Chapter

Enterococcus Present in Marine Ecosystems and Their Potential to Degrade Azo Dyes

Ganiveth María Manjarrez Paba and Rosa Baldiris Ávila

Abstract

Azo dyes are frequently used at an industrial level to restore the color of raw materials once it has faded away, make an original color more vibrant or with the purpose of giving a material a different color that is considered more attractive. These processes however, have a negative impact on the environment, evidenced in colored wastewater that is subsequently dumped into water bodies, causing disruptions in the natural balance of ecosystems and deteriorating human health. Traditional strategies for the treatment of effluents contaminated with azo dyes are limited to physical and chemical processes that have a high energy and economic cost. For these reasons, current challenges are focused on the use of microorganisms capable of transforming dyes into less toxic products. This chapter will present a description of the main characteristics of azo dyes and the different methods used for their treatment, with special emphasis on the benefits associated with biological treatment. Likewise, it will provide relevant information about *Enterococcus* and show its potential to degrade azo dyes.

Keywords: Enterococcus, marine, azo dyes

1. Introduction

Annually, more than a million tons of synthetic dyes are produced around the world for use in the leather, textile, pharmaceutical, food, cosmetic, paint, plastic and paper industries [1], of which, at least 60% represent azo dyes [2]. In addition to being recalcitrant towards various degradation processes [3], azo dyes produce dangerous chemical substances such as aromatic amines, known for their toxic, allergenic, carcinogenic and mutagenic effect on living organisms [4].

The impact of azo dyes on the environment is related to the enormous amounts of hazardous waste associated with industrial processes, which in most cases, is then released directly to water bodies without proper treatment. A further aggravating factor is that due to the inability of at least 35% of azo dyes to adhere to substrates, heavy metals have been incorporated during the dyeing process, and these act as mordants, favoring the fixation of the dye [5].

Colorants associated with metals such as copper, cobalt and especially chromium, are difficult to degrade and represent an important source of environmental contamination due to their increased presence in organic load. They generate adverse and irreversible eco-toxicological effects, bioaccumulation phenomena and biomagnification in flora and aquatic fauna and alteration of biogeochemical cycles [6].

This powerful metal-dye complex has carcinogenic and mutagenic properties for humans exposed to effluents contaminated with dyes. It can lead to skin cancer due to photosensitization, photodynamic damage, allergic contact dermatitis, renal, reproductive, hepatic, cerebral dysfunction, irritation of the respiratory tract and asthma [7].

Traditionally, physicochemical methods have been used to treat effluents contaminated with azo dyes, but their high economic and energy cost and the environmental effects associated with their use have changed the focus, in recent years, on the use of microorganisms. These are successful biological alternatives due to their survival properties, adaptability, enzymatic activity and chemical structure. Additionally, hybrid technologies have been developed, which are able to take the best of each technology and surpass the limitations of current conventional treatments [8].

Enterococcus sp. are gram-positive cocci, facultative anaerobes capable of growth in environments with low nutrient concentrations, persistent temperature fluctuations, and are resistant to desiccation, UV radiation, freezing, pH changes, high salinity and predation [9]. According to phylogenetic studies, this genus includes 50 species of clinical and environmental importance [10].

The environmental importance of *Enterococcus* sp. has to do with the fact that, since 1986, the US Environmental Protection Agency included them as part of the parameters for evaluating the quality of marine waters. Likewise, the World Health Organization considered them more important than thermotolerant coliforms, due to their ability to resist the physical and chemical conditions of seawater, and for being an excellent indicator for waters impacted by fecal contamination [11].

While international organizations consider *Enterococcus* sp. as indicators of fecal contamination of marine waters, another relevant aspect and a novelty of this article has to do with taking into consideration beaches as well. Beaches are complex ecosystems where there is a dynamic of continuous transport between water and sand. For this reason, it was considered decisive not only to evaluate *Enteococcus* sp. in water, but also in beach sand, where animal feces and residues generated by anthropogenic activities are generally found [12].

