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An innovate approach of fungal pigments as inducing the oxidase activity applied to bioelectrode systems

Uma abordagem inovadora de pigmentos fúngicos como indutores da atividade oxidase aplicada a sistemas de bioeletrodos

Um enfoque innovador de pigmentos fungicos como indutores de la actividad oxidasa aplicada a sistemas de bioeletrodos

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Abstract
The use of enzymes as part of bioelectrodes in Biofuel Cells (BFC) has been studied more often every day, aiming to reduce the operational and manufacturing high costs due in part to the use of metallic conventional catalyst. The *in-situ* production of biocatalysts can reduce even further these costs. However, some biocatalysts need the supplementation of external electrochemical mediators to achieve good coulombic efficiencies when these are used attached to bioelectrodes. In this work, two filamentous fungi were isolated from the soil of the Brazilian Caatinga Biome, that showed high oxidase activity in media containing two synthetic electronic shuttles, compared with four natural fungal pigments. Parameters such as substrate consumption, oxidase activity and microbial growth were evaluated. As was observed, all natural mediator pigments induced the enzyme production, observing an increase on enzyme production of more than 50% especially in two of them.
Keywords: Biofuel cell; Oxidases; Fungal pigment; Caatinga soil.

Resumo
O uso de enzimas como parte de bioeletrodos em Biocélulas a combustível (BC) tem sido estudado cada vez mais, visando reduzir os altos custos operacionais e de fabricação devido, em parte, ao uso de catalisador metálico convencional. A produção *in situ* de biocatalisadores pode reduzir ainda mais esses custos. No entanto, alguns biocatalisadores precisam da suplementação de mediadores eletroquímicos externos para obter boas eficiências coulombicas quando utilizados acoplados a bioeletrodos. Neste trabalho, dois fungos filamentosos isolados de solos do Bioma Caatinga Brasileira apresentaram elevada atividade oxidativa, foram avaliados em meios contendo dois mediadores eletroquímicos sintéticos, comparados com quatro pigmentos naturais produzidos por fungos. Parâmetros como consumo de substrato, atividade oxidativa e crescimento microbiano foram avaliados. Como foi observado, todos os pigmentos naturais induziran ao aumento da produção enzimática, observando-se um aumento de mais de 50% principalmente em dois deles.
Palavras-chave: Biocélulas a combustível; Oxidases; Pigmentos fúngicos; Solo da Caatinga.

Resumen
El uso de enzimas como parte de bioelectrodos en BioFuel Cells (BFC) ha sido cada día más estudiado, con el objetivo de reducir los altos costos operativos y de fabricación debido en parte al uso de catalizadores metálicos convencionales. La producción in situ de biocatalizadores puede reducir aún más estos costes. Sin embargo, algunos biocatalizadores necesitan la suplementación de mediadores electroquímicos externos para lograr buenas eficiencias

culómbicas cuando se utilizan unidos a bioelectrodos. En este trabajo, se evaluaron dos hongos filamentosos aislados de suelos de lo Biome de la Caatinga brasileña, que mostraron una elevada actividad oxidasica en medios que contenían dos sustancias mediadoras electrónicas sintéticas, en comparación con cuatro pigmentos fúngicos naturales. Se evaluaron parámetros como consumo de sustrato, actividad oxidasica y crecimiento microbiano. Como se observó, todos los pigmentos mediadores naturales no inhibieron la producción de enzimas, observándose un incremento en la producción de enzimas de más del 50% especialmente en dos de ellos.
Palabras clave: Biofuel cells; Oxidasas; Pigmentos fúngicos; Suelo de la Caatinga.

1. Introduction

Nowadays one of the biggest concerns is to how generate energy cheaply, sustainably and environmentally friendly. In this context, biological based technologies have been showing a possibility of chance, from conventional not renewable sources such as fossil fuels or the novel renewable ones using, air, water, and nuclear fusion.

