Benedito Rodrigues da Silva Neto
(Organizador)

Pesquisa Científica e Tecnológica em Microbiologia

Atena Editora
Ano 2019

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2019 *by Atena Editora*

Editora Chefe: Profª Drª Antonella Carvalho de Oliveira
Diagramação: Lorena Prestes
Edição de Arte: Lorena Prestes
Revisão: Os Autores

Atena Editora
Ano 2019

Dados Internacionais de Catalogação na Publicação (CIP)
(eDOC BRASIL, Belo Horizonte/MG)

P474 Pesquisa científica e tecnológica em microbiologia [recurso eletrônico] / Organizador Benedito Rodrigues da Silva Neto. – Ponta Grossa, PR: Atena Editora, 2019.

Formato: PDF
Requisitos de sistema: Adobe Acrobat Reader
Modo de acesso: World Wide Web
Inclui bibliografia
ISBN 978-85-7247-772-7
DOI 10.22533/at.ed.727191111

1. Microbiologia – Pesquisa – Brasil. I. Silva Neto, Benedito Rodrigues da.

CDD 579

Elaborado por Maurício Amormino Júnior – CRB6/2422

Atena Editora
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Atena Editora
Ano 2019

APRESENTAÇÃO

A microbiologia é um vasto campo que inclui o estudo dos seres vivos microscópicos nos seus mais vaiados aspectos como morfologia, estrutura, fisiologia, reprodução, genética, taxonomia, interação com outros organismos e com o ambiente além de aplicações biotecnológicas. Como uma ciência básica a microbiologia utiliza células microbianas para analisar os processos fundamentais da vida, e como ciência aplicada ela é praticamente a linha de frente de avanços importantes na medicina, agricultura e na indústria.

De forma integrada e colaborativa a nossa proposta apoiada e certificada pela editora Atena é apresentar aqui a obra “Pesquisa científica e tecnológica em microbiologia” contendo trabalhos e pesquisas desenvolvidas em diversos institutos do território nacional contendo análises de processos biológicos embasados em células microbianas ou estudos científicos na fundamentação de atividades microbianas com capacidade de interferir nos processos de saúde/doença.

A microbiologia como ciência iniciou a cerca de 200 anos, entretanto os avanços na área molecular como a descoberta do DNA elevou a um novo nível os estudos desses seres microscópicos, além de abrir novas frentes de pesquisa e estudo, algumas das quais pretendemos demonstrar nesse primeiro volume da obra “Pesquisa científica e tecnológica em microbiologia”. Sabemos na atualidade que os microrganismos são encontrados em praticamente todos os lugares, e a falta de conhecimento que havia antes da invenção do microscópio hoje não é mais um problema no estudo, principalmente das enfermidades relacionadas aos agentes como bactérias, vírus, fungos e protozoários.

Acreditamos no potencial dessa obra em primeiro lugar pela qualidade dos trabalhos aqui apresentados, e em segundo pelo campo em potencial para futuras novas discussões, haja vista que enfrentamos a questão da resistência dos microrganismos à drogas, identificação de viroses emergentes, ou reemergentes, desenvolvimento de vacinas e principalmente a potencialização do desenvolvimento tecnológico no estudo e aplicações de microrganismos de interesse.

Temas ligados à pesquisa e tecnologia microbiana são, deste modo, discutidos aqui com a proposta de fundamentar o conhecimento de acadêmicos, mestres e todos aqueles que de alguma forma se interessam pela saúde em seus aspectos microbiológicos. Portanto a obra propõe uma teoria bem fundamentada nos resultados práticos obtidos em alguns campos da microbiologia, abrindo perspectivas futuras para os demais pesquisadores de outras subáreas da microbiologia.

Assim desejo a todos uma ótima leitura!

Benedito Rodrigues da Silva Neto

SUMÁRIO

CAPÍTULO 7

ENTEROCOCCUS SP ISOLATED FROM AQUATIC ENVIRONMENT : RESISTANCE TO TOXIC METALS

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ABSTRACT: Environmental pollution by toxic metals began due to the accelerated development of industrial activities through the irregular disposal of waste generated by activities such as mining, metallurgical processes, chemical industries, agriculture and also by contaminated effluents. This pollution can affect human and other animal health through the accumulation of toxic metals in water, sediment and soil. Microorganism bioremediation stands out as a tool for the decontamination of toxic metal environments. The use of biosorption or bioaccumulating bacteria has gained much attention from researchers because of their potential to provide an effective and cost-effective means for the remediation of toxic metals. Therefore, the use of bacteria with proven remediation potential and survivability in the contaminated environment is of utmost importance for successful bioremediation. The objective of this project is to identify the biosorption capacity of toxic metals by bacteria of the genus *Enterococcus* sp isolated from the aquatic environment, by the analysis of toxic metal resistance by the Gradient Plate technique, following the maximum concentrations required by CONAMA Resolution 357/2005. The results showed that 93% of the isolates showed growth for zinc metal; 97.2% for copper and 98.6% for lead. These results reveal that the *Enterococcus* bacteria analyzed have resistance to the tested toxic metals characterizing a potential for bioremediation, considering that many bacteria found naturally in the environment may be closely related to the bioremediation process reducing the toxicity of the metals.