As a contribution to this discussion and element for further research, this article presents a review of the potential of *Enterococcus* to become an optimal biological alternative in the treatment of effluents contaminated with Azo dyes. This is due to its ability to survive in aquatic environments with adverse environmental conditions, its development of multi-resistance mechanisms for antibiotics and heavy metals, as well as its enzyme systems associated with the degradation of dyes.

1.1 The potential of bacteria for the degradation of azo dyes

For the degradation of azo dyes, bacteria have an efficient enzymatic system that allows them to carry out a series of catabolic activities, with azoreductase and laccase enzymes being responsible for the transfer of electrons to the azo bond of the dye and the production of aromatic amines [13].

The mechanism of degradation by azoreductase enzymes consists of two phases. The first, called the reducing phase, begins with the cleavage of the azo bond (-N=N-) by catalyzed reduction of the enzyme under anaerobic/anoxic or microaerophilic conditions, where NADH molecules, derived from carbohydrate metabolism are used as electron donors [13]. In the second phase, as a result of this division, relatively simple intermediate aromatic amines are generated, which are deaminated or dehydrogenated by bacteria through aerobic processes under aerobic conditions, which leads to complete degradation of azo dyes [14].

Laccases, on the other hand, are copper oxidases that degrade dyes in the presence of oxygen through mechanisms that involve direct or indirect oxidation using

redox mediators to accelerate the reaction. This involves the removal of a hydrogen atom from the hydroxyl and amino groups, replacing it with phenolic substrates and aromatic amines [15].

Bacterial action in the degradation of azo dyes is increased due to their ability to act through consortiums or synergistic associations that act as biological inducers. The union of the catabolic functions of each microorganism makes them even more useful alternatives to improve the discoloration rate of effluents contaminated with dyes, as they have greater resistance to abiotic conditions and lower rates of enzyme inactivation, especially in large-scale operations [16].

1.2 Enterococcus, potentially degrading bacteria of the complex azoic dyes - heavy metals

One of the bacteria identified as an effective biological alternative for the removal of metal-dye synergy is *Enterococcus* sp., recognized for its ability to thrive in environments with low nutrient concentrations, persistent to temperature fluctuations and resistant to desiccation, UV radiation, freezing, pH changes, high salinity and predation. Furthermore, they are considered catabolically versatile microorganisms, capable of using a wide range of unusual substrates as carbon source [17].

For a long time, the environmental importance of *Enterococcus* sp. had to do with it being an excellent indicator of fecal contamination in waters [11]. However, new potential uses of this microorganism have emerged recently. It can be exploited for the benefit of the environment, such as for its ability to metabolize xenobiotics, among which are azo dyes, and it has an affinity to bind and resist heavy metals. Furthermore, the genome of these bacteria also reveals the presence of phages, which in large-scale industrial processes could be useful in improving its general bioremediation capacity and could also prove to be a viable option in transferring the ability to degrade azo dyes to other *Enterococcus* through genetic engineering from hybrid strains [18].

The ability of *Enterococcus faecalis* to metabolize azo dyes is associated with the presence of the azoA gene. This encodes the production of the aerobic azoreductase enzyme, which is not secreted outside the cell, has a wide substrate specificity, requires flavin mononucleotide (FMN) as a cofactor and uses NADH as an electron donor [19].

The ATCC 6569 *Enterococcus faecium* strain possesses the enzyme azoreductase (AzoEf1) which shares 67% identity with the azoreductase of *Enterococcus faecalis* (AzoA). However, there are differences related to coenzyme preference, residues associated with FMN binding, substrate specificity, and specific activity. The AzoEf1 sequence is found in GenBank: GQ479040.1 [20].

Enterococcus casseliflavus, by the action of an enzyme which acts similarly to that of azoreductase, is not only able to discolor a wide range of azo dyes under micro-aerophilic conditions, but also to catabolize by desulfonation and deamination the intermediaries generated as a consequence of the reductive cleavage. The genome of this microorganism also reveals the presence of regulatory systems possibly involved in the biodegradation of aromatic contaminants [21].

