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Microbial community evolution in the biofilm attached to sponge carriers in pulp mill effluent treatment

Evolución de comunidad microbiana desarrollada en biofilm en biosoportes durante tratamiento de efluentes de celulosa

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KEYWORDS
Bioreactors; moving bed biofilm reactor; industrial effluent; wastewater treatment; gene sequencing
Biorreactores; reactor de biofilm de lecho; efluente industrial; tratamiento de aguas residuales; secuenciación genética.

ABSTRACT: This study investigates the evolution of the biofilm matrix responsible for treating effluent from a pulp mill and identifies the microbial community by 16S rRNA gene sequencing. In addition, a biocarrier promising a functional structure with better specific features for biofilm formation than traditional polyurethane carriers was explored. The average removal efficiencies were 43.7% for chemical oxygen demand (COD) and 62.7% for biochemical oxygen demand (BOD_5). The color increased during the treatment, indicating anoxic zones being formed in the inner part of this type of carrier. Periodic micrographs showed the evolution of extracellular polymeric substances and materials like fungi and bacteria adhered to the carriers. Genetic sequencing confirmed the presence of *Bacillus* sp. and *Paenibacillus glucanolyticus,* species with the potential to degrade and discolor pulp industrial effluents. Results offer a potential basis to enhance treatment facilities of pulp and paper mills based on microbial activities.

RESUMEN: En este estudio se investigó la evolución de la matriz de biopelícula responsable del tratamiento de efluentes de una planta de celulosa y se identificó la comunidad microbiana mediante la secuenciación del gen 16S rRNA. Además, se exploró un biosoporte que prometía una estructura funcional con mejores características específicas para la formación de biofilm que los medios convencionales de poliuretano. Las eficiencias de remoción promedio fueron 43,7% para la demanda

química de oxígeno (DQO) y 62,7% para la demanda bioquímica de oxígeno (DBO_5). El color aumentó durante el tratamiento, lo que indica que se están formando zonas anóxicas en la parte interna de este tipo de soporte. Las micrografías periódicas mostraron la evolución de sustancias poliméricas extracelulares y materiales como hongos y bacterias adheridos a los portadores. La secuenciación genética confirmó la presencia de *Bacillus* sp. y *Paenibacillus glucanolyticus*, especies con potencial para degradar y decolorar los efluentes industriales de pulpa. Los resultados ofrecen una base potencial para mejorar las instalaciones de tratamiento de las fábricas de celulosa y papel en función de las actividades microbianas.

1. Introduction

The wastewater generated by pulp industries has a significant impact on the environment. The sector is responsible for consuming large volumes of freshwater and, discharging it as wastewater with a high potential to adversely affect the environment in case of incomplete treatment [1]. In general, this type of wastewater contains high chemical oxygen demand (COD), biochemical oxygen demand (BOD), chlorine compounds, and about 700 other organic and inorganic compounds [2,3]. Such effluents were identified as potential sources of endocrine disruption in aquatic ecosystems, affecting the growth and reproduction of exposed organisms [4,5].

Biological methods generally have economic and environmental advantages over most physical and chemical treatments [1,5]. A variety of treatments are under constant development to treat pulp mill wastewater. Activated sludge (AS) systems replaced the aerated lagoons due to the less area and lower hydraulic retention time (HRT) required. However, the AS also has its efficiency limited by the settling rates reached by the particles. Furthermore, sludge production involves additional costs for treatment and final disposal [6]. In order to solve these problems and optimize the treatments, the moving bed biofilm reactor (MBBR) was developed.

In recent years, the MBBR system has gained popularity as a stable wastewater treatment, providing a compact and highly efficient treatment to remove COD and BOD. Although it is reported as a simple and flexible technology, it produces less sludge than other treatments [6,7]. The basic principle of MBBR is to allow the biomass to grow attached to small carriers that are moving around in the reactor due to aeration flow [8]. Therefore, knowledge about biofilm development is essential for the efficient operation of the system.

Biofilm is a three-dimensional microbial community structure embedded within a self-produced matrix of extracellular polymeric substances (EPS). This architecture provides mechanical stability and excellent resistance to the microbial community [9]. The possibility that microorganisms grow immobilized on a carrier allows them to stay close and favor intense interactions such as cell-cell communication and synergistic microconsortia [10].