The research field of fuel cells is one of the newest being explored for clean energy generation, however, the high cost of noble metals such as Au, Pt, Rh and Os, commonly used in coated electrodes as catalysts, is still considered one of the limiting factors for scaled-up applications of microbial fuel cells (MFC) and conventional fuel cells. Even though, abiotic cathodes that use oxygen as electron acceptor are frequently adopted for MFC. New research has pointed the use of enzymes attached to electrodes as biocatalyst to perform redox reactions, and this constitute a tangible horizon for the replacement of expensive metallic catalyst in MCF and Conventional ones (Morant et. *al*, 2014 [a]; La Rotta et. *al*, 2014 [a]; Mashkour, Mehrdad, et *al*, 2021).

On the other hand, since most of the enzymes are very sensitive to small changes in physical-chemical properties and reaction conditions, it is mandatory to improve their purity and stabilization to guarantee proper used and half-life. These procedures still correspond to a very hard-working and time-demanding activity, which at the end will increase the price of the biocatalyst. Having in mind this fact, the *in-situ* production of enzymes could help eventually to keep them in a micro-environmental conserved stated of activity and stability without no further purification or stabilization steps. (Morant et. *al*, 2014 [a], [b]; Jadhav, Dikpak et. *al*, 2021).

Another factor that could enhance energy production from bioelectrodes, is helping the way electrons are transported from the reaction medium to the enzyme active-site, and from this to the electrode surface. Many electron shuttle systems have been developed to achieved maximum electron transportation, by direct means (Ex. Nanowiring) or using soluble and very diffusible substances, so called electronic mediators (Ex. Synthetic Dyes) (Brunel *et al.*, 2007; La Rotta et *al*. 2011; Sorrentino et *al*, 2022). To be characterized a molecule as an electro active compound, this has to exhibit at least one redox moiety, such as a metal ion (Ex. Fe^{+2} or Fe^{+3}), a oxidable moiety (Ex. C-OH to C=O) or a reducible group (Ex. C-NH_2 to C-NH_3^+). A wide range of molecules that include one or more electro active moieties can be chosen: Organo-metallic compounds (Ex. Ferrocene), Quinone-like derivatives (Ex. Hydroquinone), Thiazinic dyes (Ex. Methylene Blue), Azo dyes (Ex. ABTS) and most recently Triphenylmethane dyes (Ex. Bromocresol green) have been used extensively in the literature as electron shuttles (La Rotta et *al*. 2011).

Nevertheless, these molecules have some limitations, especially due to their high toxicity and cost. As an alternative for these synthetic mediators, some biomolecules have also been explored, including bacterial and fungal pigments. Among the last ones, we can find Azaphillones, Antraquinoes, Oxypolyenes, Carotenes, Chlorophils, Melanins and Xantophils. All these fungal metabolites are known to participate in several biochemical processes including protection from the potentially damaging effects of bright sunlight and in particular, some UV wavelengths. Enhance the survival of both dispersal spores and resting spores, providing protection from radiation and desiccation. Reserve of energy during spore and sporangial delimitation or formation. Enzymatic inhibitors that can interfere with fungal growth processes. Regulation of redox processes acting as antioxidants and during oxidative stress (Zhou e Liu, 2010; Da Silva et. *al*, 2014 [b]; Chowdhury, A et *al*, 2022).