Enterococcus gallinarum offers an effective ecological alternative for the remediation of environments contaminated with structurally complex and recalcitrant azo dyes such as Reactive Red 35. This is done through enzymatic mechanisms that involve the presence of oxidoreductases, such as laccases, tyrosinases and azoreductases, under a wide range of pH, temperature and with high concentration of salinity. Therefore, its use on a large scale is recommended by using a suitable microaerophilic-aerobic sequential bioreactor [22].

The binding affinity of *Enterococcus* sp. to heavy metals has been attributed to the capsular polysaccharide, which contains different monomers such as glucose, galactose, mannose and fructose, and is capable of participating in the redox reaction of remediation processes of waters contaminated with heavy metals and dyes [23]. Recently, these monomers have been used for the synthesis of silver nanoparticles (AgNP) that, combined with advanced oxidation processes (AOP), have shown good results in the degradation of azo dyes such as methyl orange and Congo red [24].

In relation to metal removal, *Enterococcus faecalis* uses mechanisms such as copper transporting ATPases, present in the inner membrane, which not only work for the homeostasis of this metal but also to resist high concentrations of nickel, mercury, cadmium, lead and copper [25].

2. Methodology

Taking as reference the results of the Environmental Quality Program of Tourist Beaches [26], samples of water and sand were taken at Bocagrande beach in Cartagena Colombia, taking as reference points the areas with the highest concentration of users and suspected of contamination from a source point of marine water dumping.

To search for *Enterococcus* sp., fifty-four (54) samples were taken: 24 of water and 30 of sand, at Bocagrande beach, in Cartagena, Colombia. The standardized protocols of the Environmental Quality Program of Tourist Beaches in the Colombian North Caribbean were taken as reference [26]. The samples were transported at 4°C to the Environmental Microbiology laboratory of the University of Cartagena-Colombia, to be processed in a period of 8 to 24 hours.

The samples were processed through the membrane filtration method. In the case of the sand samples, 10 g of these were diluted in 90 mL of deionized water, the supernatant being considered as a filterable material. Filters were transferred to Slanetz & Bartley agar and incubated for 48 hours at 35 ± 0.5° C [27]. After the incubation period, the colony count was performed and the results were reported in CFU/100 ml of sample. To confirm the identification of *Enterococcus* sp., colonies were placed on blood agar supplemented with 5% defibrinated lamb blood and stored in BHI broth supplemented with 20% glycerol at −80°C.

Biochemical tests were carried out for the confirmation of the genus *Enterococcus* sp. such as: catalase, hydrolysis of esculin bile, reduction of potassium tellurite, vogues proskauer and growth tolerance in the presence of 6.5% NaCl at 9.6 pH. Gelatinase production was evaluated using Columbia agar plates containing gelatin (30 gr/l) incubated at 37°C for 48 hours. A clear area around the colonies was considered as a positive result [28]. The strains that were used as controls for all tests were: *E. fæcalis* ATCC 29212, *K. pneumoniae* 700603, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923.

For the identification of microorganisms by MALDI-TOF Mass Spectrometry, it was necessary to extract ribosomal proteins, using the formic acid extraction method. The analysis of the mass spectra was performed using a Microflex LT mass spectrometer, using the MALDI Biotyper 3.4 software package from Bruker Daltonik [29]. The interpretation of the results was based on accepting scores between 2.0 and 1.7 for the identification of genus and species. Scores below 1.7 were considered unreliable.

Advanced proteomic computational techniques were used; Their advantages lie not only in the speed and economic cost of the process, but also in the effectiveness for the structural and functional analysis of the azoreductase enzymes present in *Enterococcus faecalis*; their properties can be used to potentiate its industrial applications [30].