Carriers with different surface characteristics have been widely investigated. Most carriers are plastics made of high-density polyethylene, polypropylene, or polyethylene and have a specific surface between 200-1,200 $m^2\ m^{-3}$ [6]. More recently, foams in the shape of sponges have been studied. They have

attracted more interest due to their high porosity and the capacity to allow a rapid and stable attachment to microorganisms' growth on the surface [7,11].

The standard polyurethane foam has a "rib structure" with high porosity but little wall area for biofilm adhesion. Facing this, a new technique developed by Nisshinbo Chemical Inc. produced an innovative sponge carrier with a "wall-structure" called Aquaporousgel (APG) [7]. The APG has a specific surface area greater than 3,000 $m^2\ m^{-3}$ and is made of polyurethane-glycol [12].

Given the potential benefits of this new sponge carrier, this study aims to investigate its performance in supporting the evolution of a biofilm matrix. A laboratory-scale of an MBBR system using this sponge carrier is applied to treat pulp wastewater from a Brazilian industry.

The system was continuously operated during 240 days. The work was divided into four successive phases (I-IV), following the four collections of effluent samples carried out in the industry. The performance of the treatment was measured by the determination of the removal of soluble organic matter (COD and BOD), color, and total phenolic compounds (TPC). The biofilm formation in each phase was analyzed by scanning electron microscopy (SEM). Moreover, the microbial community present in the biofilm matrix at the end of treatment was isolated, and DNA strands were sequenced.

2. Methodology

Wastewater samples were obtained from a Brazilian pulp industry. Four sample collections were carried out in the industry throughout the research period. The referred industry employs two different pulping processes, kraft and chemi-thermomechanical pulping (CTMP), including bleaching steps. Therefore, the raw wastewater presents high-quality variation.

The raw wastewater was supplemented with nutrients in the proportion C:N:P of 100:5:1 using NH_4Cl as nitrogen and K_2PO_4 as phosphorus sources. Furthermore, the pH was adjusted to 7.0 to optimize biomass growth. The characteristics of the pulp wastewater used in each study phase are presented in Table 1.

Table 1 Pulp effluent characteristics

Parameter	Phase			
	I	II	III	IV
COD (mg L^{-1})	788.1	821.9	3287.2	2668.8
BOD (mg L^{-1})	213.9	288,8	345,2	301.2
BOD/COD	0.27	0.35	0.11	0.11
TPC (mg L^{-1})	256.4	307.9	583.1	263.3
Color (Abs.)	0.27	0.39	0.42	0.43

In phases I and II, the potential of biodegradability, indicated by the BOD/COD ratio, was 0.30 and 0.39, respectively. In phases III and IV, the effluent presented less biodegradability, and the ratio decreased to 0.10 and 0.12, respectively. This high variability is due to the industry's different pulping processes. It is known that these values suggest that the effluent has high recalcitrance and should not be sent to

biological treatments. However, the hazardous wastewater was chosen to be used in the study because it is a real scenario within the pulp industry and to ascertain the stability of this kind of biological system.

2.1. Experimental setup of the MBBR system and sponge carrier specifications

An MBBR was built on a laboratory scale with a utile volume of 1 L (Figure 1). The sponge carrier Aquaporousgel (APG) [13] was used to support the growth of adhered biomass. The carrier is manufactured using a technique that gives it a wall structure, favoring the adhesion of microorganisms, and providing high hydrophilicity. In addition, the carrier has high hydrophilicity, swelling, and sinking in water rapidly, allowing immediate flow with the aeration of the system [7].

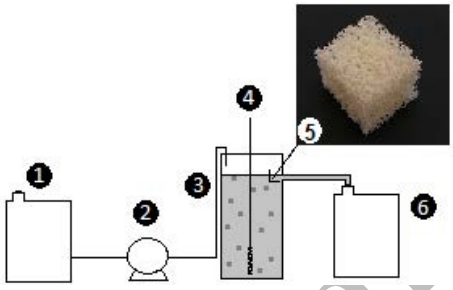

Figure 1 MBBR system with (1) feed tank (influent), (2) peristaltic pump, (3) reactor with (4) oxygen supply (mixing and aeration), (5) APG carriers, and (6) treated effluent

The samples of APG used in this study are cubic with dimensions of 1.8 cm. The biocarrier filling ratio was 10%, as recommended by the manufacturer. This amount is less than commonly used by other authors (70%) [14]. The MBBR was continuously operated for 240 days. The system was fed by a peristaltic pump (Milan® BP600/1) to keep the organic loading rate (OLR). The dissolved oxygen was maintained at around 8,0±1,0 mg L^{-1} with a diffused aeration system (Master® Junior Full). Table 2 shows the operational conditions.

