ROLE OF EXTRACELLULAR VESICLES FROM STRAIN C7.1 IN THE DEGRADATION OF TEXTILE INDUSTRY DYES

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ABSTRACT

Extracellular vesicles (EVs) have the potential to carry enzymes that degrade various toxic compounds. This study aims to investigate whether EVs from the bacterial strain C7.1 can carry enzymes that degrade textile dyes, especially azo dyes, which are major pollutants from the textile industry with carcinogenic and mutagenic properties. The Bacillus sp. C7.1 strain was cultivated in both liquid and solid media, with and without dye enrichment. The results indicated that the strain has the capacity to degrade up to 80% of a type of azo dye under static conditions and that liquid cultures with dye produced significantly more EVs compared to solid media, with no detectable EVs in solid cultures without dye. The findings suggest that dye-induced stress increases EV production. This research aims to develop marketable enzymatic nanotools for treating dye-contaminated wastewater, contributing to more sustainable industrial practices.

Keywords: Extracellular vesicles. Textile dyes. Bioremediation. Azo dyes. Textile industry

1 INTRODUCTION

Bacterial extracellular membrane vesicles (BMVs) are nanometric particles produced by bacteria and excreted by the cell into the extracellular environment. The literature describes these vesicles in two main groups: Gram-negative bacterial vesicles called outer membrane vesicles (OMVs) and Gram-positive bacterial vesicles called membrane vesicles (MVs). BMVs are primarily composed of lipids, such as phospholipids, which form a lipid bilayer similar to the cell membrane of the originating cell[1]. Regarding biogenesis, it is important to highlight that several factors determine the production of extracellular vesicles in bacteria during their life cycles, such as nutritional status, growth phase, and environmental conditions. Different biogenesis models have been proposed by genetic and biochemical studies. Most studies present primary formation factors such as the loss of connection between the outer membrane and the peptidoglycan layer, turgor pressure caused by the accumulation of PG fragments in the periplasmic space, and vesicle formation due to membrane curvature.BMVs perform various functions within cell biology, including significant transport capacity, biofilm formation, and horizontal gene transfer. Bacterial membrane vesicles have wide biotechnological applicability. It is possible to analyze the different uses of vesicles for clinical procedures, such as vaccine production, cancer therapy, and high-precision drug delivery systems[2]. As studies on extracellular vesicles are relatively recent, it has become evident that further research is needed to elucidate the role of extracellular vesicles from an environmental perspective, for example, in the bioremediation of contaminated environments. This project aims to contribute to these discoveries by studying the potential of the C7.1 strain and its ability to produce redox enzymes that act in the degradation of dyes from textile industry effluents, as well as its BMVs containing these same enzymes.

Among the many proposed functions for extracellular vesicles is their ability to transport various enzymes[3]. These have become attractive for biological treatments of xenobiotic compounds that require a cascade of enzymatic reactions until mineralization. From this perspective, it is important to highlight that the textile and clothing industry is among the most polluting sectors in the world, with considerable waste generation throughout its extensive production chain. Among these wastes are textile dyes, especially azo dyes. These dyes constitute the largest class currently used, and studies have already reported their carcinogenic, genotoxic, mutagenic, and allergenic potential. Additionally, these dyes persist in the aquatic environment and can bioaccumulate at trophic levels. Therefore, it is necessary to rethink the environmental impacts caused by the activities of this industry and propose solutions to minimize damage and make the sector more sustainable. This study aims to contribute to the development of sustainable solutions by exploring the potential of BMVs as enzymatic tools for treating dye-contaminated effluents. Thus, the overall objective is to develop marketable enzymatic nanoferramentas based on extracellular vesicles to treat effluents containing textile dyes. Specifically, the objectives include evaluating the potential of the C7.1 strain in producing azoreductase enzymes for the degradation of azo dyes used by the textile industry, starting with methyl orange dye, and subsequently analyzing the BMVs of the C7.1 strain, as well as investigating methods of cultivation and vesicle extraction to obtain viable enzymes.

2 MATERIAL & METHODS

The C7.1 strain, of environmental origin, was chosen due to its superior performance in dye decolorization, previously recognized as a producer of bacterial extracellular membrane vesicles. This strain demonstrated promising results in previous experiments, achieving the ability to decolorize 80% of the tested dyes, with methyl orange being used as the model dye for this experiment. Additionally, methyl orange is employed as a stressing agent to potentially increase vesicle production. The decolorization assays with the C7.1 strain involved culturing the strain in Luria Bertani (LB) medium for 2 days. The broth was centrifuged to precipitate the cells, which were then resuspended in saline. Aliquots of 1 ml were transferred to Erlenmeyer flasks containing 40 ml of decolorization medium artificially contaminated with a stock solution of the dye: methyl orange (final concentration in the medium: 15 mg/L). All decolorization assays were performed in duplicate under static and shaking conditions for 7 days. To evaluate the decolorization efficiency by the bacteria, 1 ml aliquots were transferred from each Erlenmeyer flask to Eppendorf tubes on the

2nd, 5th, and 7th days of cultivation. The clear supernatant obtained after centrifugation was taken to the spectrophotometer configured for scanning readings at wavelengths between 400-800 nm. The wavelength corresponding to the absorbance peak of each dye was determined, and for the purpose of calculating decolorization rates, the results obtained from the culture supernatants were compared with those of the controls (culture medium without microbial inoculum). To calculate the percentage of decolorization, an equation known in the literature is applied.