3. Results and discussion

Of the 54 samples analyzed by the membrane filtration method, in 36 samples (64.86%) colony growth was obtained on Slanetz and Bartley agar, presumptive of the genus *Enterococcus* [27]. Greater data dispersion was observed in the results of the samples of sand, with values ranging from 0 CFU / gr to 200 CFU /gr. The results in the water samples ranged from 6 CFU / 100 mL to 47.3 CFU/100 mL, as shown in **Figure 1**. The higher percentage of positive samples in sand could be explained by the relationship between the ability of *Enterococcus* sp. to form biofilm and to persist in hostile environments such as beach sand [31].

This is due to the ability of microorganisms to adhere to particulate materials, from which they obtain protection against predation and adverse environmental conditions such as: solar radiation, pH, temperature or bioavailability of nutrients. At the same time, this provides them with a food source that allows them to survive for longer periods, favoring their multiplication [32].

Confirmatory biochemical tests were performed to the 36 samples in which growth of presumptive colonies of the genus *Enterococcus* sp. was obtained. These included hydrolysis of esculin, absence of gelatinase activity, growth in 6.5% NaCl and catalase. 100% of the strains that were identified as presumptive for *Enterococcus* sp. due to their growth on Slanetz & Bartley agar showed the ability to hydrolyze esculin in the presence of bile salts. According to literature, this positive result confirms the presence of *Enterococcus* sp. [33].

Another feature that characterizes *Enterococcus* sp. is its ability to grow at a concentration of 6.5% NaCl, and its inability to break down hydrogen peroxide into water and oxygen through the enzyme catalase. However, the results obtained in these tests do not coincide with this fact about *Enterococcus* sp. reported in literature. Rather, they suggest the presence of bacteria of the genus Streptococcus sp. due to the resistance of some strains to growth in high concentrations of NaCl, or bacteria of the genus *Staphylococcus* sp. in the case of those strains whose catalase results were positive [34].

Regarding the tests for the determination of species, the enzymatic activity of gelatinase was not expressed in any of the 36 strains identified as presumptive for *Enterococcus* sp. due to growth in Slanetz & Bartley agar; This indicates loss of the gelE phenotype, which according to literature is produced in *E. faecalis* isolates [35].

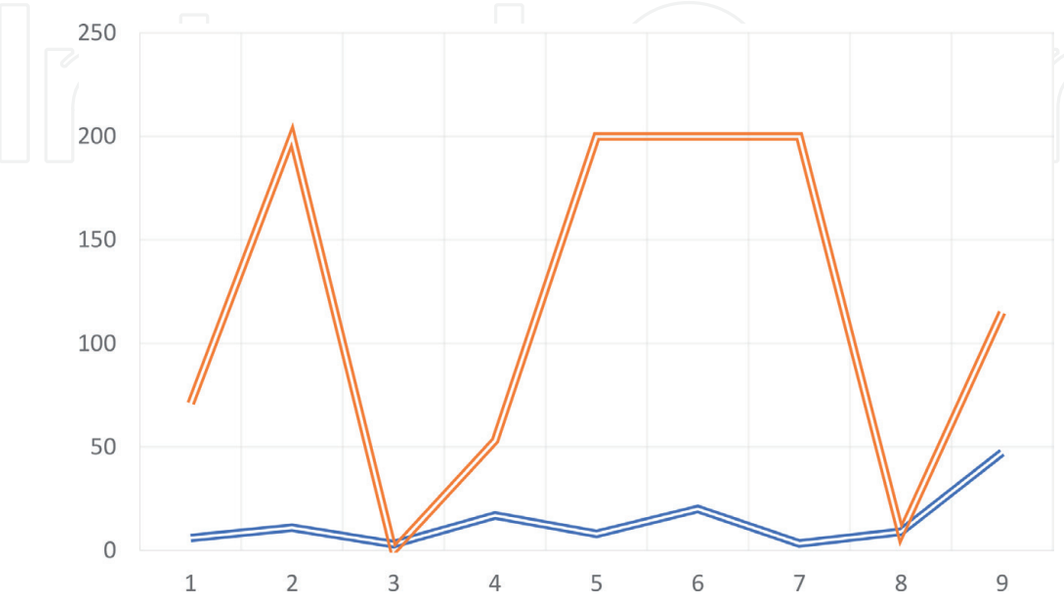

Figure 1.
Enterococci spp. levels in water and sand. Blue line: Enterococcus *in water, Orange line:* Enterococcus *in sand, Y axis: colony forming units, X axis: Weeks of monitoring.*