To minimalize or eliminate possible electrode poisoning or enzyme inactivation, we are studying the use of natural occurring pigments, whose efficacy as electronic shuttles have already been observed in our research. In addition to that it is worth pointing out that such dyes have lower toxicity and are also readily biodegradable, avoiding the deleterious effects of synthetic dyes (Da Silva et. *al*, 2014 [a]; Sharma Sunanda, 2022). Having in mind the isolation of novel electro active molecules, and founded on the immense Brazilian biodiversity, we choose the Caatinga niche as a target for the isolation of filamentous fungi. They must be capable to produce not just oxidase enzymes, but also able to produce separately or simultaneously electrogenic pigments (La Rotta et. *al*, 2014 [b]; Da Silva et. *al*, 2014 [a]). With based in previous studies from LA Rotta et. *al*, 2014 [b]; Da Silva et. *al*, 2015; Da Silva et. *al*, 2014 [b], which have proven the bioltecnological potential of several fungal species, we suggested the concomitant production of oxidase enzymes applied to the *in-situ* generation of energy in biofuel cells, coupled to the used of fungal pigments with proven electrogenic capacity. As such, in this manuscript, we describe the effect of this pigments have on the enzyme production and activity in-vivo, and we compared them to the effect caused by synthetic dyes already used as electron shuttles.

2. Methodology

2.1 Microorganisms

Indigenous fungal strains were obtained from biome Caatinga´s soil samples (Serra Talhada – Pernambuco – Brazil). From these, only 3 showed the highest extracellular oxidase production: *Aspergillus* sp. UCP 1283, *Penicillium* sp. UCP 1286 and the *Rhizopus microsporus var. chinensis* UCP 1296. In addition, 3 strains showed the highest production of pigments: *Talaromyces* sp. UCP 1324, orange pigment; *Penicillium* sp. UCP 01152, green pigment; *Aspergillus* sp. UCP 01349, red pigment; and *Penicillium* sp. UCP 1286, yellow pigment. Here, we used just *Penicillium* sp UCP 1286 and *R.microsporus var. chinensis* UCP 1296 as enzyme producers. Fungi were identified and deposited in the Culture Collection of the Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, Brazil, registered in the World Federation Culture for Collection (WFCC). The strains were kept in Sabouraud Agar medium (consisting of agar 20 g, peptone 10 g, glucose 40 g, distilled water 1000 ml, and pH adjusted to 6.0), at 5 °C.

2.2 Culture media and cultivation conditions

- Solid media for inoculum generation (Modified Nutrient Media): (g L^{-1}) – Glycerol, 20.0 g; Yeast Peptone, 6.0 g; Meat extract, 4.0 g; Agar, 20.0 g.

- Liquid media for pigment production: same as above, in absence of agar.

- Liquid medium for enzyme production (Previously described by Viswanath, B *et al.* 2008 and modified by e Morant *et al.* 2015): (g L^{-1}) – Glucose, 10.0 g; Yeast Peptone, 3.0 g; KH_2PO_4, 0.6 g; $ZnSO_4$, 0.001 g; K_2HPO_4, 0.4 g; $FeSO_4$, 0.0005 g, $MnSO_4$, 0.05 g; $MgSO_4$, 0.5 g; e Agar, 20,0 g.

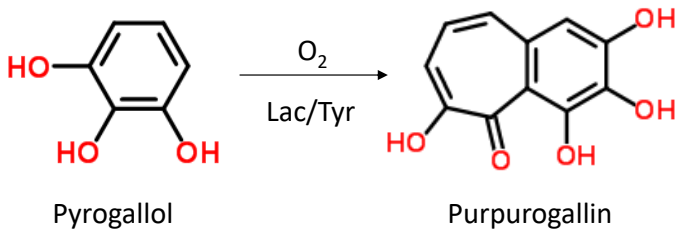
The microorganisms were inoculated in petri dishes containing solid medium and incubated for 48 h a 28º C. Growth mycelia was divided and disrupted by vortex in 10 mL of liquid media used as pre-inoculum. After 48h of incubation at 28º C and 150 rpm for enzyme production and 180 rpm for pigment production, the pre-inoculum was transferred to 250 mL Erlenmeyer flasks and kept under the same conditions of temperature and agitation for 240 h, for both productions.

