Biological Diversity in Aerated Facultative Lagoon Treating Kraft Cellulose Effluent Through Bioaugmentation

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Abstract

Background: This study analyzes the microbiological diversity in an aerated facultative lagoon system with volumetric organic loading rates of 0.2 and 0.6 $kgCODm^{-3}d^{-1}$ treating effluent from the kraft pulp industry through bioaugmentation. The samples for the study of biological diversity were taken from a laboratory-scale lagoon at steady state treating kraft pulp effluent and operated with 0.2 and 0.6 $kgCODm^{-3}d^{-1}$ for 120 days. This analysis was performed by identifying the 16s DNA sequencing, through DNA extraction, polymerase chain reaction and agarose gel electrophoresis at 1%. Next, the autochthonous bacteria were named through statistical similarity obtained from the National Center for Biotechnology Information database. The lagoon performance was assessed based on the removal efficiency of specifics compounds.

Results: In the biomass samples collected at steady state, 9 and 12 species of bacteria were identified and the species *Bacillus cereus*, *Bacillus thuringiensis* and *Paenibacillus glucanolyticus* found in this matrix presented significant removal of the parameters in the kraft effluent. In the treatment, it was possible to observe that the removal of organic matter above 50% for chemical demand and above 95% for biochemical demand. The specific compounds were not significantly removed, but this is a characteristic of biological treatments.

Conclusions: We found that the three referred species show great promise in the removal of specific parameters in a lagoon biological treatment system using bioaugmentation.

Background

Biological treatments comprise bioremediation processes, which are defined by the American Society for Microbiology as the use of living organisms to reduce or eliminate environmental risks arising from the accumulation of toxic chemicals and other hazardous waste [1]. Within the bioremediation process, the following remediation classifications can be found: natural attenuation, biostimulation and bioaugmentation.

Natural attenuation is a biological process that transforms pollutants into substances that are less harmful to the environment through biodegradation carried out by microorganisms [2].

Biostimulation is the process of improving the conditions of the environment for microorganisms through the addition of nutrients, oxygen, temperature adequacy, pH, aeration or reduction potential. Thefore, there is an improvement in the performance of microorganisms in the treatment process [2].

Bioaugmentation is related to the introduction of allochthonous bacteria, which are not native to the environment, or autochthonous bacteria, which are native to the environment, isolated or in consortium. This process is based on the spontaneous and controlled action of microorganisms to increase their quantity, allowing them to degrade pollutants in the soil, water bodies, and in industrial and domestic

effluents [1]. The microorganisms used can also act in synergy with the local native species, without interfering with natural biogeochemical processes [3].

According to [4], the application of biotechnological processes involving microorganisms in consortium or individually has been on the increase. In this sense, the great motivation of researchers involved in biodegradation studies is the continuous search for versatile microorganisms capable of efficiently degrading many pollutants at a low operating cost [5].

Among the biological systems used in the treatment of effluent from the pulp and paper industry, aerated facultative lagoons (AFL) stand out [6-8]. In Brazil, such systems are widely used due to climatic conditions, their simple maintenance, low cost and greater stability against shock loads than other systems, such as activated sludge [9, 10].

Before 2007, the bioaugmentation technique was not used in Brazil since it depended on the agreement and authorization of government agencies and environmental inspection agencies, such as the *Companhia de Tecnologia de Saneamento Ambiental*[1] (CETESB). However, on June 22, 2007, CETESB, through Board Decision No. 103/2007/C/E, authorized the use of allochthonous microorganisms in bioaugmentation [11].

Thus, bioaugmentation started to be allowed in Brazil, more specifically in the State of São Paulo, following the specific guidelines presented in the referred document. Among the standards to be followed, CETESB's technical standard No. L1.022 stands out for referring to the use of biotechnological products, which consist of microorganisms intended for the treatment of liquid effluents, solid waste and soil and water remediation [11].

Bioaugmentation can also be used with genetically modified organisms to improve the treatment system. However, since these are allochthonous microorganisms, there is a Brazilian legislation that regulates their use: Biosafety Law No. 11.105/05, in effect since March 24, 2005 [12].

The autochthonous bacterial species, commonly present in biological treatments of cellulose effluents, can live in extreme environments of temperature, pH, biochemical oxygen demand (BOD_5), chemical oxygen demand (COD) and low oxygen concentration [13]. Therefore, due to such characteristics, they are of significant importance to the treatment of kraft effluent [14].

The objective of this paper is to analyze the microbiological diversity in an AFL system treating effluent from the kraft pulp industry with volumetric organic loading rates (OLR) of 0.2 and 0.6 $kgCODm^{-3}d^{-1}$ through bioaugmentation.

[1] Environmental Sanitation Technology Company.