Table 2 MBBR system operational conditions

Phase	Operational conditions		
	Period (d)	OLR (kg_{COD} m^{-3} d^{-1})	HRT (d)
I	1-60	0.6	1.2
II	61-120	0.6	1.1
III	121-180	2.1	2.7
IV	180-240	2.1	2.2

2.2. Analytical methods

The treatment removal efficiencies were determined through soluble COD, BOD, TPC, and color. The analysis of organic matter was conducted according to the Standard Methods for the Examination of Water and Wastewater [15]. TPC was determined by UV with absorbance at 215 nm and converted into

concentration using an analytical curve with a phenol solution as standard [16]. The color was measured at pH 9.0 by VIS absorbance at 440 nm. All analyses were performed on filtered samples (membrane with 0.45 µm pore size). DO concentration was measured every workday with a DO meter.

2.2.1 Scanning electron microscopy (SEM)

The samples of the biocarrier were completely freeze-dried for the SEM photomicrographs. The analyses were performed in a Zeiss microscope model EVO MA 15 located at the Multi-User Center for Materials Characterization-CMCM of UTFPR-CT.
Magnifications from x50 to x20000 were applied. Scanning was done to the APG samples before starting treatment (day 0) and after each operation phase (day 0 to day 240).

2.2.2 Microbial community analysis

At the end of phase IV, samples of the sponge carrier APG were collected from the reactor and sectioned to isolate the cultures at the Microbiology Laboratory of the Federal University of Paraná-UFPR. The colonies were isolated in Petri dishes on solid culture media composed of nutrient agar and Luria-Bertani (LB) medium [17]. The dishes remained incubated for 24 hours at 37 °C in the absence of luminosities and CO_2. Then, the isolated bacteria were classified as Gram-positive and Gram-negative [18].
The method to extract the 16S rDNA strand is based on three stages [19]: membrane lysis, contaminants cleaning, i.e., proteins and other macromolecules, and DNA precipitation. Polymerase chain reaction (PCR) was conducted by examining the products in gel electrophoresis in 1% agarose at 108 V for 1 h, and the bands were evaluated using the PhotoDoc-It^{TM} Imaging System [19,20].
The DNA sequences were investigated in the Laboratory of Biochemistry of the Department of Biological Sciences-UFPR. Fragments of DNA that correspondeds to the 16S DNA strand were purified and the sequences were analyzed to conform to the National Center for Biotechnology Information and obtain statistical similarity with NCBI-BLAST [19,21]. Phylogenetic trees were generated based on nitrogen base alignments made through the bootstrap method using the MEGA software, providing reliable results according to the genetic evolution of species.

3. Results and discussions

3.1. Performance of the biological treatment

Figure 2 illustrates the performance of the MBBR. During phases I and II (OLR 0.6 kg_{COD} m^{-3} d^{-1}), the average COD and BOD removal rates were 45.4% and 62.6%, respectively. In phases III and IV (OLR 1.2 kg_{COD} m^{-3} d^{-1}), the removal efficiencies for COD and BOD were 42.3% and 62.7%, respectively. Review studies about MBBR treating pulp mill effluent using traditional carriers report removals between 21.5-81% for COD and 33.5-96% for BOD [6,22]. For instance, when pulp effluent with a BOD/COD ratio of 0.35, which is more favorable for biological treatment than the approximately 0.11 ratios observed in the effluent used in phases III and IV, was used, a 20% COD removal was achieved [23]. Moreover, it is important to highlight that the influent applied in the present study was less biodegradable than the effluent used by other authors, indicating the stability and robustness of the MBBR.

