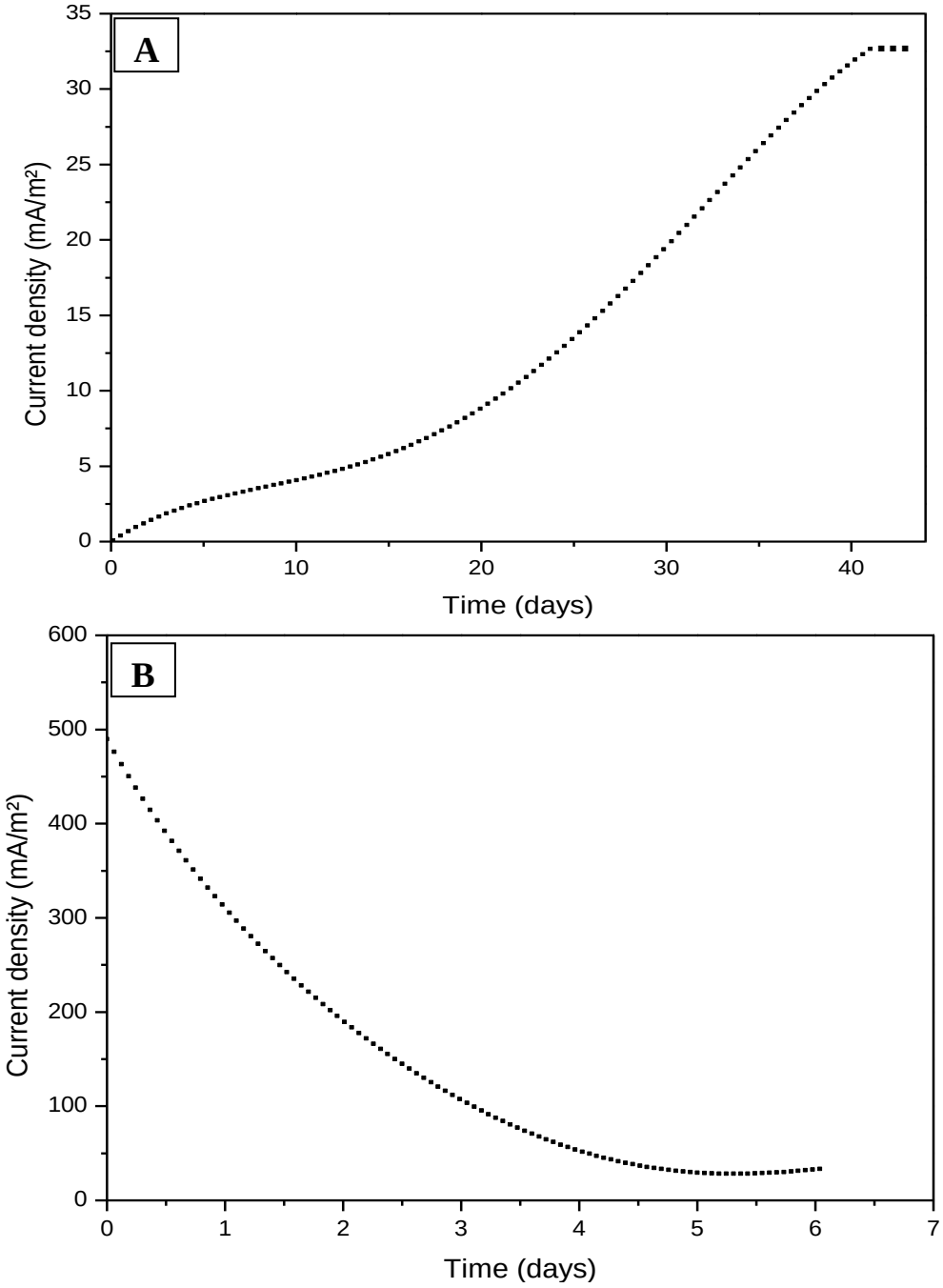
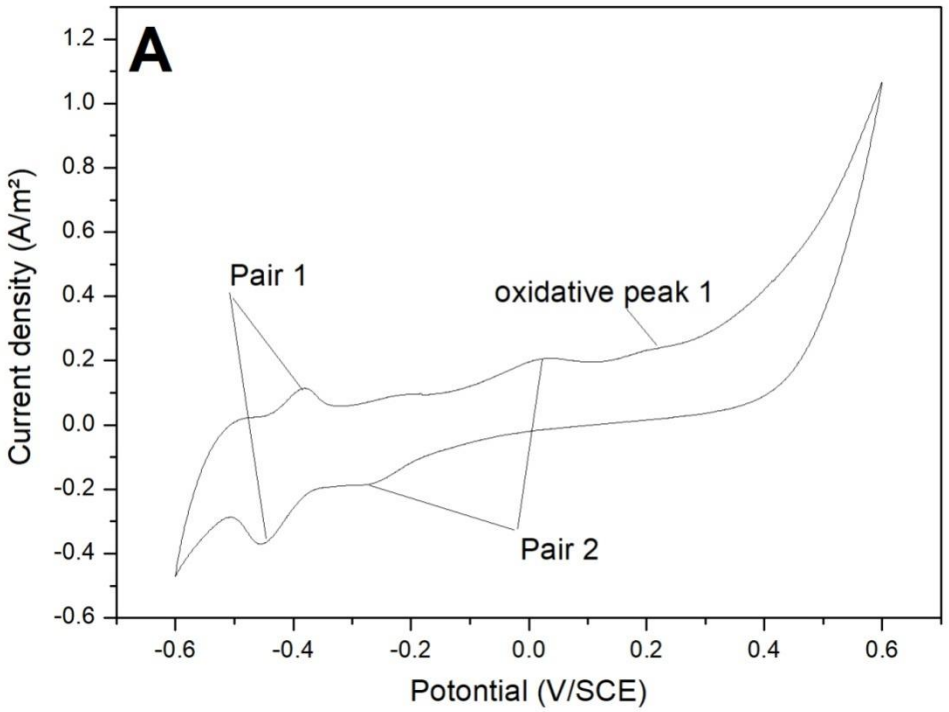