Method

Collection, biological treatment and performance analysis of aerated facultative lagoon

Aerated facultative lagoon treatment system

The industrial effluent used for continuous treatment in the AFL system was kindly provided by an unbleached kraft pulp mill based in the metropolitan region of Curitiba, in the state of Paraná, Brazil. The aforementioned mill treats its effluents through biological systems with aerated facultative lagoons and tertiary treatment to reach discharge criteria.

The continuous biological treatment used in this research was carried out in a bench-scale AFL reactor, as shown in the scheme in Figure 1, in transparent acrylic material with 1 L of useful volume and a sedimentation zone, to which the OLRs of 0.2 and 0.6 $kgCODm^{-3}d^{-1}$ were applied for a period of 120 days and separated into the two lagoons. Aeration was promoted by air pumps with a flowrate of 35 L/h and the dissolved oxygen was measured in parallel with the temperature inside the reactor, in the aeration zone and in the sedimentation zone [15].

For the composition of the AFL system biomass, we used sludge from the bottom of the aerated lagoon of the mill that provided the effluent for the study. The biomass was inoculated to a final concentration of 70 $mgVSSL^{-1}$, an intermediate value among those used in biological systems [16].

The efficiency of the treatment system was assessed as a function of the reduction in the following parameters: BOD_5, COD, total organic carbon (TOC), lignin-derived compounds, total phenolic compounds (TPC), aromatic compounds, color and turbidity [17–19], whose analyses were performed at the Multiuser Laboratory of Chemical Analysis of the Federal Technological University of Paraná (LAMAQ-UTFPR).

Microbiological analysis

a) Isolation, cultivation and gram staining

The microbiological analyses to identify the groups of bacteria that were present were carried out at the Microbiology Laboratory of the Federal University of Paraná (UFPR). The isolated and analyzed samples came from the final stabilization period of each OLR in the lagoons.

Thus, to separate the cultivated bacteria, we used the technique of isolation of colonies grown in culture media. The method is based on the seeding of microorganisms on the surface of solid culture media in Petri dishes [20]. The culture media used were nutrient agar and Luria-Bertani medium. After the seeding, the dishes were incubated in an oven at 37°C, where they remained for 24 hours in the absence of light and CO_2.

The isolated bacteria were morphologically characterized through the characteristics of the bacterial colonies, classifying them as Gram-negative and Gram-positive [21]. Gram stain slides were analyzed

under an optical microscope at 400x magnification to classify bacteria into Gram-negative and Gram-positive.

b) DNA extraction

In order to analyze the microbiological diversity, DNA extraction from the bacteria was initially performed by adapting the method employed by [22], which consists of three phases: membrane lysis, cleaning of contaminants (proteins and other macromolecules) and DNA precipitation.

c) Polymerase chain reaction (PCR)

After extracting DNA from the bacteria, we performed a polymerase chain reaction, known as PCR. Initially, the reaction was prepared and later, the amplification of the DNA strands was carried out in a thermocycler, following the technique adapted from [22].

d) Agarose gel electrophoresis at 1%

After PCR amplification, the products were analyzed through the technique of agarose gel electrophoresis at 1%, which had been previously prepared, and the samples were placed inside the electrophoresis tank at 108 V for approximately 1 hour. The bands generated were evaluated through the PhotoDoc-It™ Imaging System.

e) Genetic sequencing

Finally, the DNA fragments corresponding to the 16s DNA strand were purified and sequenced in the Laboratory of Biochemistry of the Department of Biological Sciences – UFPR. Such a sequence was analyzed with the help of the database of the National Center for Biotechnology Information (NCBI) to obtain statistical similarity with NCBI-BLAST. In this respect, the statistical similarity result with a value above 97% reveals a specific species of bacteria [23].

f) Phylogenetic tree

The alignments of the nitrogenous bases, which were previously obtained in the genetic sequencing step, were done through the bootstrap method using the MEGA software in order to provide greater reliability to the result according to the genetic evolution of the species, which were later displayed in a phylogenetic tree. When the number of replicas is 100, the species whose replica is closest to 100 is the one identified in the sample, originating from a common ancestor [24].

Results And Discussion

Performance of the treatment system

Figure 2 presents the organic matter removal data in terms of BOD_5 and COD in the two lagoons.

In Figure 2 (a) and (b), it can be observed that the average BOD_5 removal values were greater than 90%, with a maximum removal of 94% in the two systems, which is in line with the literature that suggests that

aerated lagoon systems can vary between 50-95% in the removal of BOD_5 in effluent from the pulp and paper industry [25, 26].

In relation to COD, it can be observed that at both loading rates, there was removal varying between 40-60% during the 120 days of operation. In general, the removal level was similar to that obtained by [25] using the same volumetric organic loading rate employed in this research for an aerated facultative lagoon.