For vesicle production, the initial inoculum followed the same pattern and was inoculated in vesicle production-inducing medium (decolorization medium). Two treatments were applied to optimize vesicle production: liquid and solid media enriched or not with dye, to determine their capacity to carry enzymes of interest. Cell growth monitoring was performed by spectrophotometry, measuring optical density at 600 nm. For vesicle extraction, specific methods adapted for liquid and solid medium cultures were employed. In liquid cultivation, vesicles were extracted using a Viva Flow Cassette, followed by ultrafiltration and ultracentrifugation. In solid cultivation, the extraction method was based on ultracentrifugation, followed by filtration to remove cellular debris. To evaluate the effectiveness of the vesicle production method (in solid or liquid media), protein dosage methods were employed using a protein dosage kit. Additionally, the characterization of membrane vesicles included analyses via scanning electron microscopy. The characterization data underwent statistical analysis to evaluate the uniformity and variability of the samples among different treatments.

3 RESULTS & DISCUSSION

The preliminary results found in this study suggest that the C7.1 strain exhibited interesting methyl orange decolorization rates, particularly under static conditions (Figure 1). This is because reductive enzymes like azoreductase are anaerobic or microaerophilic, meaning they function better under low-oxygen conditions. Agitation can increase the concentration of dissolved oxygen in the medium; thus, the presence of oxygen can inhibit these enzymes' activity and consequently the dye decolorization[5]. Moreover, the strain also produced a significantly higher amount of extracellular vesicles (EVs) in liquid culture with dye compared to extraction in solid medium with dye, which showed limited vesicle production. Extraction in solid medium without dye did not result in detectable vesicles under microscopy. Size distribution analyses indicated that, in the solid medium culture (Figure 2), most vesicles were concentrated in the 60 to 80 nm diameter range, with a significant peak around 60 nm, representing about 40% of the vesicles. In the liquid medium culture (Figure 4), the vesicles were also mainly concentrated in the 40 to 80 nm range, but with a more pronounced peak around 40 nm, corresponding to approximately 50% of the vesicles, indicating that this strain may have the characteristic of forming small vesicles. The variation in vesicle size between the two types of culture could have significant implications for their biological functions and applications. Smaller vesicles, such as exosomes, generally in the 30-100 nm range, play a crucial role in intercellular communication, molecule transport, and maintenance of cellular homeostasis. The predominance of smaller vesicles in both types of culture suggests that these cultures may be efficient for studies of cellular communication and drug delivery.

It was also observed that the C7 strain produced a significantly higher amount of extracellular vesicles in liquid culture with dye compared to the extraction in solid medium with dye, which showed limited vesicle production. Additionally, the extraction in solid medium without dye did not result in detectable vesicles under microscopy. This phenomenon can be contextualized by the fact that the production of extracellular vesicles is often induced by cellular stress conditions. In our case, the stress agent was the dye added to the medium. This observation suggests that the presence of the dye may be causing stress to the cells, leading to an increase in vesicle production. To investigate further, we plan to conduct growth curves in LB medium, where the cells are in non-stressed conditions, and in dye decolorization medium. This study aims to determine if vesicle production is related to the need for dye in the medium and if there is significant damage to the cells in the stressed condition. These growth curves will be conducted in the next stages of the project. As can be observed in the microscopy results of the solid and liquid cultures (Figures 4 and 5) in the dye decolorization medium, both cultures showed the formation of extracellular vesicles. In the liquid medium (Figure 4), by analyzing the microscopy images, a greater variety of vesicles could be observed. These images serve as evidence that the extraction methods used are effective and that the extraction shows a certain level of purity. These results reinforce the validity of the techniques employed and suggest that different culture methods can influence the diversity and quantity of vesicles produced.

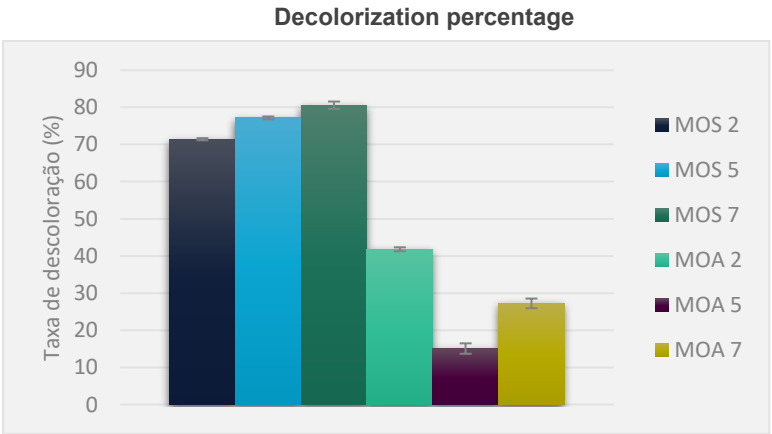

Figure 1. Decolorization Percentage of Methyl Orange (MO) by the C7.1 Strain on Days 2, 5, and 7. MOS: MO Static Condition, MOA: MO Aeration Condition.