INTRODUCTION

Bacteria of the genus *Enterococcus* are Gram-positive cocci that are arranged in pairs or short chains. They are facultative anaerobes and their growth can occur at different temperatures (10 to 45ºC) and in high salt concentrations. They are often found in the gastrointestinal tract of humans in high concentrations in human and animal feces. They are opportunistic pathogens that cause thousands of infections, which pose great risks to human health. (AHMED et al, 2012; BOEHM, SASSOUBRE, 2014).

Kimiran-Erdem et al. (2006) described that a characteristic of these microorganisms is their resistance to physical and chemical stress, such as the presence of antibiotics and toxic metals, unlike other fecal bacteria that are found in the environment. Microorganisms have developed metal resistance and detoxification mechanisms for their survival due to the presence of toxic metals in the environment. Microbial resistance to metals can be divided into three categories: intrinsic resistance mechanisms (which do not require metal stress, resistance mechanisms that require metal stress, and resistance mechanisms that depend on the type of metal and its activation. Resistance determinants are encoded on the chromosome, but some can be encoded on mobile genetic elements such as plasmids and transposons (ROANE, et al., 2009).

Anthropogenic activities involving mining, improper waste disposal, effluents containing metals, application of pesticides and fertilizers in abundance, industries (metallurgical, batteries, chemicals, etc.) are the main causes of environmental pollution by toxic metals. Although metals are natural constituents of the earth and some of them are essential to the life of organisms, excess can have harmful effects on human health (Table 1) (KANG et al., 2016; KAR et al., 2008; MORAIS et al. , 2012; NAIK, DUBEY, 2013).

Conventional methods for removing toxic metals from the environment include precipitation, flocculation and filter membranes, but are costly processes. New technologies have been developed to reduce or recover environmental contamination by toxic metals, such as bioremediation by microorganisms. This process includes the degradation of the pollutant by biochemical reactions (HALTTUNEN, et al., 2006; PEREIRA and FREITAS, 2006).

Heavy Metals	Main polluting sources	Harm to human health
Cooper (Cu)	Mining, Pesticide Production, Chemical Industries, Pipe Corrosion, Household Sewage, Algaecides, Fungicides and Metal Refining.	Anemia, Stomach and intestinal irritation, Liver and kidney damage and Poisoning.
Lead (Pb)	Pesticides, fumes, automotive emissions, mining, coal burning, painting, industrial effluents and tobacco.	Mental retardation in children, Encephalopathy, Congenital paralysis, Neuronal deafness, Nervous system damage, Gastrointestinal, liver and kidney damage, and Epilepsy.
Chrome (Cr)	Industrial Effluents, Aluminum & Steel Production, Inks, Explosive Pigments and Photography.	Allergies, Cancer and Poisoning.
Zinc (Zn)	Refineries, Mining, Waste Incineration and Metal Coatings.	Respiratory, Gastric and Cardiac Changes

Table 1: Heavy metals, their main polluting sources and their effect on human health.

adapted from Singh et al. (2011), Nies (1999) e Biondo (2008)

Bacteria are capable of remediating metal contaminated environments, including aquatic environments, sediment and soil. In soils and sediments such remediation may occur in situ or ex situ through metal oxidation or leaching, such as acid and sulfate production and volatilization. Bacteria utilize immobilization strategies including metal sequestration due to the ability of some intracellular or extracellular complex metal bacteria. Another way of immobilization is to create an anaerobic reduction or condition in the environment that results in the reduction or precipitation of the metal. In aquatic environments, the most common way of toxic metal bioremediation may be by the formation of microbial biofilm (ROANE, et al., 2009).

Bacteria adapt to toxic metals through a variety of resistance systems, mediated by chromosomes, transposon and plasmids. Some mechanisms of bacterial resistance to metals are: permeable barrier exclusion, intra- and extracellular sequestration, active transport or efflux pumps, and reduced sensitivity of cellular targets to metallic ions (Figure 1) (BRUINS, et.al, 2000).

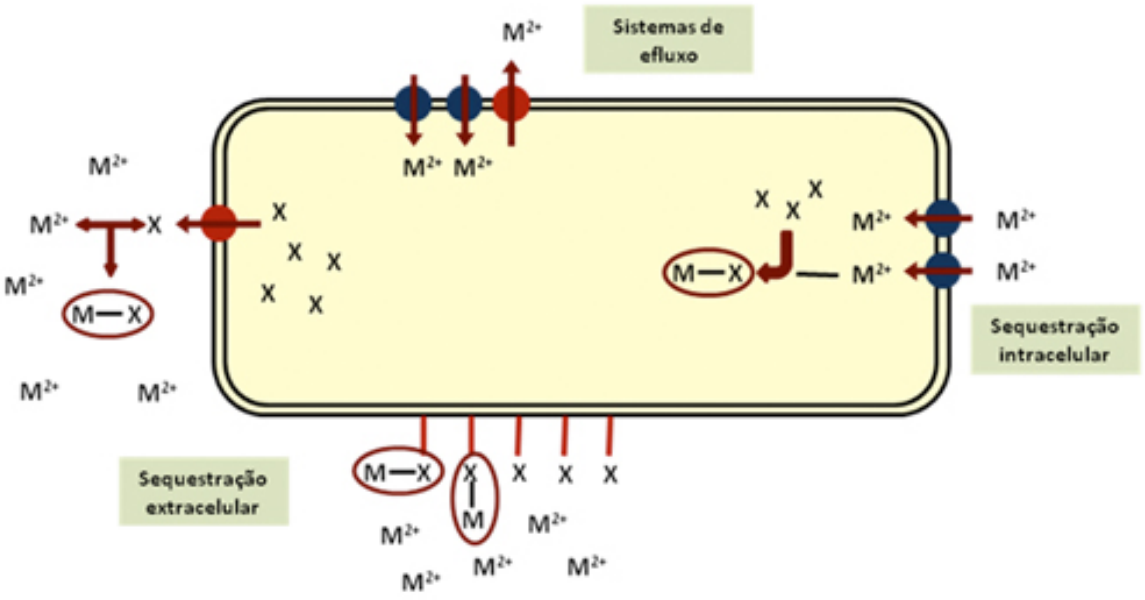

Figure 1 - Representative scheme of the main mechanisms of tolerance in bacteria; M +: toxic metal; X: Cell constituents that interact with metals.

