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Identification of Ascomycetes Recovered From Petrol Stations in the Metropolitan Region of João Pessoa-PB, Brazil

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ABSTRACT
Petroderivatives from petrol stations constantly contaminate the urban environment and are responsible for significant negative impacts on it. Fungi are one of the main groups responsible for the degradation process of these compounds in soils. The aim of this work was to isolate and identify filamentous fungi from three petrol stations in the Metropolitan Region of João Pessoa, a municipality in Northeast Brazil. The microorganisms, isolated from the soil and diesel oil samples, stored in the tanks, were identified by coverslip technique using Sabouraud-Dextrose Agar. Eight fungal isolates were obtained from three different genera of the Ascomycota: *Paecilomyces* (strains TP01 and TP08), *Penicillium* (strains TP02 and TP07) and *Aspergillus* (strains TP04, TP05, TP06 and TP13). A Simple test of hydrocarbonoclastic activity indicated that the strains recovered from soil and fuel samples were recognized for their potential as bioremediation agents, so that they can be used in further tests aimed the removal of petroleum hydrocarbons, particularly in soil.
Keywords: Hydrocarbonoclastic fungi, Paraíba, Urban pollution.

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I. INTRODUCTION

Petroleum Is A Generic Term Often Used To Denote A Complex And Toxic Mixture Of Aliphatic, Aromatic And Heterocyclic Hydrocarbons. It Also Constitutes A Small Fraction Of Naturally Formed Metals And Organometallic Compounds From The Anaerobic Conversion Of Organic Matter Deposited In Low Permeability Sediments Under Specific Pressure And Temperature Conditions [1]. Given This, Petroderivatives Represent One Of The Most Important Classes Of Xenobiotics, Responsible For Negative Impacts On The Environment, Especially Biota, Water Bodies And Soil [2][3].

Petrol Stations Are The Largest Source Of Hydrocarbon Contamination In Urban Areas. This Environment And Its Surroundings Exert Significant Selective Pressures On The Biota Present, Due To The Recalcitrant And/Or Persistent Nature Of Most Of The Organic Compounds Involved, As Well As The Degree Of Variable Mutagenicity, Which Promotes Changes In The Number And Metabolic Activity Of The Present Microbiota [4].

Filamentous Fungi Present Two Major Advantages Over Other Microorganisms When Used As Soil Bioremediation Agents. The First Advantage Refers To The Morphological Characteristics Inherent To The Group, Such As Size And Structure, Enabling Greater Mechanical Contact With Neighboring Areas And On The Surface Of The Soil, Resulting From An Average Displacement Of Hyphae By About 1 Cm^2 Per Day [5][6].

The Second And No Less Important Advantage Of Filamentous Fungi On Bacteria Concerns The Non-Specificity Of Their Enzymes. It Is Known That The Enzymatic System Of Filamentous Fungi Involves Three Main Groups: Lignin Peroxidases, Manganese Peroxidases And Laccases. They Are Needed For The Degradation Process Given The Complex Molecules, Such As Carotenoids, Lignin, Alkaloids And Terpenoids, Occurring In Vegetal Material, Which Is The Main Substrate Of These Microorganisms [7][8][9].

Enzymes Are Released In High Concentrations In An Attempt To Overcome The Sorption Or Complexation Losses Of The Substrate. Given This, The Filamentous Fungi Promote The First Attack On Petroleum Hydrocarbons, Conferring An Ecological Role Of Extreme Importance By Allowing The Formation

Of Less Toxic And/Or More Soluble Metabolites That Can Be Used By Other Microorganisms, Including Other Fungal Species, As Well As By Bacteria [10][11].

Thus, The Aim Of This Work Was To Isolate And Identify Potentially Hydrocarbonoclastic Filamentous Fungi Recovered From Soil And Diesel Oil Stored In Tanks Of Petrol Stations.

II. EXPERIMENTAL MATERIALS AND METHODS

2.1. Samples And Collection Points

The Study Included Two Types Of Samples, Collected In Appropriate Sterilized Containers And Under Aseptic Conditions, At Three Petrol Stations In The Metropolitan Region Of João Pessoa, A Municipality Located In The Northeast Region Of Brazil (Table 1). For The Soil Samples, About 50 g Were Taken Between The Surface And At A 15 cm Depth, Near The Pumps Or In The Surrounding Area. Samples Of 1L Of Fuel Were Purchased.