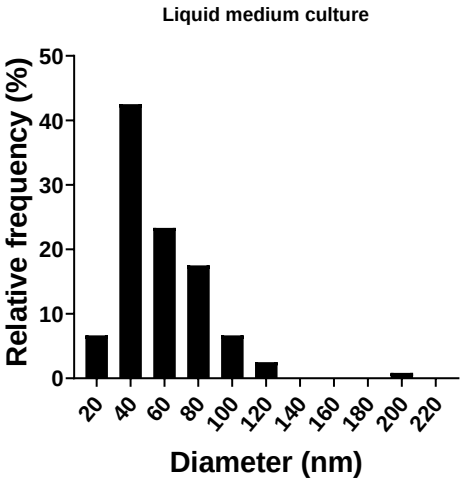

Figure 2. Relative frequency, obtained by Transmission Electron Microscopy, of the size of EVs in liquid medium.

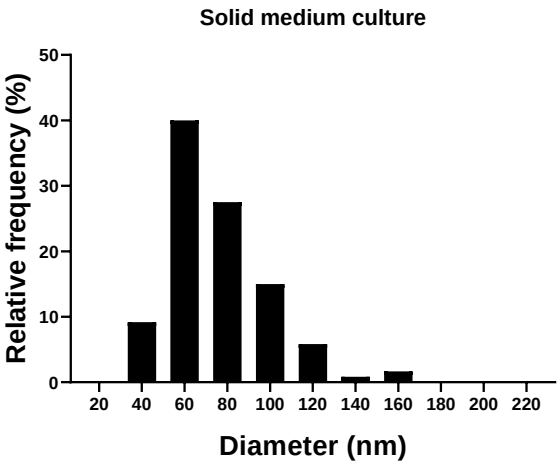

Figure 3. Relative frequency, obtained by Transmission Electron Microscopy, of the size of EVs in solid medium.

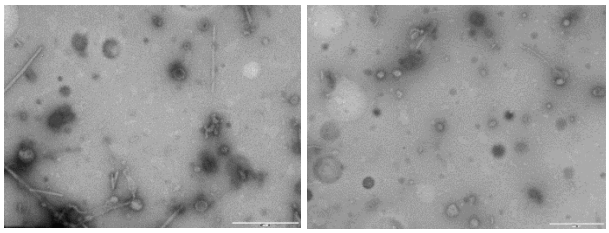

Figure 4. Image of EVs in solid medium. Transmission Electron Microscopy. Scale of 500 nm

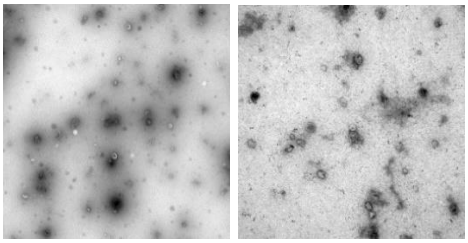

Figure 5. Image of EVs in solid medium. Transmission Electron Microscopy. Scale of 500 nm

4 CONCLUSION

It is important to conclude by emphasizing that there are ongoing experiments in the project, whose results will be presented in the future. Among the activities to be carried out are the procedures for detecting and evaluating enzymes in extracellular vesicles, including azoreductase, laccase, lignin peroxidase, and manganese peroxidase. These experiments will follow specific protocols and will be conducted in 96-well plates using a SpectraMax microplate reader for kinetic readings, with blank assays and repetitions to ensure the accuracy of the results. Additionally, in future assays, decolorization tests based on the carrier potential of the vesicles with methyl orange will be conducted, calculating the decolorization percentage based on the absorbance of the samples. Residual toxicity assays will also be conducted to assess the environmental safety of the dyes after treatment. It is expected that these characterization analyses will be complemented by proteomic methods of nanoparticle identification to identify the specific proteins present in the vesicles. To summarize, it is essential to emphasize that there are ongoing experiments in the project, whose results will be presented in the future. Among the activities to be carried out are the procedures for detecting and evaluating enzymes in extracellular vesicles, including azoreductase, laccase, lignin peroxidase, and manganese peroxidase. These experiments will follow specific protocols and will be conducted in 96-well plates using a SpectraMax microplate reader for kinetic readings, with blank assays and repetitions to ensure the accuracy of the results. Additionally, in future assays, decolorization tests based on the carrier potential of the vesicles with methyl orange will be conducted, calculating the decolorization percentage based on the absorbance of the samples. Residual toxicity assays will also be conducted to assess the environmental safety of the dyes after treatment. It is expected that these characterization analyses will be complemented by proteomic methods of nanoparticle identification to identify the specific proteins present in the vesicles.

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