2.3 Biological parameters

2.3.1 Enzyme activity determination

Samples were taken every 24 h along the fermentation time. The centrifuged at 4500 rpm, supernatant was recovered and then frozen for further determinations. Glucose was determined using an enzymatic kit from BIOCLIN®. Oxidase activity was determined using the adapted method from MORANT *et al.* 2015; TRIANA *et al.* 2005 based on the oxidation of pyrogallol to purpurogallin (E_{420nm} = 26.6 $mmol^{-l}.L\ cm^{-1}$), and UI were defined as the amount of purpurogallin mmol mL^{-1} per minute formed at 25º C e pH 5,0 (Figure 1).

Figure 1. Reaction of pyrogallol oxidation catalyzed by oxidases (Lacasse, Tyrosinase or Peroxidase).



Source: Own authors (2022).

2.3.2 Fungal growth and substrate consumption.

Fungal growth was estimated form the final mycelium volume obtained at the end of each fermentation, dried at 50º C until constant weight. Pigment producer fungi were cultivated, and pigments were purified according to the methodology previously described by Da Silva *et* al. 2015. Glucose was enzymatically determined using an enzymatic Kit based on the coupled reaction of Glucose oxidase GOX and Horse-radish peroxidase HRP), in the presence of 4-chlorophenol and 4-aminoantipyrina to from red derivatives of aminopropyl-quinoneimines (APQI) that can be quantified at 540 nm. Concentration of glucose was stoichiometrical to the amount of APQI produced after GOX/HRP reactions.

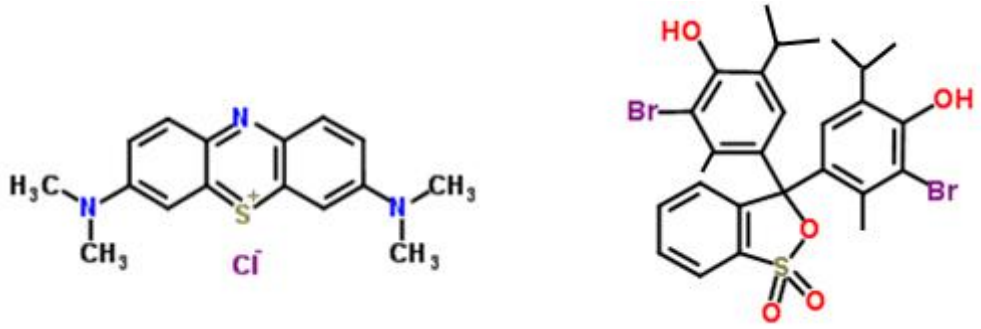
2.3.3 Fungal Pigments production and purification

Pigments producer fungi were cultivated according to the above-described condition. At the end of the fermentation, cells were separated from the pigment. Containing media, and then solutions were processed according to the methodology described by Da Silva *et* al. 2014[b], that includes freeze-dry of liquid phase using a liophylizer LabConco® FreezeZone 4.5 until complete dryness.

2.3.4 Effect of fungal pigments over enzyme activity

An amount of 10 ppm of each pigment was added to the culture media used for enzyme production, and their effect on the enzyme activity or inhibitory effect was compared with to synthetic dyes commonly used as electron shuttles for oxidase enzymes in bioelectrode (Figure 2). one tiazinic dye: 3,7-Bis(dimethylamino)phenothiazin-5-ium chloride or Methylene blue (MB) and one Triphenylmethanic dye: 4,4'-(1,1-Dioxido-3H-2,1-benzoxathiole-3,3-diyl) bis(2,6-dibr omophenol) or Bromothymol Blue (BPB). All pigments were added to the fresh media prior to the inoculum addition (Wu, K. et *al*. 2022). All experiments were performed in duplicate.

Figure 2. Structure of the synthetic dyes MB (Left) and BPB (Right).



Source: Own authors (2022).