The TOC removal analysis had an average of 49% for the AFL with an OLR of 0.2 $kgCODm^{-3}d^{-1}$ and 41% removal for the one with an OLR of 0.6 $kgCODm^{-3}d^{-1}$. The results obtained at these loading rates were similar to the result obtained by [8] in an aerated facultative lagoon.

Figure 3 shows the data on the removal of specific compounds, namely: total phenolic compounds, lignin compounds, aromatic compounds, lignosulphonic compounds, in addition to the parameters of color and turbidity.

It is possible to observe in Figure 3 (a) that the TPC had an increase during the AFL treatment with an average of 26%, and in Figure 3 (b) there was a TPC removal of approximately 11%. Some studies with kraft effluent have shown an increase in total phenolic compounds in aerated biological systems [19, 25, 27–29].

In relation to the other specific compounds of the kraft pulp effluent, it was observed that the removal of lignin compounds was approximately 13% and 27% at the OLRs of 0.2 and 0.6 $kgCODm^{-3}d^{-1}$, respectively. For the aromatic compounds, the removal average was 16% and 18% at the OLRs of 0.2 and 0.6 $kgCODm^{-3}d^{-1}$, respectively. The lignosulphonic compounds had an average removal of 8% at both OLRs.

Possible increments of specific compounds derived from lignin in aerated lagoons were also observed by [25, 28], and were related to biotransformation processes of high-molecular-weight molecules during biological treatment in these systems.

As shown in Figure 3 (a) and (b), it is possible to verify that there was no expressive color removal, reaching 4% and 10% at the OLRs of 0.2 and 0.6 $kgCODm^{-3}d^{-1}$, respectively. According to [8, 29], the increase in color may be related to the process of biotransformation of chromophore units and the condensation of color-forming compounds without mineralization of the effluent. In other studies, low color removal was also verified during treatment through aerated lagoons [7, 29].

In relation to turbidity removal, the system showed an average removal of 94% and 87% at the OLRs of 0.2 and 0.6 $kgCODm^{-3}d^{-1}$. In general, the AFL system, in both phases, showed significant removal in this parameter, indicating potential for clarification of the effluent in the AFL sedimentation zone.

Microbiological analysis

Table 1 shows the bacteria identified at the steady state of the two applied OLRs.

The genetic sequencing of the bacteria was performed through a comparison of information from the NCBI database and statistical similarity analysis, naming the bacteria in the sample by their high similarity with the microorganisms in the database.

Table 1
Cultivable bacteria identified in AFLs

OLR 0.2 kgDQOm^{-3}d^{-1}	Statistical similarity (%)	OLR 0.6 kgDQOm^{-3}d^{-1}	Statistical similarity (%)
Acinetobacter junii	98	*Acinetobacter junii*	98
-		*Aeromonas hydrophila*	97
Bacillus anthracis	99	*Bacillus anthracis*	99
Bacillus cereus	98	*Bacillus cereus*	98
Bacillus thuringiensis	98	*Bacillus thuringiensis*	98
Brevibacillus reuszeri	98	*Brevibacillus reuszeri*	98
-		*Brevundinomas diminuta*	97
-		*Comamonas testosteroni*	98
Bacillus kochii	98	*Bacillus kochii*	98
Lysinibacillus sphaericus	98	*Lysinibacillus sphaericus*	98
Paenibacillus glucanolyticus	98	*Paenibacillus gluconolyticus*	98
Sphingomonas koreensis	97	*Sphingomonas koreensis*	97
Total = 9 species		Total = 12 species	

The total number of microorganisms identified was 9 species of bacteria at the OLR of 0.2 kgCODm^{-3}d^{-1} and 12 species at the OLR of 0.6 kgCODm^{-3}d^{-1}.

In the literature, two aspects were observed regarding the study of biological diversity. The first for those observed in biological treatment systems which are related to reactor performance, especially regarding specific parameters such as phenolic compounds and color; and the second those from studies in which there was isolation of bacteria with subsequent bioaugmentation treatment utilizing these selected groups to improve the removal of specific parameters from cellulose effluents.

In the investigation conducted by [30], the removal of parameters such as BOD_5, COD and color was 66%, 61% and 90%, respectively, using *Bacillus cereus*. [31] also used *Bacillus cereus* associated with *Serratia marcescens* and *Serratia liquifaciens* and obtained a removal of color (65%), TPC (63%), COD (63%) and BOD_5 (64%).

[32] used *Bacillus thuringiensis* in a treatment system and obtained removal of BOD_5 (93%), COD (89%), TOC (82%) and color (73%). [33] used *Bacillus thuringiensis* and verified a removal in terms of COD and TPC of 61% and 64%, respectively.

[34] used *Paenibacillus* sp. and obtained removal of color (68%), lignin compounds (54%), total phenol (86%), BOD_5 (83%) and COD (78%). In research by [35], the authors used *Paenibacillus glucanolyticus* and verified the potential to degrade cellulose, hemicellulose and lignin.