Adapted from www.esb.ucp.pt/en/projects.

The exclusion mechanism impermeable barrier associated with changes in the cell wall and membrane. This mechanism is characterized as an attempt by the body to protect essential cellular components that are sensitive to metals (BRUINS, et.al, 2000).

Biosorption is a type of toxic metal bioremediation that can occur through the physicochemical interaction between the metal and the constituents of the membrane or cell wall of microorganisms, providing an alternative for treatment and recovery of contaminated areas (OISHI, 2014).

Some factors influence the biosorption mechanism of metals, such as biomass (living cells or non-living cells), types of biomaterials, chemical properties of metal solutions and environmental conditions such as pH and temperature. The metals biosorption process in living cells occurs into two procedures. The first is through the adsorption of metal ions on the surface of cells, before the metal ions adhere to the cell membrane or the cytoplasm adhere to cell wall containing a variety of polysaccharides and proteins which provide local assets that make ionic bonds. The second step, the metal ions penetrate the cell membrane and enter into the cells. The mechanisms involved in biosorption are coordination, complexation, ion exchange, physical adsorption and microprecipitation inorganic (Das et al., 2007).

The use of bacteria with proven remediation potential and survivability in the contaminated environment provides an alternative for treatment and recovery of contaminated areas. The present study aims to analyze the possible ability of *Enterococcus* bacteria to carry out biosorption of toxic metals, due to their survival characteristics in extreme environments.

OBJECTIVE OF PROJECT

This work aims to evaluate the resistance of *Enterococcus* sp. isolated from the aquatic environment to toxic metals (lead, copper, chrome and zinc) with potential for bioremediation.

METHODOLOGY

Study area

The municipality of Apucarana is located in the north central region of the state of Paraná. According to the Brazilian Institute of Geography and Statistics (2014), this municipality has an area of 558,389 km2, BORDERING the municipalities of Arapongas, California, Cambira, Londrina, Mandaguari, Marilândia do Sul, Novo Itacolomi, Rio Bom and Sabáudia. The municipality still covers in its territory the districts of Correia de Freitas, Pirapó, São Pedro and Vila Reis.

The region has several rivers, streams and lakes used by the population for leisure and supply system (Figure 2). The studies concentrated on the Ivaí rivers (Corrego do Jaboti); Pirapó and Rio Bom (Ribeirão Biguaçu), because they cover the urban and rural areas.

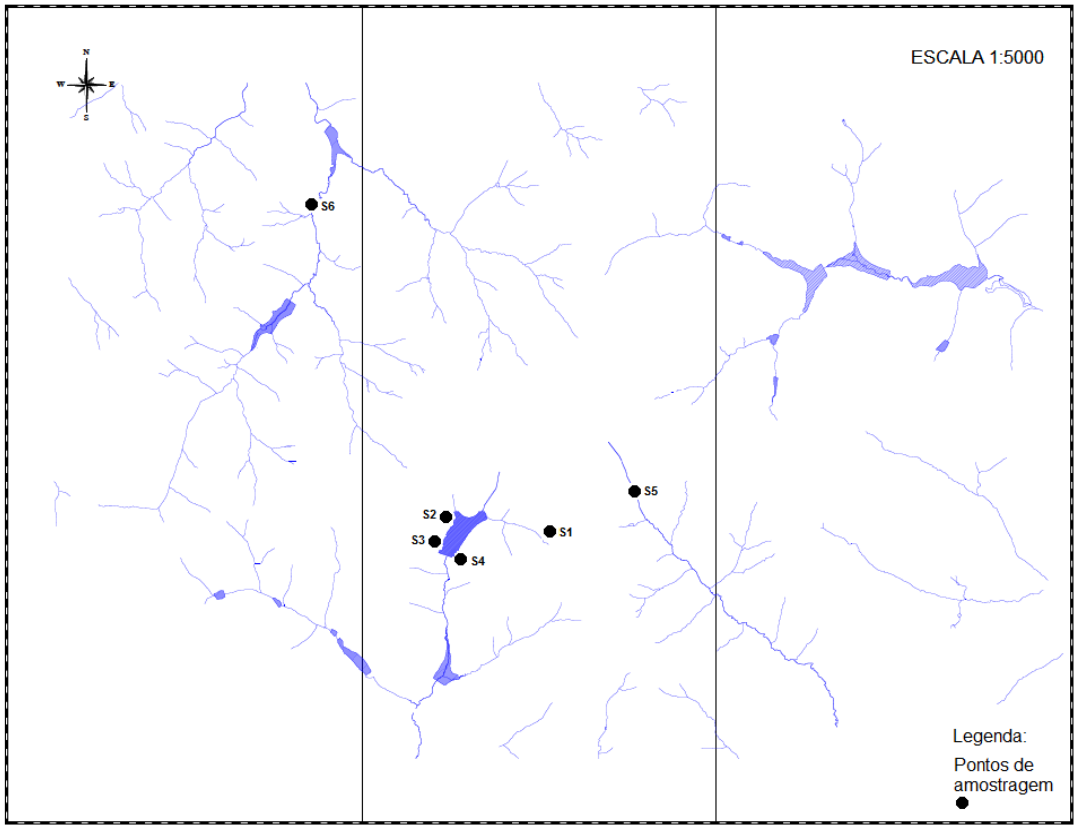

Figure 2 - Hydrographic map of the municipality of Apucarana / Paraná - Brazil. Source: Portal of the Apucarana City Hall, 2015