On the other hand, the ability to reduce potassium tellurite is one of the tests that allows differentiation of *Enterococcus faecalis* from other *Enterococcus* species. Seven (7) strains isolated from water samples and 24 strains isolated from sand samples were positive for tellurite reduction. Negative tellurite strains may suggest the presence of *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus gallinarum*, or *Enterococcus casseliflavus* [36].

Taking into account the discrepancy in the results obtained, high-precision confirmatory tests were performed using matrix-assisted laser ionization mass spectrometry or MALDI TOF. Unique mass peaks are considered specific biomarkers for each genus and species. In species discrimination, MALDI-TOF MS allowed the identification of 12 strains belonging to three different species of *Enterococcus* sp. as follows: *E. faecalis* (8/32), *E. faecium* (3/32), *E. hirae* (1/32). The spectrogram of the identified *Enterococcus* is shown in **Figure 2**.

The in-silico analysis showed a low amount of cysteine residues and a high amount of aliphatic amino acids in the primary structure, which indicated that Azoreductase (2HPV) is some intracellular proteins. The hydrophobicity condition of cysteine suggests that the enzyme is nonpolar and hydrophilic in nature. The presence of a high percentage of α helices indicates that Azoreductase (2HPV) is considered thermostable, as shown in **Figure 3**.

More than 90% of the amino acids were located in the allowed region of the Ramachandran graph, which indicates their stability in nature. The results obtained by SAVES showed that the enzyme have stable crystallography and the SWISS-MODEL QMEAN, ANOLEA and ERRAT analyzes confirmed their good quality. The structural analysis established that Azoreductase (2HPV) have better thermal stability and a superior quality model than other enzymes degrading dyes such as peroxidases and laccases [37], as shown in **Table 1**.

The STRING analysis (protein –protein) identified that the proteins that interact with the azoreductase studied had an unknown 3D structure. However, the