The three identified species (*Bacillus cereus*, *Bacillus thuringiensis* and *Paenibacillus glucanolyticus*) show great promise in removing specific parameters in an AFL biological treatment system using bioaugmentation.

Phylogenetic tree

Figures 4, 5 and 6 represent the phylogenetic tree of the species *Bacillus cereus*, *Bacillus thuringiensis* and *Paenibacillus glucanolyticus*, identified at the two OLRs employed in the AFL, whose data were obtained using the MEGA software and the results from the application of the Bootstrap method closest to 100 indicates the species of bacteria present in the sample.

The microorganisms identified in the kraft effluent were also found in other studies with bacteria, such as the ones by [6, 30–32, 34]. The species with the greatest potential for removing the specific parameters of pulp and paper effluents is *Bacillus cereus*, especially regarding color removal in cellulose effluent treatment, as analyzed by [30, 36].

Conclusions

In this study, we analyzed the microbiological diversity in an aerated facultative lagoon system treating kraft pulp industry effluent at OLRs of 0.2 and 0.6 $kgCODm^{-3}d^{-1}$ utilizing bioaugmentation treatment.

The bacterial species identified from the samples collected in the aerated and sedimentation zones of the lagoons were named through BLAST, which showed high statistical similarity for *Acinetobacter junii* (98%), *Aeromonas hydrophila* (97%), *Bacillus anthracis* (99%), *Bacillus cereus* (98 %), *Bacillus kochii* (98%), *Bacillus thuringiensis* (98%), *Brevibacillus reuszeri* (98%), *Brevundimonas diminuta* (97%), *Comamonas testosteroni* (98%), *Lysinibacillus sphaericus* (98%), *Paenibacillus glucanolyticus* (98%), and *Sphingomonas koreensis* (97%).

We found that the identified species, namely *Bacillus cereus*, *Bacillus thuringiensis* and *Paenibacillus glucanolyticus*, show great promise in the removal of specific parameters in an AFL biological treatment

system using bioaugmentation.

Abbreviations

AFL - aerated facultative lagoons; AC - aromatic compounds; BOD - biochemical oxygen demand; COD - chemical oxygen demand; CETESB - environmental sanitation technology company; GTEF - effluent treatment research group; LAMAQ - multiuser laboratory of chemical analysis; LC - ligninic compounds; LSC - lignosulfonic compounds; NCBI - national center for biotechnology information; OLR - organic loading rates; PCR - polymerase chain reaction; TOC - total organic carbono; TPC - total phenolic compounds; UFPR - Federal University of Paraná; UTFPR - Federal Technological University of Paraná.

Declarations

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Availability of data and materials

The dataset supporting the conclusions of this article is available in the Institutional Repository of the Federal Technological University of Paraná (RIUT) repository, [unique persistent identifier and hyperlink to dataset in http://repositorio.utfpr.edu.br/jspui/handle/1/25541].

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Authors' contributions

JV Nunes and CR Xavier designed the research; JV Nunes and MWB Silva programmed the task and collected the samples; JV Nunes and IC Flôr analyzed the data and drafted the manuscript; GH Couto, VA Vicente, JD Almeida and F Celinski revised the manuscript. All authors reviewed and approved the manuscript.

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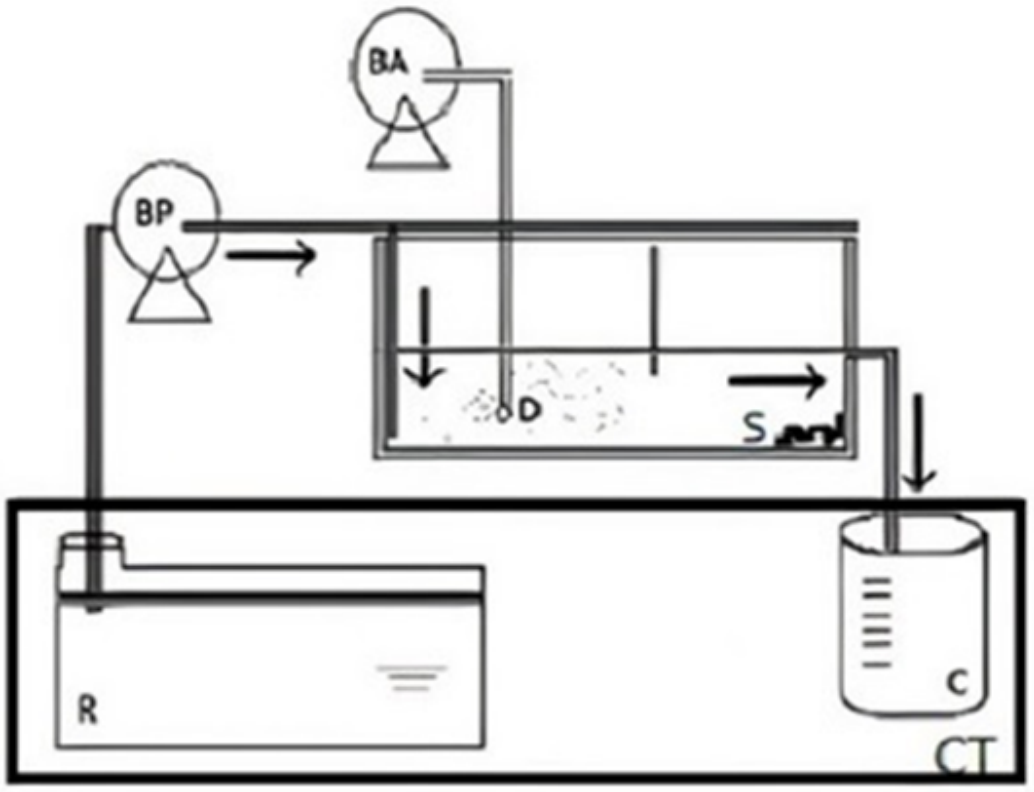
Figures