CHARACTERIZATION OF COLLECTION PLACES

Córrego Jaboti - Santo Expedito Echologic Park

The Jaboti stream (Santo Expedito Ecological Park) rises near the Cristo Rei Cemetery, leading to Jaboti Lake. At the stream site, Apucarana City Hall installed Parque Santo Expedito, which is characterized by religious tourism. Jaboti stream follows a valley bordered on its left bank by an agricultural area, and a partially urbanized area on its right bank.

The park has a playground, square, ecumenical chapel, stage, grotto with the image of Santo Expedito, gazebo with the image of Jesus Christ, sand soccer field, parking, water spout approximately 5m from the source of the stream. Jaboti, one of the spouts being the point of study (Figure 3); and due to this short distance it is considered as spring. The park also has natural water pools (PORTAL OF APUCARANA MUNICIPAL PREFECTURE, 2015).

Figure 3 - Location of the collection point (Jaboti stream) (A) and stream overview (B). The red dot indicates the location of the Jaboti stream at the coordinates 23º34'3.83"S / 51º27'46.7'W; altitude 773m.

Source: Google Earth.

Jaboti Lake

Three points were chosen for collection in Lake Jaboti, named points A, B and C (Figure 4). The Jaboti Park has afforestation and infrastructure, as well as a playground, outdoor gym, events room, toilets, snack bars, hiking trail, bike path, public telephone, watercraft, known as "pedal boat", and fishing is allowed in this area. local, all to attract visitors to the area; in addition to the Apucarana Cultural and Sports Association (ACEA), this entire area being limited by Avenida Jaboti.A barragem para formação do lago foi construída na bacia do ribeirão Barra Nova, com

altura de 23m, comprimento de crista de 16m e largura da base de 155m. O local de vazão da barragem está situado na porção sul do lago (PORTAL DA PREFEITURA MUNICIPAL DE APUCARANA, 2015).

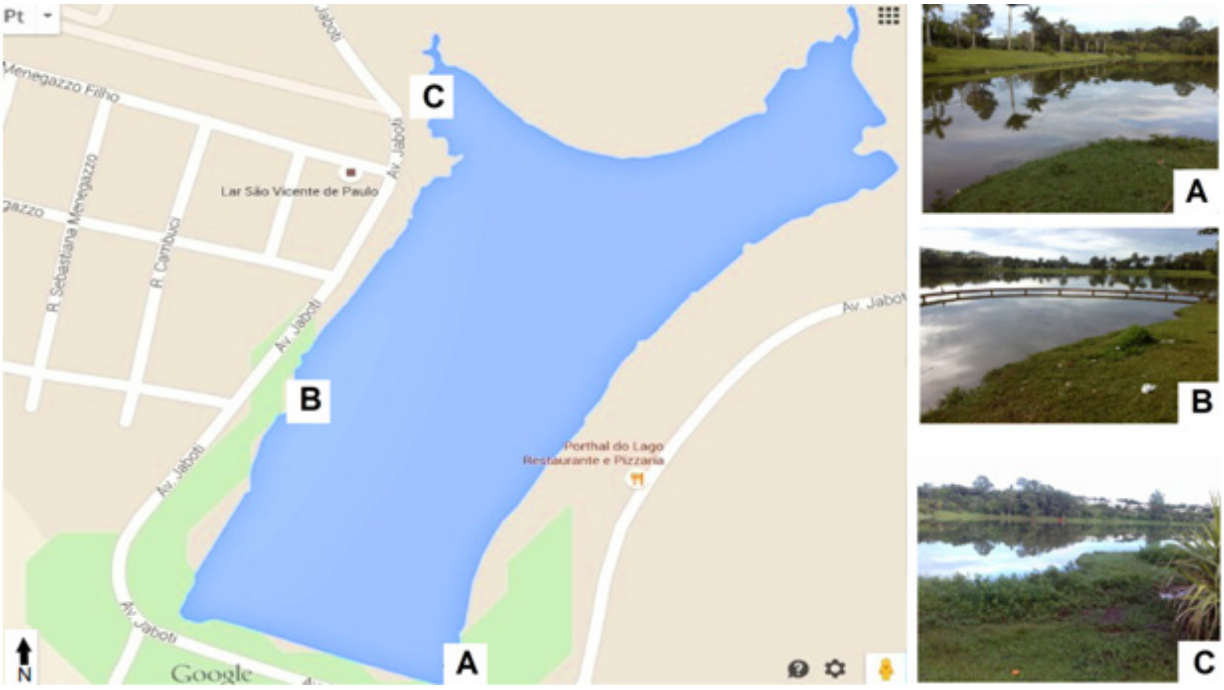

Figure 4 - Location and overview of collection points on Jaboti Lake. (A) at coordinates 23º34'9.95 '' (S) / 51º28'25.54''W; altitude 742m. (B) at coordinates 23 ° 34'2.36 '' S / 51 ° 28 '31.2 '' W; altitude 742m. (C) at coordinates 23º33'52.92''S / 51º28'25.81''W; altitude 742m.