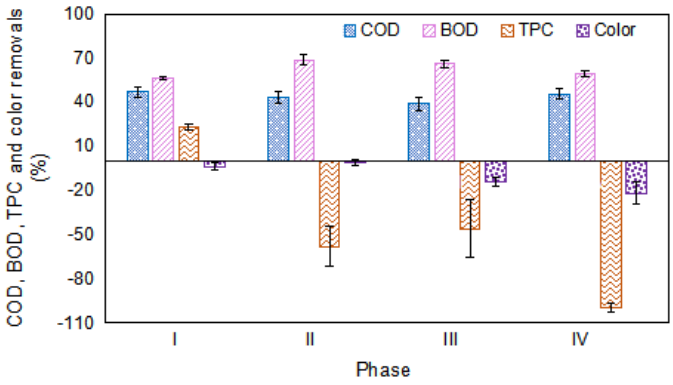

Figure 2 Removal efficiencies of the biological system

Regarding color, it was observed to have an overall increase instead of its removal. In phase IV, the maximum increase of color reached 33% in the effluent after being treated, which could be caused by the arrangement of the APG carriers.

The color increment was also observed in studies of aerated lagoons used in pulp wastewater treatments [24–26]. The authors report that it probably occurs due to the conversion of high-molecular-weight organic matter to smaller chromophores. This phenomenon is assigned to the facultative microorganisms living in the anoxic zones formed in the sludge sedimentation regions. The MBBR system in the present study was maintained under intense aeration. However, the structure of the sponge carrier may have made the diffusion of oxygen difficult, enabling the formation of anoxic zones inside the sponges.

The average of TPC removal in phase I was about 23%. After this initial phase, the removal of total phenolic compounds declined until it started to increase in the treated effluent. In phase IV, the average increase of TPC was close to 100%. Increments of this parameter in biological treatments of pulp wastewater are known and are assigned to the biotransformation of lignin derivates into compounds with phenol groups when under aeration [19,23,27,28].

3.2. Biofilm formation in the biocarrier

Figure 3 presents the evolution of the biofilm matrix through 240 days of biological treatment.