SUBTITLE

BA – Aeration pump
BP – Peristaltic pump
CT – Cool box
D – Air diffuser
S – Sedimentation zone
→ - Flow direction
C – Treated effluent collector
R – Affluent reservoir

Figure 1

Bench AFL scheme

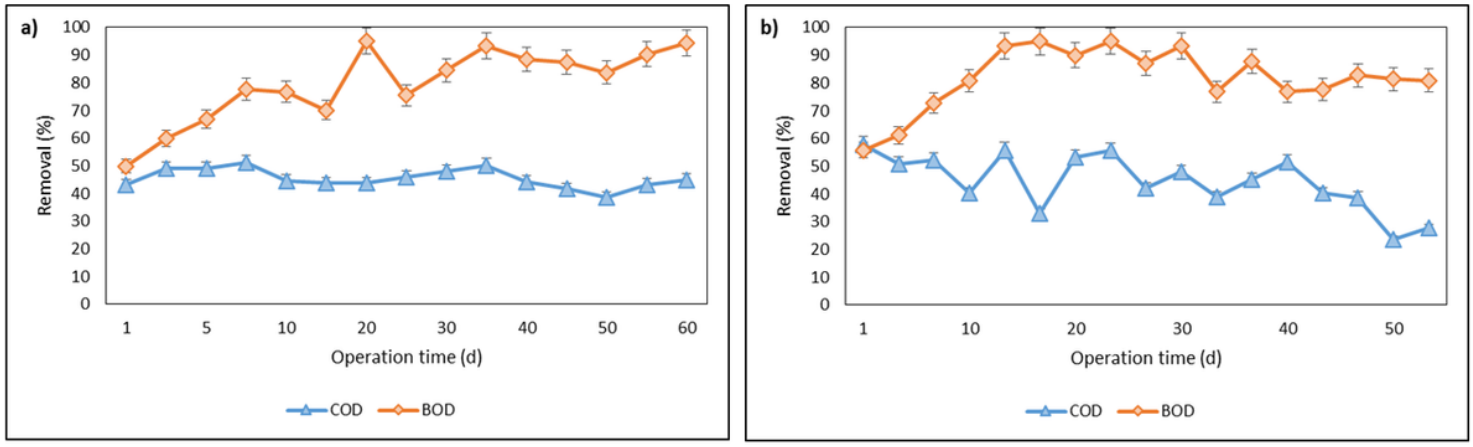


Figure 2

Removal of organic compounds as BOD5 and COD Note: a) OLR 0.2 kgDQOm-3d-1. b) OLR 0.6 kgDQOm-3d-1. BOD5 – biochemical oxygen demand. COD – chemical oxygen demand.