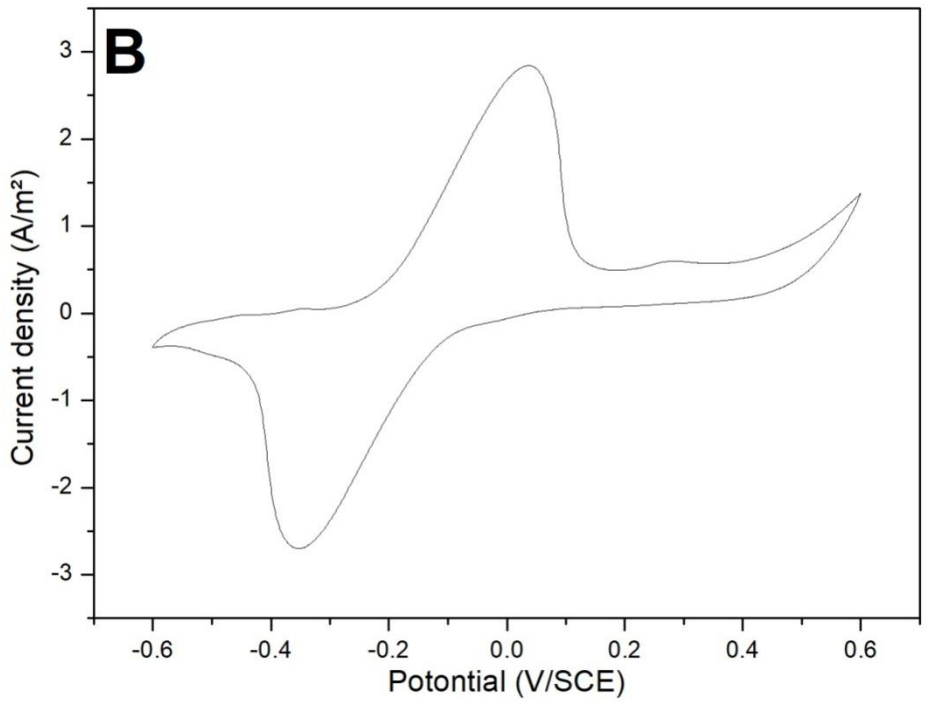
Figure 3. Evolution of the current density on carbon felt electrode polarized at -0.1 V vs. SCE in YPG liquid medium inoculated with *Hansenula fabianii* A/ without methylene blue and B/ with 300 mM of methylene blue.

3.4.2. Cyclic voltammograms

To elucidate how electrons are shuttled from living *Hansenula fabianii* cells to the anode surface, the bioelectrochemical activity of the anode in the presence and absence of methylene blue in addition of non inoculated YPG sterile liquid medium were examined by cyclic voltammetry (CV). The CV performed on carbon felt electrode in sterile YPG medium shows no electroactivity. However, the

reactor inoculated by *Hansenula fabianii* in the absence of a mediator (Fig.4A), showed two pair of redox peaks and one individual oxidative peak, while large pairs of redox peaks and one individual oxidative were observed in the presence of methylene blue (Fig. 4B).