2.3.5 Cyclic voltammetry

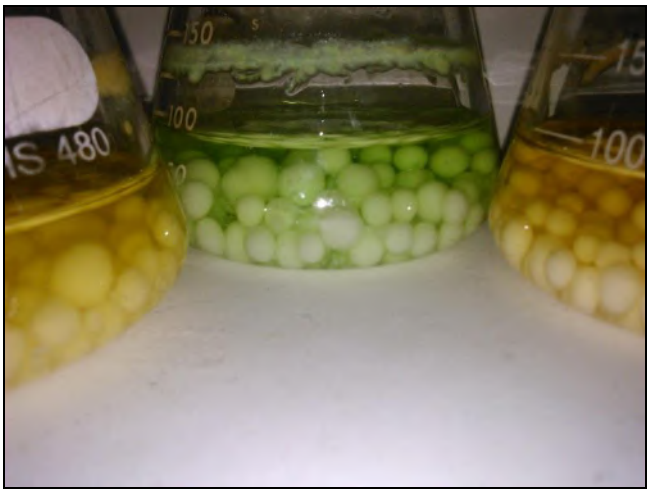
To perform the cyclic voltammetry, to obtain oxidation and reduction peaks of a substance, electrochemical cells were used containing as supporting electrolytes, potassium chloride (KCl) and phosphoric acid (H_3PO_4), both with 10 mmol L^{-1}. The electrochemical analysis system consisted of a glassy carbon working electrode, a platinum wire counter electrode and Ag|AgCl as a reference electrode. Potential sweeps were performed from -1.0 to 1.0 V with speeds of 0.1 $V.s^{-1}$ (Di Noto, et *al*. 2022; Wang H. W., et *al.* 2021)

3. Results and Discussion

3.1 Pigments: Effects on cellular morphology and decolorization

Fungus *R. microsporus var. chinensis* had the capacity of decolorization of media containing BM, though no significant changes in microbial growth were observed. On the other hand, BPB caused a decrease on microbial growth of 50% compared with control experiment, and dimorphism was observed in most of the samples. No experiment with *R. microsporus var. chinensis* showed pellet formation, since all biomass remained suspended during agitation, with the consequent precipitation to the bottom when agitation stopped. On the other hand, *Penicillium* sp. UCP 1286 did not showed any capacity for pigment decolorization. However, pigment addition does not cause in the fungus the inhibition of its own yellow pigment production (Figure 3). Equally to the observation made over *R. microsporus var. chinensis* growth, the strain *Penicillium* sp. UCP 1286 also diminished its growth with the addition of pigments, especially when BPB was added, however kept its original morphology. In the presence of the other pigments, *Penicillium* sp. UCP 1286 showed the formation of uniformed, rounded pellets.

Figure 3. Colored pellets were formed by *R. microsporus var. chinensis* UCP 1296 in the presence of MB. The fungus naturally produces yellow pigment that mixed with MB produce green color.



Source: Own authors (2022).

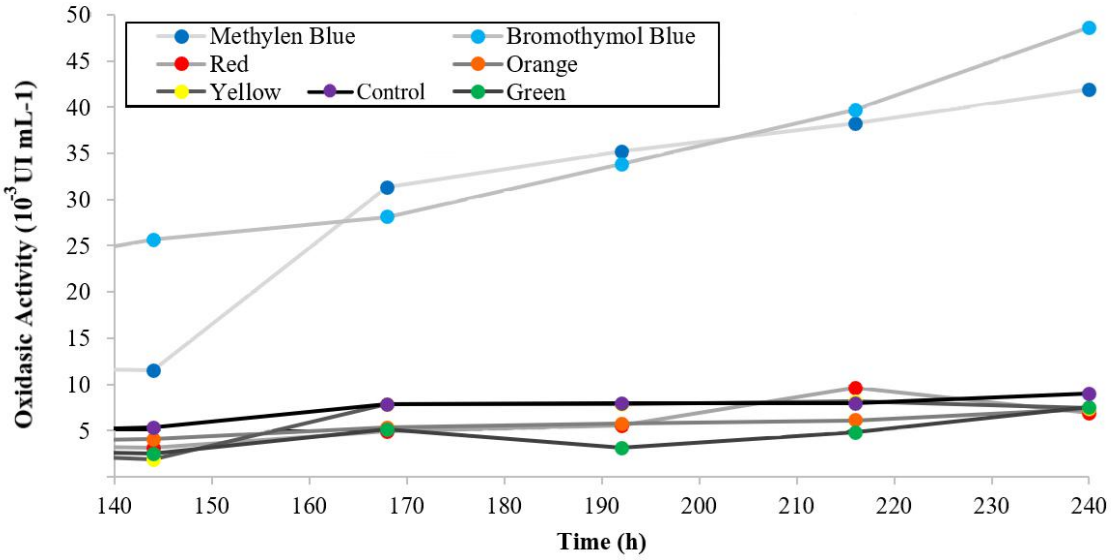
As we already showed in previous studies (Morant et. *al* 2015 e Morant et. *al* 2014[b]), the enzyme production of *Penicillium* sp. UCP 1286 was not related with the pigment excretion, and the morphology showed to be the same when no pigment was added. Based on the observations made by Da Silva et. *al* 2015, we also confirmed that fungal pigment addition did not inhibit fungal growth during cultivation in solid medium