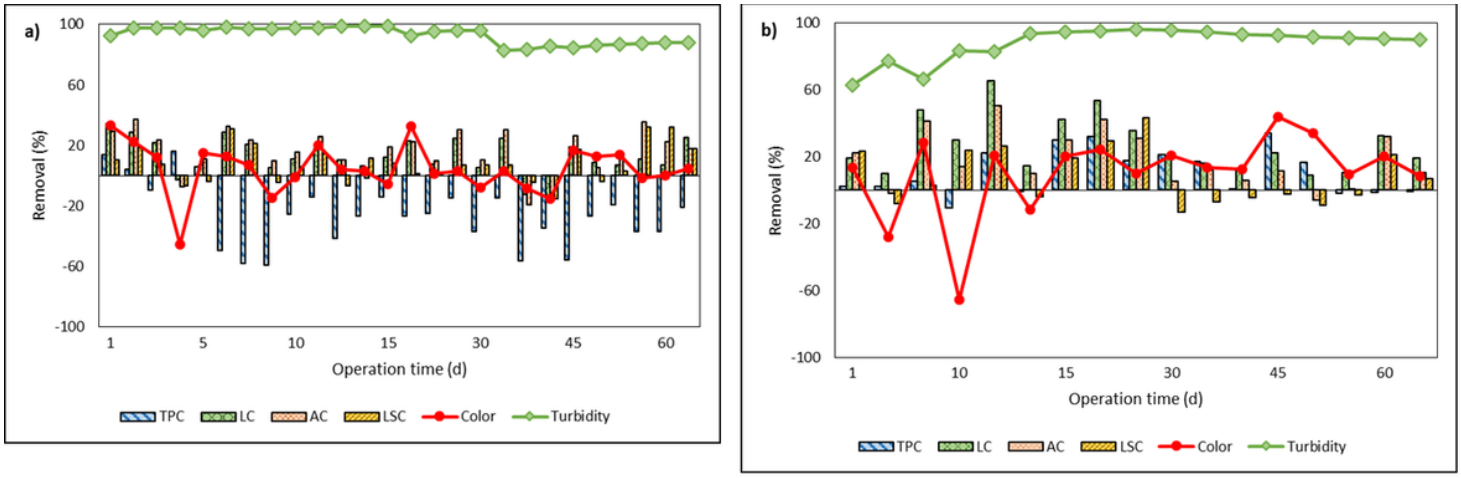


Figure 3

Evaluation of removal of specific compounds, color and turbidity Note: a) OLR 0.2 kgDQOm-3d-1. b) OLR 0.6 kgDQOm-3d-1. AC – aromatic compounds. TPC – total phenolic compounds. LC – ligninic compounds. LSC – lignosulfonic compounds.