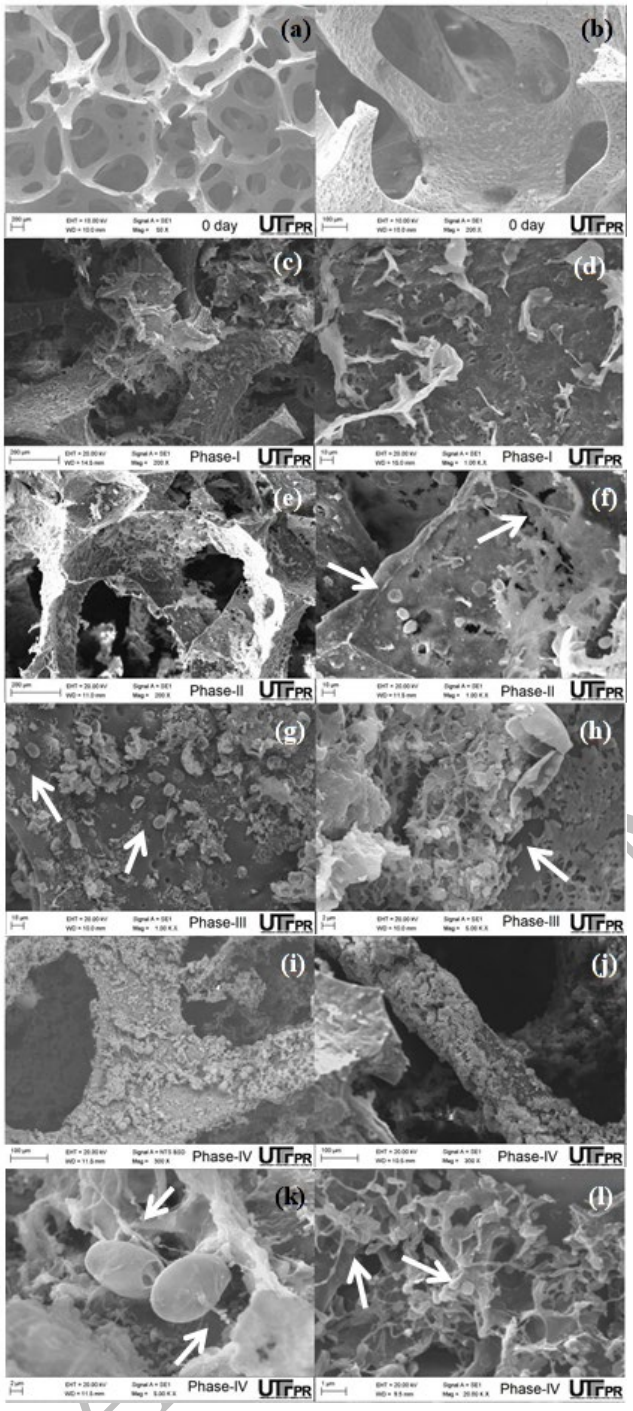

Figure 3 Scanning electron microscopy for magnifications from x50 to x20000 of sponge carriers APG on (a-b) 0 day and at the end of (c-d) phase-I, (e-f) phase-II, (g-h) phase-III, and (i-l) phase-IV

Figure 3(a-b) shows the sponge carrier APG before being used in the treatment (day 0). The APG presents a three-dimensional structure with high porosity. It is possible to observe the interconnected pores and a

better structure than regular polyurethane foams, as observed in micrographs available in the literature [7,29].

Figure 3(c-d) shows a sample of sponge carriers employed during phase I (days 1-60), and it is already possible to observe levels of coverage on the carrier surface. This rapid absorption capacity of the sponge may be evidence of the initial removals and subsequent release of the chemical compounds in the effluent (e.g., the initial removal of TPC and its increase in the following phases).

Figure 3(e-f) shows the SEM micrographs from the APG at the end of phase II (day 120). It is possible to see an increase in biofilm coverage. Additionally, the arising of spherical materials and filamentous microorganisms is observed.

At the end of phase III, Figure 3(g-h), there was an increase in these spherical materials. In Figure 3(h), with a magnification of x5000, it is possible to note the matrix of EPS, consistent with findings in the literature [30]. In Figure 3(k) (x5000 magnification), it is possible to observe in detail a microorganism involved within extracellular substances, as reported in previous studies [31]. Figure 3(l) (mag. of x20000) confirms the presence of smaller microorganisms.

Comparing the micrographs of the beginning and the end of the treatment period, the biofilm structure's evolution is evident, and it is possible to note that it presents a diversity of microorganisms on an irregular surface. The microstructure of the biocarrier APG presented an excellent place for microorganism immobilization and survival. Also, it can be verified that the pores remain open, allowing the diffusion of organic matters and suggesting long-term use of the carriers.

3.2.1 Identification of microorganisms

Preliminary results of the microorganism's characterization confirmed the presence of fungi, larger materials, and bacteria, smaller materials, revealed in the micrographs (subtitle 3.2). Fungus *Trichoderma* was the most abundant microorganism in the sponge carrier, especially the specimens *T. reesei* and *T. atroviride*. In literature [32] is reported that *T. reesei* has properties that could be used to reduce the use of bleaching chemicals for the pretreatment and hemicellulose breakdown processes. Specimens of the fungus *Aspergillus fumigatus* and *Aspergillus ibericus* were also found.

It was possible to isolate and conduct the gene sequencing of three bacteria samples from the microbial community living in the biofilm. Two of them were identified as *Bacillus* sp. (Figure 4 and Figure 5). The genus *Bacillus* sp. are facultative anaerobic bacteria. This attribute may be aligned with the hypothesis of an anoxic zone formation within the biocarrier and the color increase in the effluent treated previously discussed.

It was not possible to define exactly the *Bacillus* species because even though their phenotypes are different, their intra and inter-phylogenetic relationships are not clear to identify by 16S rRNA gene sequence [33]. Nevertheless, different studies evidence the relevance of *Bacillus* sp. Concerning pulp and paper effluent. Previous studies [34] isolated different strains from pulp and paper effluent and explored a novel species named *Bacillus* sp. IITRDVM-5. The authors varied the bacterial inoculum between 2 and 8% v/v during a sequential batch biological treatment and achieved a reduction of 89.5% COD and 93.3% BOD under 72 h of treatment. In addition, the degradation of 73.0% of color, 88.5% of

total phenol, and 64.1% of lignin was observed. Subsequently, *Bacillus* sp. IITRDVM-5 was studied in the treatment of pulp and paper effluent by activated sludge with paper mill sludge powder and sewage sludge powder instead of using urea-diammonium phosphate as an environmentally safe solution source of nutrients [35]. The treatment showed satisfactory reductions in COD, BOD, lignin, and total phenol, demonstrating the potential of using *Bacillus* sp. in approaches that are more sustainable for treating industrial effluents.