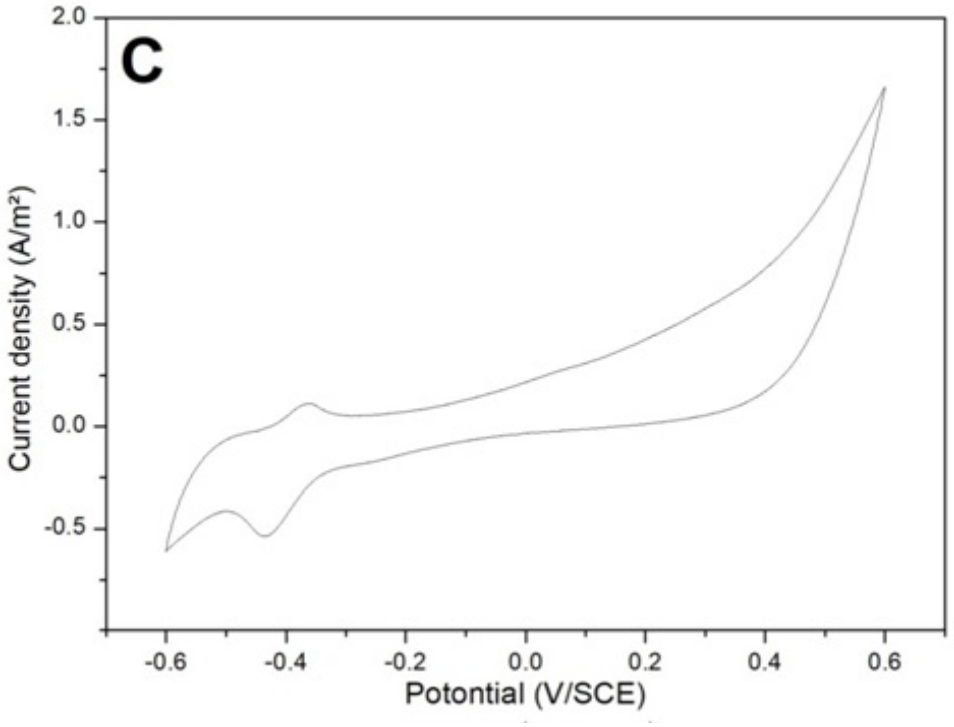


Figure 4. Cyclic voltammetries recorded using carbon felt in YPG liquid medium. A/ without methylene blue, B/ with methylene blue and C/ supernatant.

In the absence of methylene blue, the oxidative and reductive peak potential were -0.41 V, +0.1V and −0.36 V, -0.25V (vs SCE), respectively, which means that *Hansenula fabianii* can produce its own mediator. To confirm these results, cyclic voltammetry (Fig. 4C) was used to examine the electrochemical activity of the supernatants obtained by centrifugation of the anolytic yeast culture. The curve shows that the culture of *Hansenula fabianii* synthesizes and secretes into the medium an electrochemically active soluble compound that should serve as an endogenous mediator of electron transfer. However, there was a large pair of redox peaks with potential at approximately-0.36 V (vs.SCE) or +0.04 V (vs. SCE) related to the presence of methylene blue, while the pair of redox peaks produced by *Hansenula fabianii* disappeared.

The cyclic voltammograms obtained under mediator and metiatorless conditions both showed that the oxidative peak were similar. The oxidative peak potential of redox pair 1 against the saturated calomel reference electrode was 0.08 V which means that *Hansenulafabianii* might be able to transfer electrons to the electrode via direct contact with the anode surface and can contribute to current generation.

Our results corroborate with the study of Haslett (2011) in which soluble electroactive molecules in *A. adeninivorans* suspension were detected in MFC anodic compartment[24]. Also,*C. melibiosica* produces electrochemically active soluble compounds under polarization in MFC, which are not formed during normal yeast cultivation [26] and the yeast electron transfer is most probably accomplished via the production and secretion of redox molecules, acting as endogenous mediators. However, it was proven that bacteria driven MFCs yield greater current than yeast driven MFCs due to the better electron

transfer of bacteria. Therefore, the presence of artificial electron mediators is essential to improve the performance of yeast-based MFCs[28].

Nowadays, Microorganisms that are able to survive well in high concentration of heavy metals are of great interest as inoculums to treat industrial wastewater loaded by heavy metal in BES. Since many work showed that heavy metal is a drawback for current generation by bioanode in BESs. In our knowledge this is the first time that a study isolated a new electroactive yeast that tolerating high concentration of different heavy metal heavy which will be useful for industrial wastewater treatment charged by heavy metal in BESs.