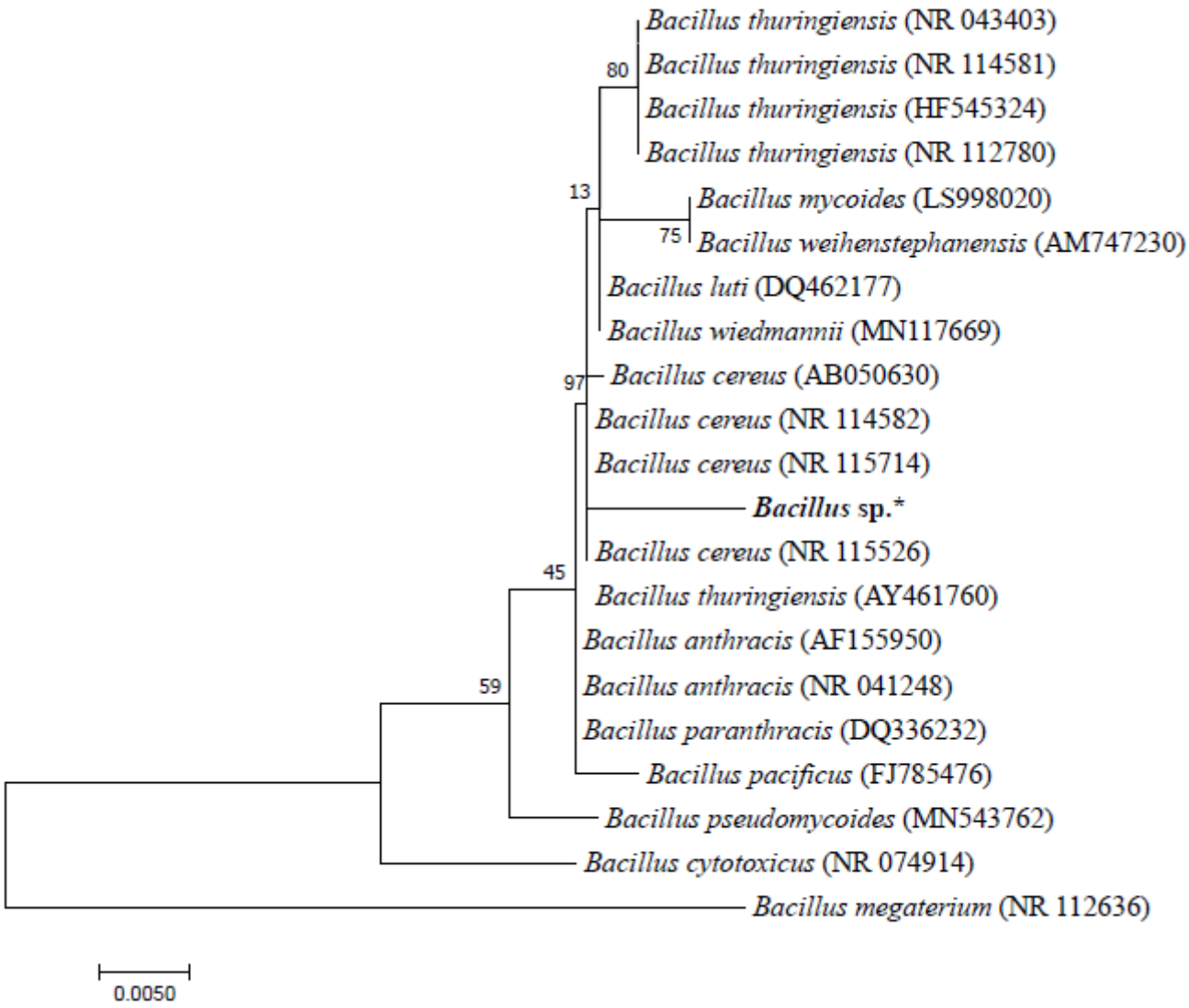


Figure 4

Phylogenetic tree of the species Bacillus cereus Note: The phylogenetic tree was obtained using the union of neighbors with Bacillus megaterium as an outer group, obtaining a replica of 97 for the Bacillus cereus.

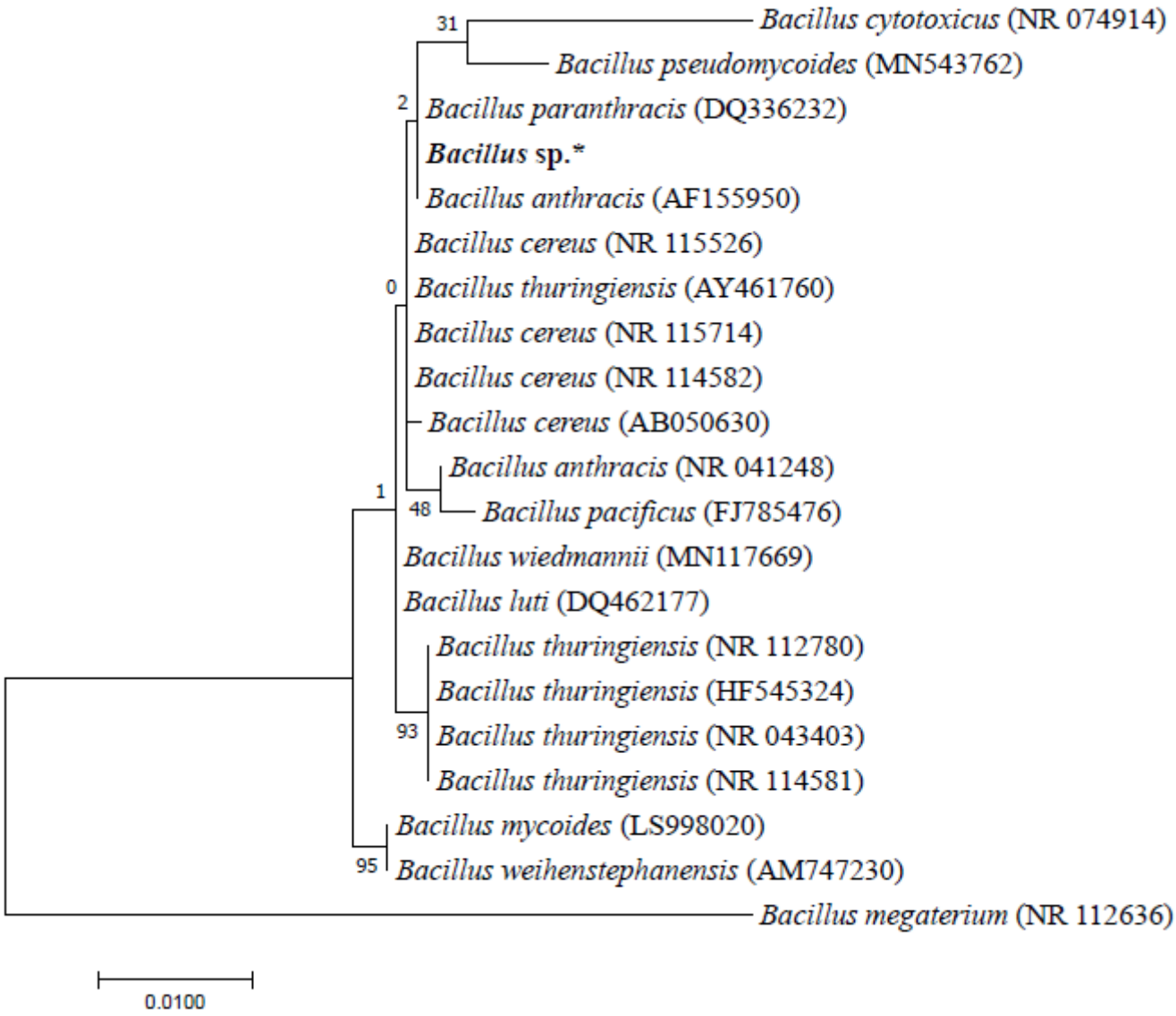


Figure 5

Phylogenetic tree of the species Bacillus thuringiensis Note: The phylogenetic tree was obtained using the union of neighbors with Bacillus megaterium as an outer group, obtaining a replica of 93 for the Bacillus thuringiensis.