3.2 Effect of pigments on enzyme activity

After the fermentation, the *Penicillium* sp. UCP1286 showed the highest value of enzymatic enriched by fungal pigments at 216 h, with the red mediator, we archive 9649 UI.min^{-1} (Figure 4). On the other hand, *R. microsporus var. chinensis* had its great value with the orange pigment at 144h, obtaining 67544 UI.min^{-1} as enzymatic activity (Figure 5).

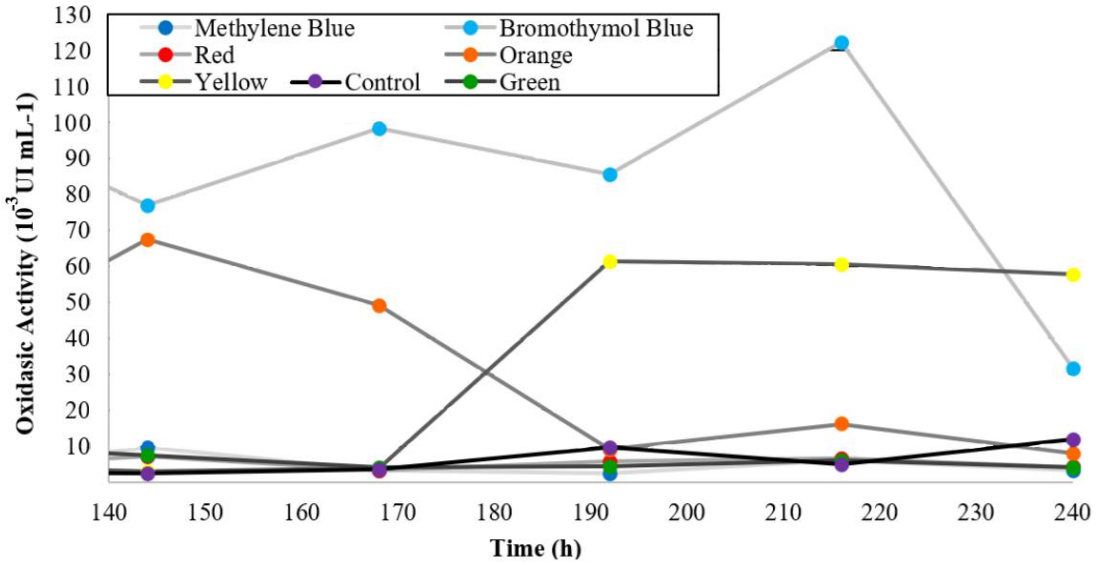
From Figures 4 and 5, it was possible to observe that there is a similar pattern for all fungal pigments and synthetic dyes evaluated. In the case of *Penicillium* sp. UCP1286, Dyes MB and BPB cause an increase almost constant on the enzyme activity. *R. microsporus var. chinensis*, higher oxidasic peaks were observed when synthetic dyes were uses, however, yellow pigment it was the one that showed the highest microbial growth at the end of the experiment. Higher oxidasic activity levels of 4600 UI.mL^{-1}, were previously found by Morant et al. (2014 [b]) *Penicillium* sp. UCP1286, but when red pigment from *Aspergillus* sp. UCP01349 an increase of 2 fold-times was observed. In the case of *R. microsporus var. chinensis*, Morant et al. (2015) observed the highest enzymatic activity of 2700 UI mL^{-1}, but when the orange pigment obtained from *Talaromyces* sp.UCP 1324, the observed oxidasic activity increased up almost 5000 UI.mL^{-1}.