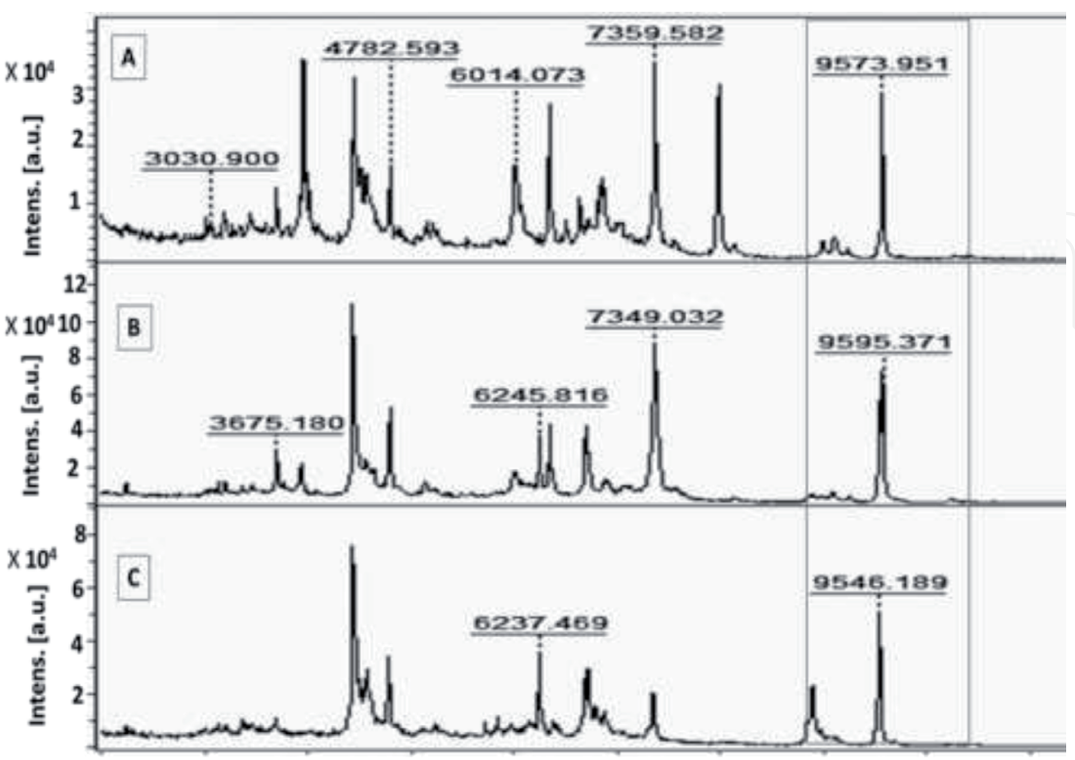

Figure 2.
Mass spectrogram of the three Enterococcus *species identified by MALDI-TOF MS analysis.* Enterococcus hirae *(A),* Enterococcus faecium *(B) and* Enterococcus fecalis *(C).*

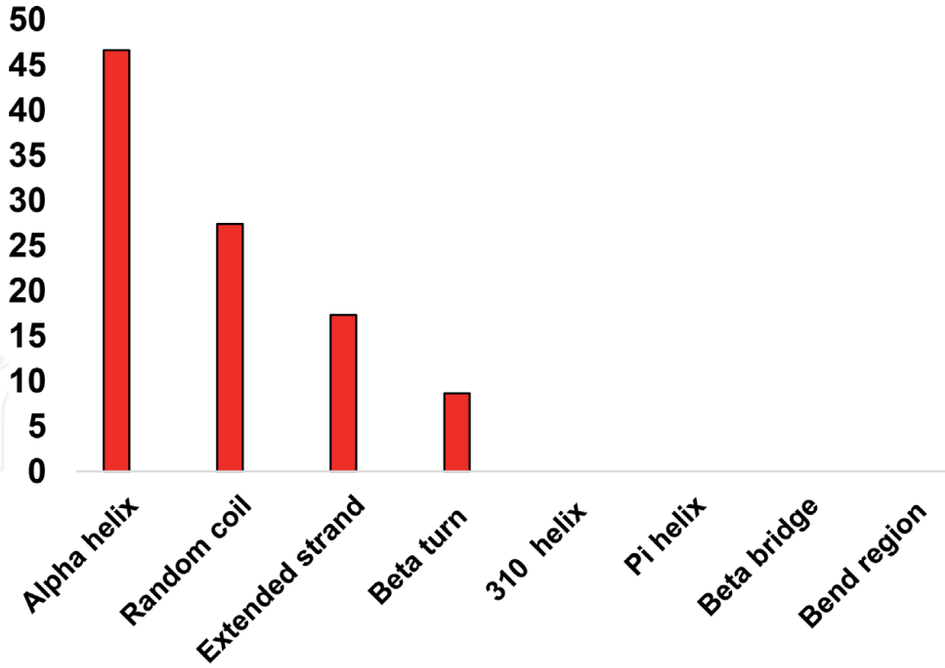

Figure 3.
Percentage of helices in Azoreductase.

Enzymes	3D-1D score (%)	ERRAT quality factor	QMEAN Z-score	AA in FR of Ramamchandran plot (%)
Azoreductase (2HPV)	99.88	95.40	1.32	97.8

Table 1.
Evaluation of structural quality of the enzyme Azoreductase (2HPV).

formation of interconnection networks was evidenced, possibly due to the interaction with bacteria that is genetically similar, which is especially favorable in dye degradation processes using bacterial consortia. At an industrial level, this improves the discoloration rate of effluents contaminated with dyes, as it has greater resistance to abiotic conditions and lower rates of enzyme inactivation, especially in large-scale operations [38].