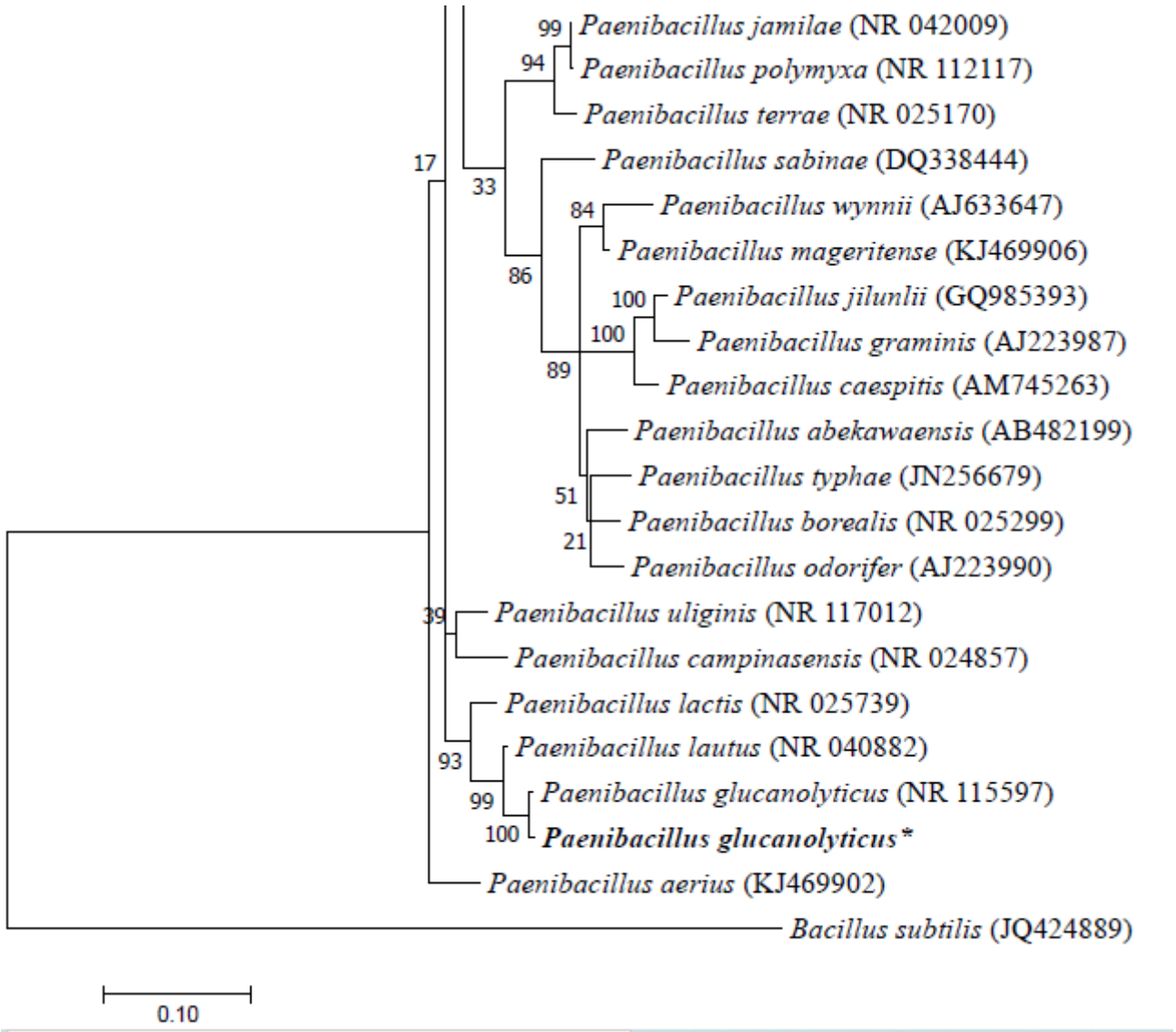


Figure 6

Phylogenetic tree of the species Paenibacillus glucanolyticus Note: The phylogenetic tree was obtained using the union of neighbors with Bacillus subtilis as an outer group, obtaining a replica of 100 for the Paenibacillus glucanolyticus.