Figure 4. Enzyme activity profiles observed for *R. microsporus var. chinensis* UCP 1296 with addition of synthetic dyes and fungal pigments.



Source: Own authors (2022).

Figure 5. Enzyme activity profiles observed for *Penicillium* sp. UCP1286 with addition of synthetic dyes and fungal pigments.



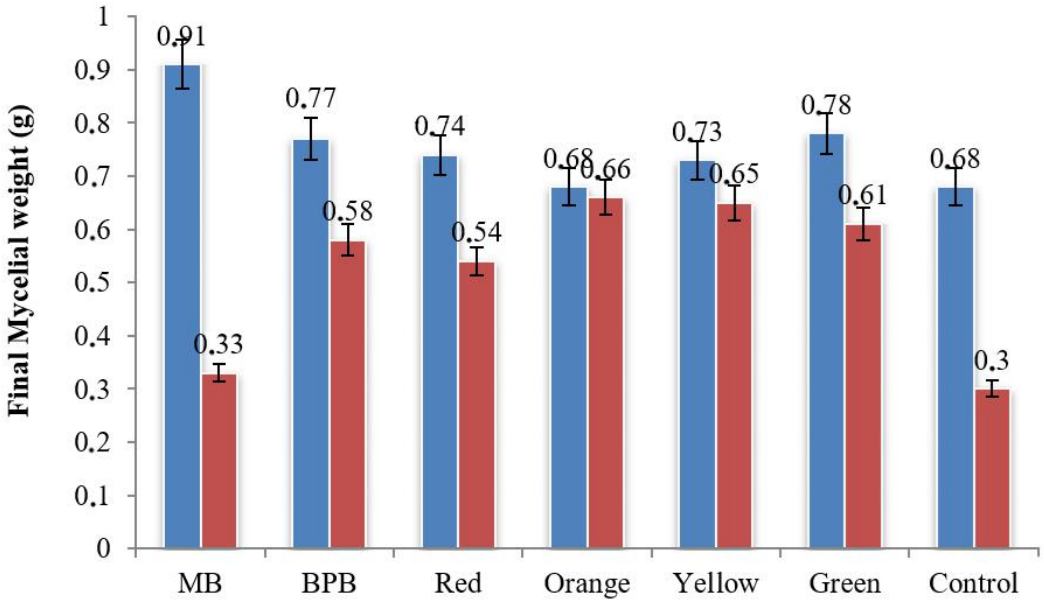
Source: Own authors (2022).

3.3 Effect over substrate consumption and microbial growth

The glucose consumption in the presence of natural fungal pigments, showed to be very efficient in most of cases, having a residual average concentration of 3 mg.dL^{-1} for *Penicillium* sp UCP1286 and 12 mg.dL^{-1} for *R. microsporus var. chinensis* UCP 1296. The addition of pigments also demonstrated the beneficial effect over the microbial growth (Figure 6). For, as such green pigment from *Penicillium* sp. UCP1286 showed to have the highest fungal effect, while for *R. microsporus var. chinensis* a noticeable beneficial effect was caused for pigments orange, yellow and green, from *Talaromyces* sp. UCP1324, *Penicillium sp*. UCP1286) and *Penicillium* sp. UCP1395, respectively. All pigments kept in general a homogeneous

average for biomass production. However, when MP was added the highest biomass production was achieved for *Penicillium* sp. UCP1286, and now significant effect was observed for this dye over *R. microsporus var. chinensis* growth.