Another study [36] demonstrated the potential of *Bacillus* sp. NG-27 for pulp biobleaching. The authors explained that these strains are known for producing thermoalkali-stable xylase, which can be used as an alternative to reduce chemicals in the paper industry. An isolated strain of *Bacillus* sp. PS-6 is presented in the literature for its application in the phytoremediation process, demonstrating satisfactory accumulation of eight heavy metals from pulp and paper industry wastewater [37]. Furthermore, other researchers have studied the role of *Bacillus* sp. in the treatment of effluents from pulp and paper mills. For example, *B. cereus* [19,38], *B. subtilis* [39,40], *B. toyonensis* [41], and *B. aryabhatti* [42].

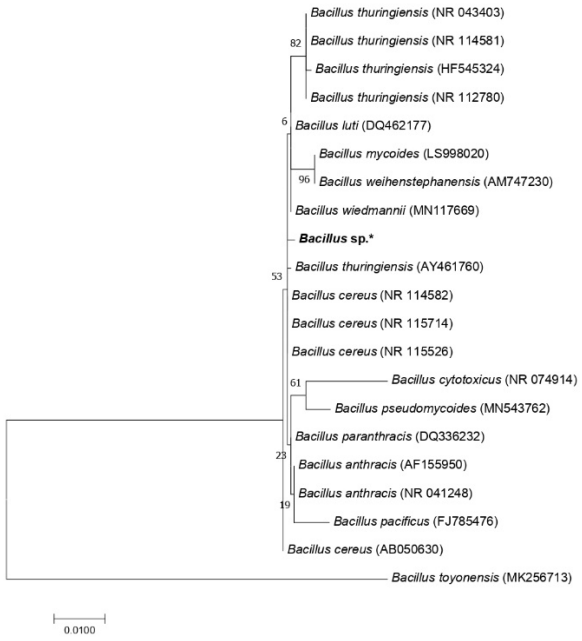

Figure 4 Phylogenetic tree of *Bacillus* sp. obtained using the union of neighbors with *Bacillus toyonensis* as an outer group.

Figure 5 Phylogenetic tree of *Bacillus* sp. obtained using the union of neighbors with *Bacillus cereus* as an outer group.