Source: Google Earth Adaptation.

Barreiro dam - redemption park

The supply of Apucarana is made through the Pirapó River, and surface springs, such as Caviúna stream, as well as Serra Geral aquifer wells. Redemption Park (Figure 5) is a religious theme park; The same is the revitalization of the degraded permanent preservation area on the banks of the Barreiro dam, belonging to the Pirapó river basin, which supplies Apucarana as previously mentioned, as well as the region. The park was established with the objective of revitalizing the surroundings of the old "dump" of the study city, as well as the recovery of the upper course of the Pirapó River.

Figure 5 - Location of the collection point (Barreiro dam) (A) and overview of the repression (B). The red dot indicates the location of the dam at coordinates 23º31'13.97"S / 51º29'22.32"W; altitude 631m.

Source: Google Earth Adaptation.

Ribeirão biguaçu - park of the bible

Ribeirão Biguaçu (Bible Park) is another collection point (Figure 6), and it is one of the tourist elements of Apucarana that attracts people from other cities to visit it, not only being restricted to the local population (TORRES, 2013). According to Faria and Faria (2006), the Biguaçu river basin belonging entirely to the municipality of Apucarana - PR, being one of the tributaries of the Bom river.

The Biguaçu Valley was channeled through the erosion control program, as one of the streets that give access to the current Park (João Antonio Braga Cortes Street), on the outskirts of the "Country Club", was taken by a huge erosion; and this situation caused great inconvenience to the residents of the region, because people had to change the route until they reached the other side of the city (FERREIRA, 2006).

In the surroundings of the Biguaçu stream there is a region that is divided into two parks, the São Francisco de Assis Park and the Bible Park. These are religious themed environmental parks and have urban afforestation and landscaping, a fountain, trash cans, a hiking trail, a water mirror, benches, lamps and a locker room.

Figure 6 - Location of the collection point (Biguaçu stream) (A) and general view of the stream (B). The red dot indicates the location of the stream at the coordinates 23º33'31.54"S /

51º27'10.57"W; altitude 778m.

Source: Google Earth Adaptation.

SAMPLE COLLECTION

One hundred twenty water samples were collected in the months of October and November 2014 and February and March 2015, predetermined in the previous items, according to occupation and use by the population. Water collection was carried out in polyethylene terephthalate bottles pretreated with 0.1% Tween 80 solution. After collection, the samples were packed in an isothermal box containing ice and sent to the UTFPR Microbiology Laboratory - Londrina campus.

QUANTIFICATION AND IDENTIFICATION OF *ENTEROCOCCUS* SP.

The densities of *Enterococcus* sp. were determined based on the filter membrane technique. To this end, 100 mL of water was filtered through a nitrocellulose membrane with 0.45 *µ*m porosity and 47mm diameter (SARTORIUS STEDIM BIOTECH®). The membranes were deposited on the surface of the kanamycin esculin azide agar (KEA) culture medium (HIMEDIA) and incubated at 37 ° C for 24 / 48h.

The bacterial isolates were submitted to phenotypic identification tests: observation of the morphotintorial characteristics by Gram staining and catalase enzyme production, following protocol described by Facklam et al. (1999). Catalase enzyme production was verified by depositing a drop of hydrogen peroxide (3% v / v H_2O_2) on the bacterial suspension. The absence of bubble formation is indicative of negative reaction, characteristic of *Enterococcus* sp.

TOXIC METAL CONCENTRATIONS

Metal concentrations were defined according to CONAMA Resolution 357 of March 17, 2015, in its Art. 5 which classifies the bodies of water used for recreation and leisure between classes III and IV. Table 2 below shows the concentrations allowed by CONAMA Resolution 357/2005.

The solutions were prepared with sterile ultrapure water and filtered with 0.22 *µ*m porosity disposable membranes and stored under refrigeration.

Toxic metal	Lead ($Pb(NO_3)_2$)	Cooper ($CuSO_4$)	Zinc ($ZnSO_4$)	Chrome ($K_2Cr_2O_7$)
concentrations (mg/L)	0,033	0,013	5	0,05

Table 2 - Toxic metal concentrations allowed by CONAMA Resolution 357/2005

GRADIENT PLATE TECHNIQUE

The gradient plate technique was used for initial tests to verify the possible resistance of the isolates to metals. According to Szybalski and Bryson (1952), this method evaluates the reduction in the amount of metals required for the experiment.

To assemble the plate, two layers of agar medium were used. The first lower layer is composed of 10 mL of Muller Hilton agar medium and is slanted gel (Figure 7A). The upper layer was composed of 20 mL of Muller Hilton agar plus metal solutions with the minimum concentrations required by the legislation described in Table 1, thus forming a metal concentration gradient (Figure 7B).

After mounting the plate, the isolates were inoculated in horizontal lines from the least concentrated to the most concentrated (Figure 7C).

Subsequently, bacterial isolates that presented resistance were inoculated in concentrations 10 to 40 thousand times higher than recommended by Conama.