Figure 6. Mycelial weight (g) produced by *Penicillium* sp. UCP1286 (blue bars) and *R. microsporus var. chinensis* UCP 1296 (red bars) with methylene blue (MB), bromothymol blue (BPB) and fungal pigments.



Source: Own authors (2022).

It was observed that *R. microsporus var. chinensis* UCP 1296 was able to degrade MB, with no apparent alteration at growth level neither change over mycelium structures. However, growth changes were evident when BMP was used, causing the decrease on biomass by a half in comparison to the control experiment, and significant dimorphism was observed. No experiment *Rhizopus* shoed pellets formation.

3.4 Capacity of electron transportation by cyclic voltammetry

Cyclic voltammetry analyzes showed in general a similar behavior with almost all pigments, being mostly observed two oxidation peaks and three reduction peaks, Indicating the presence of an irreversible reduction peak (Tables 1 and 2). On the other hand, the yellow pigment when evaluated in H_3PO_4 and the red pigment in KCl, proved to be quite efficient in terms of reversibility of the observed redox peaks. In general, it is observed that all pigments have a good capacity for charge transfer and transport, even though the irreversibility of one of the peaks is observed.

Table 1. Cyclic Voltammetry response to redox peaks fungi pigments red, green, orange, and yellow to KCl saline bridge

CYCLIC VOLTAMMETRY REDOX PEAKS - KCL (V)				
RED PIGMENT	Oxidation peak	-0.12	-0.87	-
	Reduction peak	1.12	-0.2	-0.5
GREEN PIGMENT	Oxidation peak	-0.12	-0.87	-
	Reduction peak	1.12	-0.2	-0.5
ORANGE PIGMENT	Oxidation peak	-0.12	-0.87	-
	Reduction peak	1.12	-0.2	-0.5
YELLOW PIGMENT	Oxidation peak	0.75	-0.12	-0.87
	Reduction peak	1	-0.2	-0.5

Source: Own authors (2022).

Table 2. Cyclic voltammetry response to redox peaks fungi pigments red, green, orange, and yellow to H3PO4 saline bridge.

CYCLIC VOLTAMMETRY REDOX PEAKS - H_3PO_4 (V)				
RED PIGMENT	Oxidation peak	0.87	0.5	-0.87
	Reduction peak	1.12	-0.1	-0.6
GREEN PIGMENT	Oxidation peak	-0.5	-0.87	-
	Reduction peak	1.12	-0.1	-0.5
ORANGE PIGMENT	Oxidation peak	-0.5	-0.87	-
	Reduction peak	1.12	-0.1	-0.5
YELLOW PIGMENT	Oxidation peak	0.37	-0.87	-
	Reduction peak	1.12	-0.1	-0.5

Source: Own authors (2022).

4. Conclusion

Despite, the results observed for synthetic dyes in terms of beneficial effects over enzyme production and microbial growth in comparison with the innovative role of the fungal pigments, the use of the last ones is preferred since their *in-situ* production. According to our observations the isolation of the very active enzymes forms the studied fungi, let us think about the possibility of a coupled system of two or more electroactive microorganism working together in a hybrid or consorted system of two or more fungi with proven electrogenic capacity. The experiments will intend to demonstrate the effectiveness of this kind of microbial consortia in microbial electrodes for MFC on the innovate future in bioeletrogenic role of induction by fungal pigments. This article gives the used ecologically unfriendly processes for induction of the oxidases by pigments as new alternatives that are in the development stage and could be important in the near future for the treatment of effluents, which reduced to the cost of the process.

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