3.5. Epifluorescence microscopy and ESEM

After 43 days of the chronoamperometry, *Hansenula fabianii* colonized carbon felts in the absence and presence of methylene blue were imaged by epifluorescence microscopy and ESEM (Fig.5).

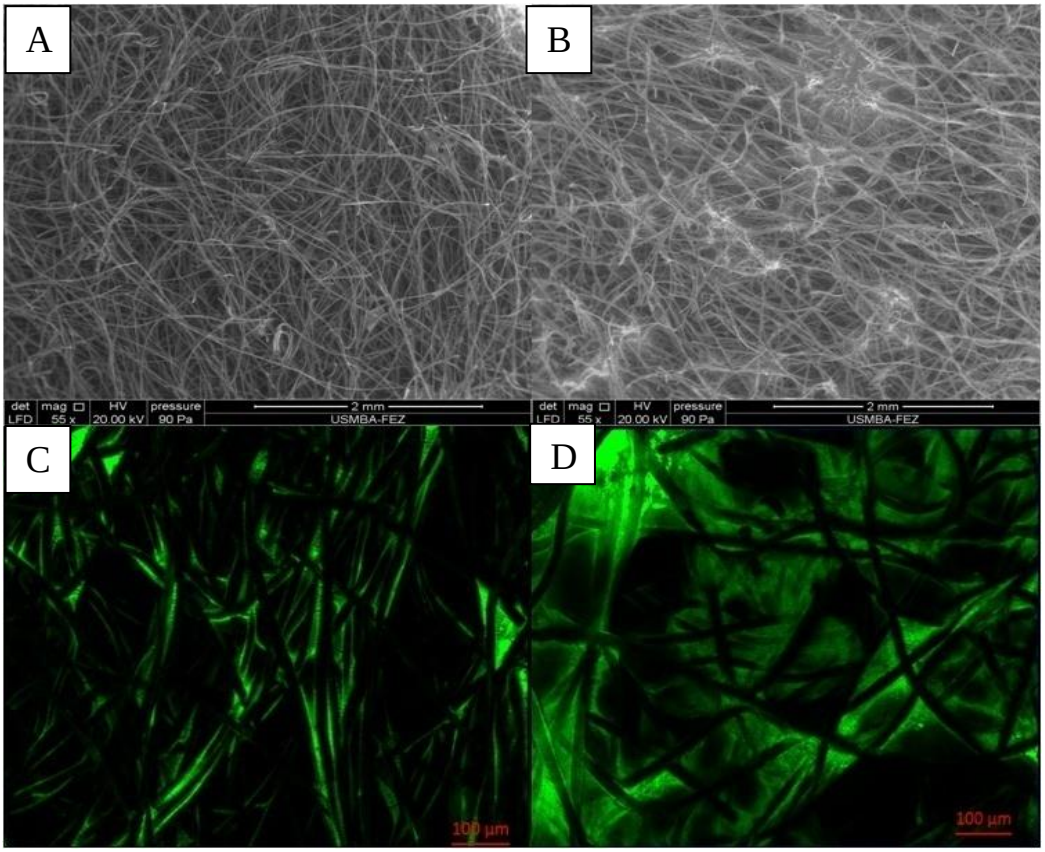


Figure 5. ESEM images of *Hansenula fabianii* bioanodes formed on carbon felt (A/ absence of methylene blue and B/ presence of methylene blue) and epifluorescence microscopy (C/ absence of methylene blue and D/ presence of methylene blue).

To evaluate the amount of electrode surface covered by biofilm, Epifluorescence images were analyzed by gray scale interpretation. The coverage rates were quantified at 4.34% and 12.50% in the absence and presence of methylene blue respectively.

The ESEM images confirmed the results obtained by epifluorescence microscopy and it showed that in the absence of methylene blue the biofilm was mainly formed by about 8 µm diameter fibres constituting the woven structure of the carbon felt. However, in the presence of methylene blue, the biofilm developed a very thin layer between the carbon fibres without changes in the porosity of carbon felt. Hubenova (DATE). illustrated the capacity of *Candida melibiosica* to form biofilm on the surface of carbon felt after 30h of inoculation.

4. CONCLUSION

Hansenula fabianii yeast strains demonstrated its ability to survive in spite of the presence of high concentration of various heavy metals (Cr^{6+},Hg^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}). On the other hand, *Hansenula fabianii*, is demonstrated to exhibit indirect electron transfer without the aid of exogenic mediators. It has also been shown that *Hansenula fabianii* can transfer electrons to electrode surfaces in the presence of glucose as carbon source leading to current generation (32 mA/m²).