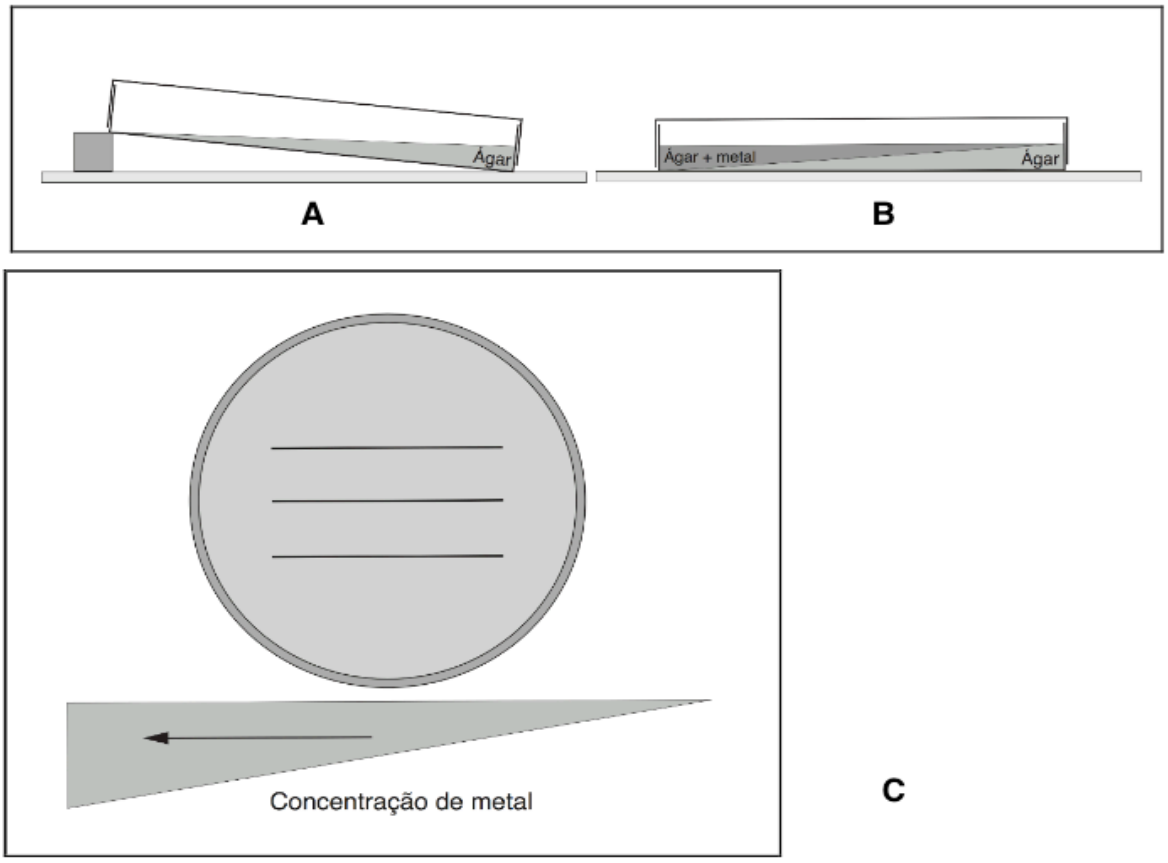

Figure 7 - Toxic metal gradient plate (A and B); C Representation of the inoculum lines on the gradient plate

RESULTS AND DISCUSSION

Characteristic colonies of *Enterococcus* sp. on KEA agar, they are black in color due to the hydrolysis of esculin in dextrose and esculetin, which reacts with ferric citrate producing a brownish black precipitate (Figure 8A). A total of 327 presumptive

Enterococcus sp. were obtained from the 120 water samples analyzed. Seventy-six (23.24%) isolates had phenotypic characteristics of Gram staining and *Enterococcus* sp. Catalase test (Figure 8).

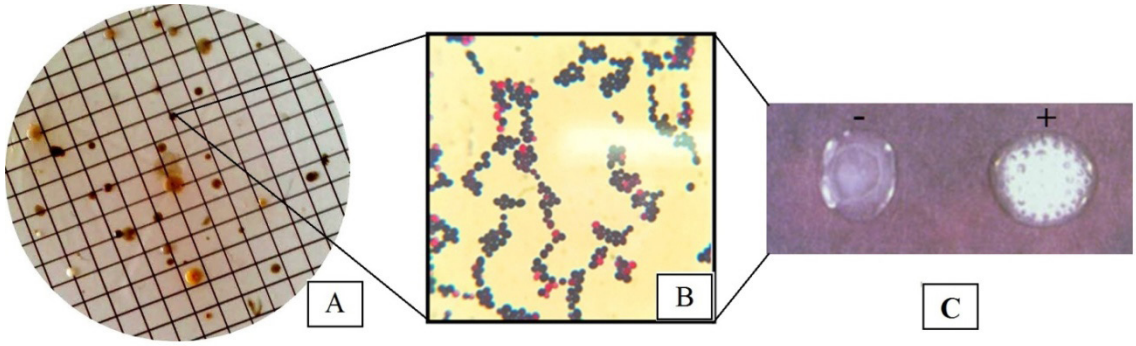

Figure 8 - Characteristic colonies of *Enterococcus* sp. using KEA culture medium (A); followed by Gram staining (Gram positive cocci) (B) and catalase test (C).

Several studies show that there is prevalence of *E. faecalis* and *E. faecium* in water samples (FERGUSON et al., 2013; YAMANAKA, 2011). However, in the present study, these were not the prevalent species, showing the genetic diversity among the isolates in the environment, depending on the geographical location.