Tab. 1 Location Of Collection Points For Soil And Fuel Samples.

Site	Global Position System
01	7°07'58"S 34°51'37"W
02	7°07'42"S 34°52'06"W
03	7°06'51"S 34°53'24"W

2.2 Isolation Of Hydrocarboclastic Filamentous Fungi

Soil Fungi Were Isolated According To The Modified Method Described By Ben Said Et Al. (2008) [12]. About 10 g Of Soil Were Transferred To Vials Of 500 mL Capacity Containing 100 Ml Of Nutrient Broth And Incubated At Room Temperature For 15 Days. Then, Samples Were Inoculated At The Central Point Of The Surface Of Sabouraud-Dextrose Agar (SDA) To Which Had Been Added 50 mg/L Of Amoxicillin. After An Incubation Period Of 3 To 5 Days At 30°C, The Colonies Were Transferred To Tubes Containing Agar And Kept Under Refrigeration At 4°C.

Isolates From Diesel Oil Were Obtained Using A System Using The Method Described By Brown Et Al. (2010) [13]. A Volume Of The Fuel (50 mL) Was Transferred To A 500 mL Vial Containing 12.5 mL Of Sterile Distilled Water And 12.5 Ml Of Mineral Broth, With The Following Composition: K_2HPO_4 (0.5 g/L); $(NH_4)_2SO_4$ (0.5 g/L); $MgSO_4$ (0.5 g/L), $FeCl_2$ (10 mg/L); $CaCl_2$ (10 mg/L); $MnCl_2$ (0.1 mg/L) And $ZnSO_4$ (0.01 mg/L), pH 7.2±0.2, To Which Had Been Added Traces Of Yeast Extract And Vitamins (7.2±0.2). The System Was Incubated For 14 Days Under Agitation Of 150 RPM At 30°C, And The Fungal Isolate Was Obtained As Described For The Soil Samples.

2.3 Identification Of Filamentous Fungi

Identification Was Performed By The Coverslip Technique [14]. Initially The Microorganisms Were Collected In Test Tubes Containing SDA, Where The Culture Was Kept At Room Temperature For 15 Days And Then Under Refrigeration At 4°C.

Fragments Of The Fungal Culture Were Inoculated In The Center Of The SDA Surface. The Plates Were Then Maintained At Room Temperature. Observations Were Performed Over 15 Days At 48h Intervals, Except On The Last Day, When The Interval Was 24h. The Measurements Were Carried Out With A Millimeter Ruler And Were Used To Construct A Graph For Radial Growth Observation.

For The Evaluation Of The Microstructures, The Coverslip Plate Culture Technique Was Performed. Fragments Of The Fungal Colony Were Strategically Placed On The Surface Of The SDA In The Petri Dishes And Covered With A Pre-Flanged Coverslip. After Growth, The Coverslips Were Removed Every 24 Hours And Placed Inverted On A Slide Containing Violet Crystal Dye And Observed Under An Optical Microscope. All The Experiments Were Carried Out At The Laboratory Of Environmental Microbiology Of The Biotechnology Center Of The Federal University Of Paraíba.

2.4. Hydrocarbonoclastic Activity Test

A Rapid Oil Drop Collapse Test Was Carried Out Using Microdilution Plates Containing 1.5 mL Of Mineral Broth, 25 mL Of Fungal Suspension (10^8 CFU/mL), 10 mL Of Oil (Diesel And Lubricating Oil) And 1,5 mL Of A 10% Solution Of 2,6-Dichlorophenol Indophenol 10% (DCPIP). The Plates Were Incubated At Room Temperature For 10 Days With Observations Each 24h Until The Vanishing Of The Blue Color [15].

III. RESULTS AND DISCUSSION

Under The Conditions Offered In This Study, It Was Possible To Obtain Eight Isolates From At Least Three Different Genera Of The Ascomycota (Table 2).

The Mycelial Growth Of Fungal Isolates, With The Exception Of TP13, Was Evaluated For 15 Days In SDA (Fig. 1). The Isolate TP01 Showed A Faster Growth. On The Eighth Day Of Growth, It Occupied The Entire Surface Of The Culture Medium Contained In The Petri Dish.