Hansenula fabianii should make a good biocatalyst for MFCs as it has high levels of tolerance to various heavy metals. The perspective role of this strain is to use it as a biocatalyst for bioaugmentation of anodic microflora to increase the electrogenic activity of bioanodes designed for industrial wastewater treatment in MFCs.

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References

1. M.A. Rodrigo, P. Cañizares, H. García, J.J. Linares, J. Lobato, *Bioresour. Technol.*, 100 (2009) 4704–4710.
2. J.C. Biffinger, J. Pietron, O. Bretschger, L.J. Nadeau, G.R. Johnson, C.C. Williams, K.H. Nealson, B.R. Ringeisen, *Biosens. Bioelectron.*, 24 (2008) 900–905.
3. L. Mao, W.S. Verwoerd, (2013) 1–18.
4. B.E. Logan, (n.d.).
5. O. Schaetzle, F. Barrière, K. Baronian, *Energy Environ. Sci.*, 1 (2008) 607–620.
6. A. Gunawardena, S. Fernando, F. To, *Int. J. Mol. Sci.*, 9 (2008) 1893–1907.
7. H.A. Videla, A.J. Arvía, *Biotechnol. Bioeng.*, 17 (1975) 1529–1543.
8. D. Brady, J.R. Duncan, (1994) 149–154.
9. A. Engl, B. Kunz, *J. Chem. Tech. Biotechnol.*, 63 (1995) 257–261.
10. C. Abourached, T. Catal, H. Liu, *Water Res.*, 51 (2014) 228–233.
11. Y. Wu, X. Zhao, M. Jin, Y. Li, S. Li, F. Kong, J. Nan, A. Wang, *Bioresour. Technol.*, 253 (2018) 372–377.
12. W. Bahafid, N.T. Joutey, H. Sayel, M. Iraqui-Houssaini, N. El Ghachtouli, *Geomicrobiol. J.*, 30 (2013) 422–429.
13. A.B. Motoyama, E.J. Venancio, G.O. Brandão, S. Petrofeza-Silva, I.S. Pereira, C.M.A. Soares, M.S.S. Felipe, *J. Clin. Microbiol.*, 38 (2000) 3106–3109.

14. L. Morales-Barrera, F. de M. Guillén-Jiménez, A. Ortiz-Moreno, T.L. Villegas-Garrido, A. Sandoval-Cabrera, C.H. Hernández-Rodríguez, E. Cristiani-Urbina, *Biochem. Eng. J.*, 40 (2008) 284–292.
15. M. Kato, H. Iefuji, K. Miyake, Y. Iimura, *Appl. Microbiol. Biotechnol.*, 48 (1997) 621–625.
16. J. Juvera-espinosa, L. Morales-barrera, E. Cristiani-urbina, 40 (2006) 114–121.
17. W. IRAWATI, *Biodiversitas, J. Biol. Divers.*, 18 (2017) 971–977.
18. A. Mowat, *Water Pollut. Control Fed.*, 48 (2015) 853–866.
19. Z. Lewandowski, *Water Res.*, 19 (1985) 589–596.
20. S. Vaňková, J. Kupec, J. Hoffmann, *Ecotoxicol. Environ. Saf.*, 42 (1999) 16–21.
21. S.V. Raghavulu, R.K. Goud, P.N. Sarma, S.V. Mohan, *Bioresour. Technol.*, 102 (2011) 2751–2757.
22. F.J. Rawson, A.J. Gross, D.J. Garrett, A.J. Downard, K.H.R. Baronian, *Electrochem. Commun.*, 15 (2012) 85–87.
23. H. Shkil, A. Schulte, D.A. Guschin, W. Schuhmann, (2011) 806–813.
24. N.D. Haslett, F.J. Rawson, F. Barrière, G. Kunze, N. Pasco, R. Gooneratne, K.H.R. Baronian, *Biosens. Bioelectron.*, 26 (2011) 3742–3747.
25. J. Williams, A. Trautwein-schult, M.A. Squire, K. Baronian, (2014) 2223–2229.
26. Y. Hubenova, M. Mitov, *Bioelectrochemistry*, 78 (2010) 57–61.
27. Y. V Hubenova, R.S. Rashkov, V.D. Buchvarov, M.H. Arnaudova, S.M. Babanova, M.Y. Mitov, *Ind. Eng. Chem. Res.* (2011) 557–564.
28. Y. Hubenova, M. Mitov, *Bioelectrochemistry*, 106 (2015) 177–185.