The presence of enterococci in environmental samples has been considered an indicator of recent fecal contamination (a few days or weeks) complementary to thermotolerant coliforms. This microorganism originates from both animal feces and human fecal origin, and the main counts of enterococci are the quality assessments of water sources and bodies (FALAVINHA; DEGENHARDT, 2014).

Due to the fact that enterococci do not survive in polluted environments, the probable source of contamination of these environments is generally related to anthropogenic activities that occurred at the time of sampling or earlier days, or another probable contamination by animals. living in the collection sites (HASMAN et al., 2006).

The main applications of *Enterococcus* counting are assessments of the quality of springs and water bodies, and the quality of treated water and the evaluation and monitoring of the hygienic conditions of industrial systems (SILVA et al., 2000).

In this study, 72 bacterial isolates from water bodies were tested. The results revealed that 97.2% showed continuous growth in the gradient plate with the tested metals (Figure 9).

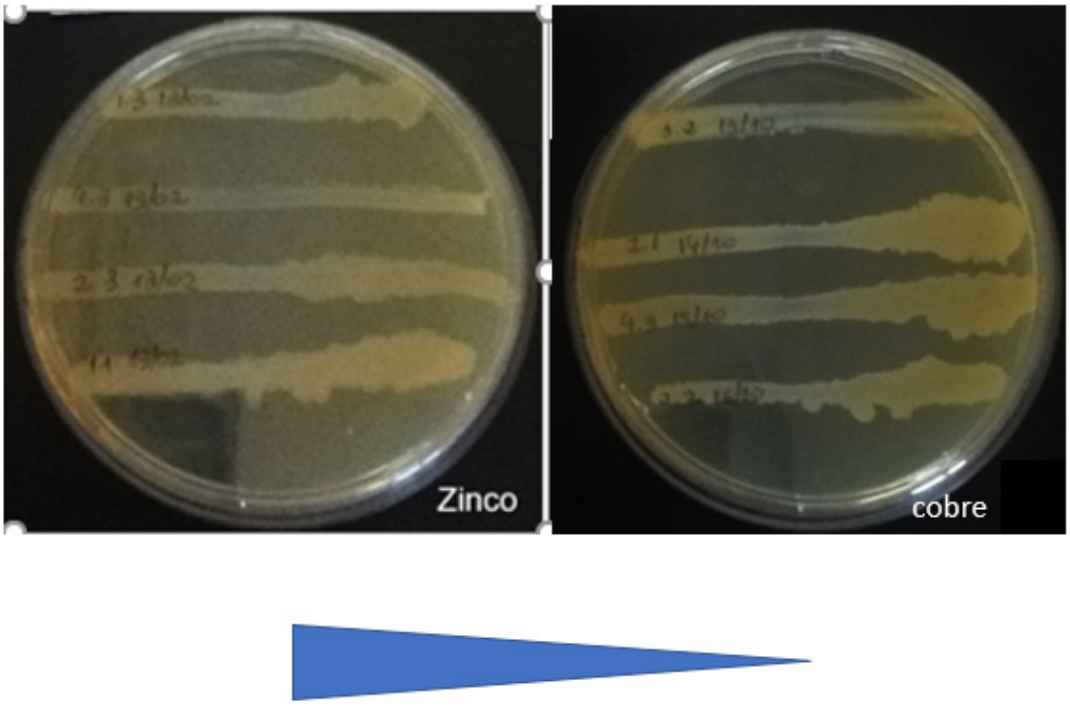

Figure 9 - Representative photo of growth on the gradient agar plate; Isolates of the genus *Enterococcus* growing in various concentrations of zinc and copper metal present in the culture medium. Arrow represents higher and lower concentration of metal in the gradient plate.

The figure 10 shows the growth of bacteria exposed to the maximum concentration allowed by CONAMA Resolution 357/2005. It can be observed that all isolates showed microbial development, with different intensities.

Figure 11 represents the growth of *Enterococcus* sp. at concentrations 40,000 times as permitted by legislation for chrome, lead and copper, and at concentrations 10,000 times for zinc metal. The results show that there was still growth for lead and copper at very high concentrations, which may characterize a bacterial resistance to these metals.

Environmental pollution by toxic metals has been growing rapidly along with industrialization as many companies still release their contaminated effluent directly into the environment. According to Banerjee et al. (2015), toxic metals accumulated in bacteria may be a more viable and cheaper alternative for wastewater treatment. Based on the preliminary results presented in this work, it is possible to identify bacteria with resistance to high concentrations of metals, which makes them a great potential for bioremediation.