Tab. 2 Origin, Identification Of Fungal Isolates And Oil Drop Collapse Time

Strains	Source	Genera	Oil Drog Colapse (Days)	
			LO	D
TP01	Fuel	*Paecilomyces* Sp	3	2
TP02	Soil	*Penicilium* Sp	2	3
TP04	Soil	*Aspergillus* Sp	3	4
TP05	Soil	*Aspergillus* Sp	2	3
TP06	Soil	*Aspergillus* Sp	2	3
TP07	Soil	*Penicillium* Sp	2	3
TP08	Fuel	*Paecilomyces* Sp	2	3
TP13	Soil	*Aspergillus* Sp	2	3

LO – Lubricating Oil, D – Diesel

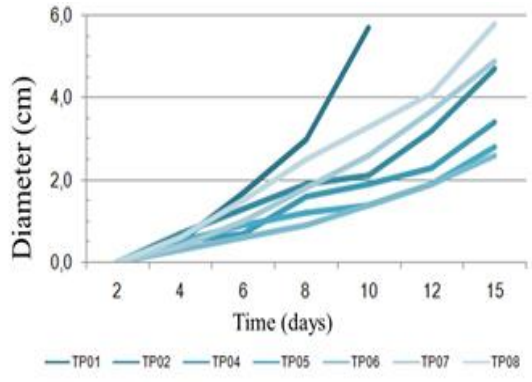

Fig. 1 Size Of Seven Fungal Colonies For 15 Days In Sabouraud-Dextrose Agar.

These Results Indicated That The Culture Medium Used Was Effective In Promoting Growth And Sporulation At Room Temperature (±30°C), As It Provided The Nutritional Supply Required For The Different Fungal Isolates, Favoring *In Vitro* Development.

Fig. 2 Illustrates The Macroscopic Appearance Of Colonies TP01 And TP05, Exhibiting Variations In Texture And Staining. The TP01 Colony Presented Aerial Mycelium Of Cottony Appearance, Containing Few Conidia And A Brown To Cream Pigmentation (Front And Back Of The Colony). The TP05 Colony Showed A Velvety Dark Green Appearance With White Borders, A Mass Of Greenish-Colored Conidia And A Creamy Pigmentation On The Back Of The Colony.

The Vegetative And Reproductive Structures Present In The Different Fungal Isolates Were Analyzed By The Coverslip Technique. For All Isolates, Only The Formation Of The Branched Mycelium Was Displayed In The First 24 Hours. From The 48 Hours, The Mycelial Differentiation Could Be Observed, Where Morphological Differences Between The Vegetative And Reproductive Structures Of The Different Isolates Were Identified.

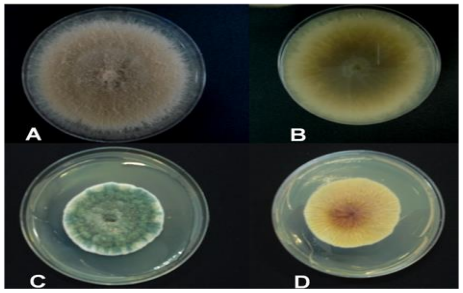

Fig. 2 Macroscopic Aspects Of Two Isolates In Sabouraud-Dextrose Agar. TP01, Front (A) And Back (B) And Soil: TP05, Front (C) And Back (D).

The TP01 And TP08 Isolates From Diesel Oil Presented Single Conidiophores And Verticillate Twigs With Two To Four Phialides. The Phialides Displayed An Intumescent Basal Portion And An Elongated Portion Which Produced And Sustained The Ellipsoid Conidia. These Characteristics Identified The Species As The Genus *Paecilomyces*.

The Isolates TP02 And TP07 (Soil) Were Biverticilated, With Conidiophores, Metulae And Rough Phialides, In Which Spherical Conidia Were Distributed. These Structures Are Characteristic Of The Genus *Penicillium*. In The TP04, TP05, TP06 And TP13 Isolates Collected From The Soil, Characteristic Structures Of The Genus *Aspergillus* Were Identified, For Example, As Simple Conidiophores Distributed In The Form Of Bunches And Arranged On A Vesicle.