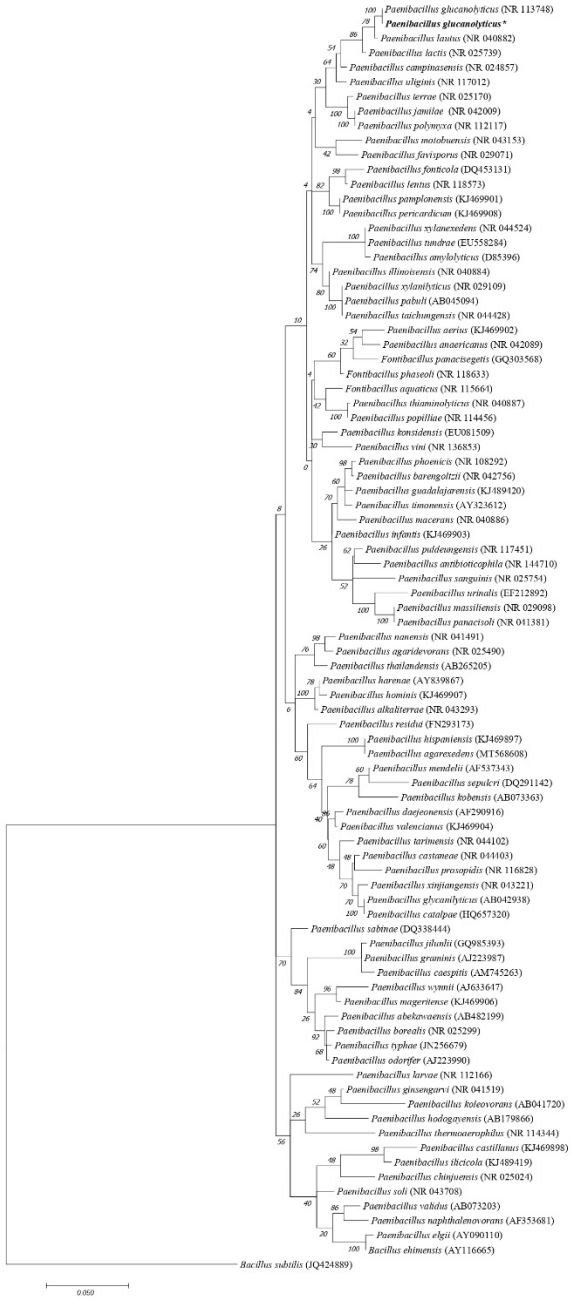

Figure 6 Phylogenetic tree of *Paenibacillus glucanolyticus* obtained using the union of neighbors with *Bacillus subtilis* as an outer group

In the present study, *Paenibacillus glucanolyticus* was identified within the biofilm matrix using 16S rRNA gene sequencing (Figure 6). A study identified Paenibacillus sp. as a species with potential for color treatment when examining the removal of specific compounds from kraft pulp effluent using

isolated bacteria [19]. By adding strains of this species to an aerated lagoon, the authors achieved removal rates of 69% for color, 37% for TPC, 53% for lignin compounds, 50% for aromatic compounds, and 49% for lignosulfonic compounds. Another study [43] also reported satisfactory results, with 68% color removal and 86% total phenol removal, by isolating *Paenibacillus* sp. from a batch reactor treating pulp effluent. Additionally, *P. glucanolyticus* isolated from black liquor has been studied for its potential to metabolize cellulosic components [44,45]. Its genome sequence was drafted to elucidate the metabolic pathways by which these bacteria break down lignocellulosic components [46]. These findings support the pulp and paper industry's efforts to become more sustainable by improving the efficiency of effluent treatment facilities and reducing harmful environmental impacts.

4. Conclusions

Despite the COD influent presenting significant differences between the phases and OLR being increased from 0.6 to 1.2 $kg_{COD}\ m^{-3}\ d^{-1}$, the MBBR with the sponge carrier APG showed satisfactory organic matter removal efficiencies. Over the 240 days of treatment, the averages were 43.8% for COD and 62.7% for BOD removals, even when treating wastewater not readily biodegradable (BOD/COD<0.10). These results evidence the stability of the MBBR.

SEM micrographs showed the APG functional structure, which was superior to traditional sponge carriers. It was possible to verify that the pores remained open, suggesting a prolonged lifetime use of the biocarrier. Furthermore, the low filling ratio applied (10%) indicates another economic advantage.

The micrographs also allowed us to evaluate the evolution of the biofilm matrix, formed by microorganisms and extracellular polymeric substances. By 16S rRNA gene sequencing, *Bacillus* sp. And *Paenibacillus glucanolyticus* were identified. The strains identified have important roles in the degradation of pulp mill effluent. In addition, the presence of facultative anaerobic bacteria and the color increases are aligned with the supposition of the formation of anoxic zones in the inner of the carriers. Although the occurrence of an anoxic zone in the inner of the sponge may be disadvantageous for the conditions applied in this study, this condition can be used as an advantage for the treatment of other types of industrial effluents. However, more studies should be developed on this topic.

5. Declaration of competing interest

We declare that we have no significant competing interests including financial or non-financial, professional, or personal interests interfering with the full and objective presentation of the work described in this manuscript. This article is an excerpt from a Master's thesis in Environmental Science and Technology.

6. Acknowledgments

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8. Author contributions

E.M. designed and performed the experiments and wrote the manuscript. C.P. helped carry out the experiments. J.V.N, M.W.B.S., and I.C.F carried out the genetic analysis. V.A.V. supervised the genetic analysis. C.R.X. conceived and supervised the project.

9. Data availability statement

The data that support the findings presented in this study are available from the corresponding author upon reasonable request.

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