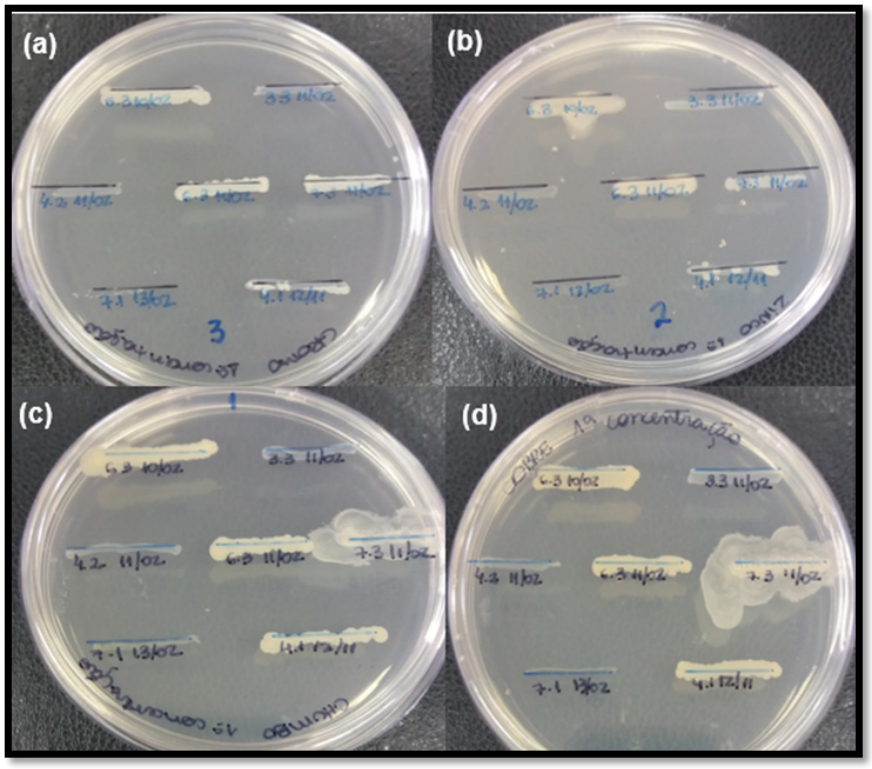

Figure 10 - Growth in plate with the concentration allowed in CONAMA Resolution 357/2005. (a) Chrome metal growth plate, (b) Zinc metal growth plate, (c) Lead metal growth plate and (d) Copper metal growth plate.

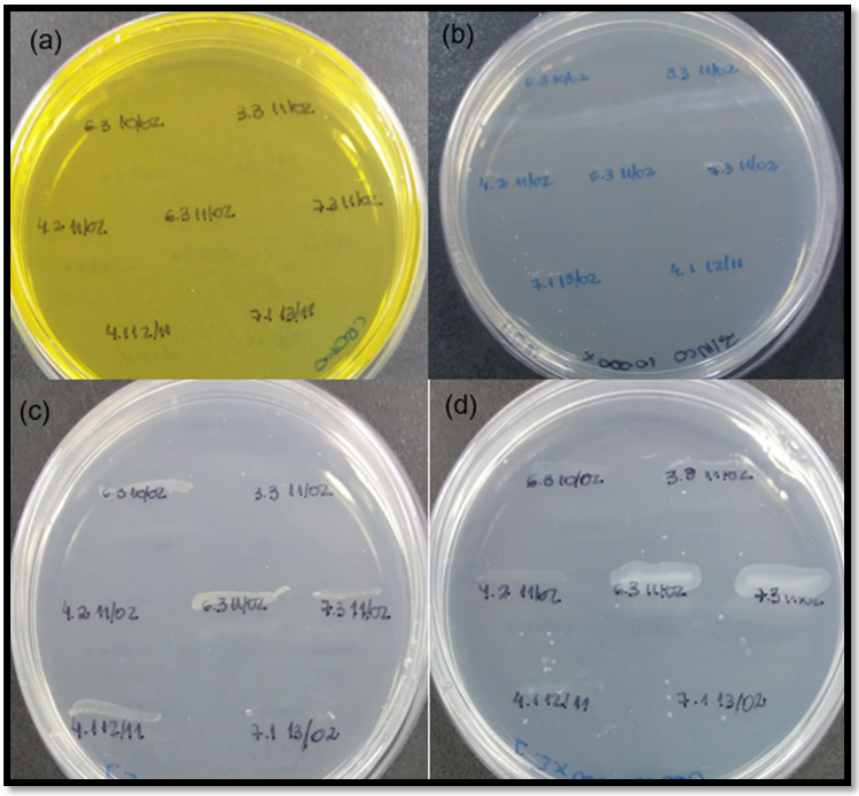

Figure 11 - Growth in plate with concentration allowed in CONAMA Resolution 357/2007. (a) 40,000 times concentrated chrome metal growth plate, (b) 10,000 times concentrated zinc metal growth plate, (c) 40,000 times concentrated metal growth plate and (d) metal growth plate covers 40 thousand times concentrated.

Bacterial resistance to toxic metals is an important factor to be considered in the study of remediation of contaminated areas, as this resistance is directly related

to the survivability and bacterial growth at high concentrations of toxic metals (KANG el al., 2016).

Environmental pollution by toxic metals has been growing rapidly along with industrialization as many companies still release their contaminated effluent directly into the environment. According to Banerjee et al. (2015), bioremediation of toxic metals by bacteria may be a more viable and cheaper alternative for wastewater treatment.

CONCLUSION

Based on the results presented in this work, bacteria with resistance to high concentrations of metals can be identified, which makes them a great potential for bioremediation. The ability of bacteria to survive extreme environments such as the presence of toxic metals clearly shows the importance of studying the resistance to these pollutants, as they can be used in the natural bioremediation process of these environments.

ACKNOWLEDGMENT

Moralez, A. T. P. for the suggestions of the experimental part of this work, as a requirement of the postdoctoral.

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ÍNDICE REMISSIVO

A

B

C

D

E

F

G

I

L

M

O

P

Q

R

S

V

Z

Agência Brasileira do ISBN
ISBN 978-85-7247-772-7