Conidia Are Reproductive Elements Of Asexual Origin Capable Of Withstanding Adverse Conditions, And Germinating Under Favorable Conditions, Ensuring The Propagation Of The Species [16]. The Different Morphological Types Of Conidia Found In The Isolates Of *Paecilomyces* sp, *Aspergillus* sp And *Penicillium* sp Are Characterized As Ectospores Because They Are Formed At The Extremities Of Special Hyphae, Called Conidiophores [17]. The Arrangement Of The Conidiophores, The Structures Where The Conidia Are Deposited And The Form Of The Conidia Are Essential Taxonomic Characteristics To Distinguish The Species Within The Different Genera [18].

Paecilomyces sp, *Aspergillus* sp And *Penicillium* sp Are Considered Eucarpic Fungi [19], With Characteristics Easily Identified In Leaflet Culture. Reproductive Structures Appeared Only In One Part Of The Somatic Structures, The Remaining Part Being The Vegetative Form. In General, The Somatic Structures Are Different From The Reproductive Ones And They Have A Diversification Of Forms On Which Species Classification Is Also Based [20].

All The Genera For Which The Isolates Were Identified Have Been Reported In The Literature As Bioremediation Agents Of Soils Contaminated By Petroderivatives Or Crude Oil, Either As Axenic Cultures Or In Consortia [21][22].

In Light Of This, Our Study Aimed To Obtain Hydrocarbonoclastic Organisms For Bioremediation Studies. The Type Of Impacted Area Chosen For The Collections Was Decided Based On The Premise That At The Petrol Stations, Where Conservation Status Was Visually Compromised, The Contamination History Would Guarantee Already Adapted Organisms.

In The Hydrocarbonoclastic Activity Test, With The Exception Of The Strains Recovered From Fuel Samples, The Lubricating Oil Drop Collapsed Before The Diesel Oil (Table 2). This Suggests A Preferential Use Of C20-C40 Aliphatic Compounds As Sole Source Of Carbon, As Well As The Possible Interference Of The Preservatives Added To Diesel Oil On The Growth Of Microorganisms Is This Fuel Mixture [11]. In Addition, The Fungal Strains Isolated From The Diesel Were More Adapted To This Fuel Than The Strains Recovered From Soil Samples.

Although Brazilian Regulates The Environmental Pollution Prevention And Control Of Petrol Stations [23], Most Of These Facilities Still Represent A Great Risk Due To The Location And The Poor State Of Conservation Of Their Storage Tanks. These Tanks Are Subject To Chemical And Microbiological Corrosion, Contributing To Leaks And Resulting In Soil Contamination And Changes In Soil Biota [24].

A Surprising Finding Of The Study Was The Obtaining Of Two *Paecilomyces* sp. In The Diesel Samples. This Is An Explicit Sign Of The State Of Conservation Of The Tanks, Signaling The Risk Of Contamination Of The Stored Product And, Consequently, Revealing An Imminent Environmental Impact [25]. According To Literature, The Occurrence Of Hydrocarboclastic Microorganisms In Storage Tanks Is Based On Two Aspects: The Presence Of Water And Ability Of The Microorganisms To Use Carbon From The Fuel [26].

The Presence Of An Intermediate Zone Between Stored Fuel And Water Carried By Rain Is A Favorable Medium For Microbial Growth In Tanks [27]. Additionally, Water May Also Originate From Other Sources, Such As The Fraction In The Composition Of The Fuel Itself, The Residue Produced In The Cleaning Operations, Ineffective Waterproofing, Condensation Of Air Droplets And Infiltration During The Replacement Procedure In The Tanks [25].

The Detection Of Filament Fungi In Diesel Also Indicates That Other Micro-Organisms, Such As Bacteria And Yeast, May Be Present, Which Implies Possible Damage To The Facility And To The Consumer. Future Studies With The Isolates Will Be Able To Characterize Them With Respect To The Biodegradable Potential For Their Use In Tests Aiming At The Removal Of Petroleum Hydrocarbons In Soils.

IV. CONCLUSIONS

All Strains Of Filamentous Fungi Recovered At Petrol Stations Were Recognized For Their Potential As Bioremediation Agents. On The Other Hand, Obtaining Fungi In The Stored Fuel Samples Reflects The Conditions Of The Storage Tanks, Where The Degree Of Contamination Is Not Known, Nor The Possible Consequences Involved, With Respect To The Environmental Impacts, Corrosion Of The Tanks And Deterioration Of Stock.

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