4. Conclusions

The Bocagrande beach in Cartagena, Colombia is one of the most visited Colombian destinations by locals, as well as national and international tourists. Its high number of users throughout the year, the dumping of domestic waste generated by tourist activity, as well as other drainage carried by rain, are all considered triggers of pollution in this ecosystem.

Matrix-assisted laser ionization mass spectrometry or MALDI TOF identified other species apart from *Enterococcus faecalis*, such as *E. faecium* and *E. hirae*. The presence of these microorganisms in tourist beaches generate health related concerns about the presence of fecal contamination, sewage drains from homes or hotels along the beach, or possible overflow of wastewater treatment plants [39].

According to the World Health Organization guideline values for recreational marine waters at risk of transmitting gastrointestinal diseases (EGI) and acute febrile respiratory disease (ERFA), the results of this study indicate that Bocagrande's beaches are in category A; This means that the concentration of

Enterococcus faecalis is less than or equal to 40 CFU/100 mL and that the estimated risk for exposure is <1% for EGI and < 0.3% for ERFA.

The current biotechnological challenges lead to the development of solutions that guarantee the quality of our ecosystems and the health of human beings exposed to environmental imbalance. In relation to the problems associated with the use of dyes in different industrial processes, there have been many technological strategies developed to reduce the polluting load in industrial effluents and in receiving water bodies.

Dye removal strategies have evolved over the years. This happened due to the development of new physical and chemical methods, which progressed towards the use of environmentally friendly and cost effective biological solutions for the industry. These biological solutions have used plants, algae and other microbial biomasses as an alternative for dye removal. However, bacteria are the most robust microorganisms that, due to their structure and genome, become potential degraders of recalcitrant contaminants such as azo dyes.

The competitive advantages of bacteria are, among others, their short life cycle, their ability to adapt, and their metabolic action; they are able to degrade and detoxify the secondary metabolites produced in the discoloration process. These properties prevail in bacterial communities present in marine ecosystems, considering that these are capable of removing, in monoculture or in consortium, individual colorants, mixtures of colorants and the metal-colorant complex. Their use, although underexploited, becomes relevant with the advent of emerging technologies connected with nanotechnology, alternative energy, circular economy and environmental sustainability.

The mechanisms involved in the simultaneous removal of dyes and the metal-dye complex, the enzyme profile and the intermediate metabolites should be the subject of future studies based on genomics and proteomics. Likewise, due to the legal and environmental limitations when monitoring industrial discharges and the distribution of azo dyes in the environment, it is necessary for the scientific community to provide innovative mechanisms in which monitoring discharges and bodies of water receptors are based on amine detection.

The results of this study suggest that the enzymes Azoreductase (2HPV) are potential degraders of azo dyes due to their stability, good quality of crystallographic structure, as they are intracellular, hydrophilic and thermostable. The high content of α helices indicates their thermal resistance, which, associated with their structural quality, makes them potential degraders of azo dyes.

The properties of Azoreductase (2HPV), whose origin is *Enterococcus faecalis*, confirm that the bacterial communities present in marine ecosystems have developed special mechanisms that allow them to resist adverse environmental conditions such as hypersalinity, pH variations and the presence of heavy metals. This makes them more stable and able to degrade recalcitrant contaminants such as azo dyes [40].

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Conflict of interest

The authors declare no conflict of interest.

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Author details

Ganiveth María Manjarrez Paba* and Rosa Baldiris Ávila
Clinical and Environmental Microbiology Group, Faculty of Natural and Exact Sciences, University of Cartagena, Cartagena, Colombia

*Address all correspondence to: gmanjarrezp@unicartagena.edu.co

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