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Resistance to Heavy Metals and Enzymatic Production of Actinomycetes and Micromycetes Isolated from Soils of Lifoula and Dolisie Landfills (Republic of Congo)

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Abstract: Heavy metals are a cause of concern worldwide due to their negative effects in soils. This work aimed to study the growth and enzymatic production of actinomycetes and micromycetes in heavy metal-polluted soils from public dumps in Lifoula and Dolisie (Republic of Congo). The soil samples were sterilely taken from a depth of 5 to 10 cm and stored in the refrigerator at 4°C. Isolation, purification and identification were carried out on ELAL and PDA media after heat treatment of the samples at 60°C for 60 mn ; resistance to heavy metals by the growth test on ELAL and PDA media enriched with Lead (Pb), Copper (Cu), Zinc (Zn), Nickel (Ni), Chromium (Cr) and Tin (Sn), and production enzymatic on LB medium. The results obtained revealed that all actinomycete isolates belonged to the genus Streptomyces and mycelial isolates to the genera *Penicillium* (C1L and C1D), *Aspergillus* (C2L and C3D), *Rhisopus* (C2D) and *Rhizomucor* (C4D). Heavy metal resistance revealed that isolate A1L (*Streptomyces sp*.) was resistant to Pb, Cu and Sn ; isolate A1D (*Streptomyces sp*.) was tested for Pb and Ni ; isolate A3D (*Streptomyces sp*) with Pb, Ni and Sn. Isolates C1L (*Penicillium sp*.), C2D (*Rhizopus sp*.) and C3D (*Aspergillus sp*.) were the most resistant to all heavy metals with inhibition percentages less than 40%. Out of 11 isolates tested, 7 isolates (A1L, A2L, C1L, C2L, C1D, C2D, C3D) produced protease : 3 cm in diameter for isolate C1L. All isolates degraded tween 80 ; produces amylase and cellulase : 3 cm in diameter for C1D and C1L isolates and lipases with 2 cm for C4D. These results show that the soils of the Lifoula and Dolisie landfills contain microorganisms resistant to heavy metals and producers of enzymes. These isolates can be used in waste management and bioremediation of environments polluted by agri-food effluents.

Keywords: *actinomycetes, enzyme, heavy metals, micromycetes, soil*

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1. Introduction

The collection, disposal and management of domestic waste remains a public health problem in African cities because it leads to heavy pollution of all vital resources, leading to food security difficulties [1,2]. In Brazzaville, domestic waste collection and disposal services which were previously provided by town halls have since 2016 been provided by the AVERDA Company through a Brazzaville household waste landfill center in Lifoula [3] while in Dolisie, they are sent by municipal services to a landfill where the only treatment they undergo is incineration. Enzymatic hydrolysis is the first step in the biodegradation processes of landfill organic matter ; it is the work of micro-organisms producing extracellular enzymes. These enzymes are responsible for the hydrolysis of macromolecules such as cellulose (main material of wood and paper), proteins and fats into smaller, soluble molecules (sugars, amino acids, fatty acids). These molecules will undergo the stages of acidogenesis, acetogenesis and methanogenesis to form methane (biogas) with a release of carbon dioxide [4] thus participating in the increase in greenhouse effect with a very negative effect on climate change.

Metals released from certain wastes into the soil constitute a problem in the environment due to their persistence. Indeed, unlike organic compounds, they cannot be degraded, either biologically or physicochemically. Although the chemical nature of metals can be changed by oxidation or reduction, their elemental nature remains the same and excludes any possibility of thermal decomposition or microbiological degradation. Therefore,

they tend to accumulate in soils or sediments, where they can develop very complex chemistry due to the abiotic (pH, organic matter, redox potential) and biotiac (microbial activity, plant root) influences of the environment [5]). Indeed, they can be adsorbed and modified in their chemical form. One of the consequences of the accumulation, persistence and behavior of metals in the environment is that complex processes must be put in place to restore contaminated sites [6].

As a result, municipal landfills become sources of environmental pollution due to the accumulation of plastics, heavy metals and organic substances. Chemical processes can be used for the decontamination of polluted sites, but the application of these methods remains costly and specific for certain toxic targets. Another approach that can contribute to the elimination of this contamination is bioremediation, which is a simple technology using microorganisms for effective, less expensive and environmentally friendly detoxification. Also, to date, several microorganisms such as Streptomyces, Aspergillus tereus, Penicillium chrysogenum, Candida utilis and Saccharomyces cerevisiae are today used in bioremediation strategies for sites polluted by heavy metals [7,8]. It is with this in mind that we were interested in the study of the resistance to heavy metals of actinomycetes and micromycetes in the soils of the municipal landfills of Lifoula and Dolisie.

2. Materials and Methods

2.1. Materials

The biological material consisted of soils from public landfills in Lifoula (Brazzaville) and Dolisie, culture media and reagents.

2.2. Methods

2.2.1. Location of Sampling Sites

The two composite soil samples used were each made from a mixture of five soil samples taken from different points of the municipal landfills of Lifoula (Longitude S 04°03'28.9"; Latitude E 015°22'06.1') and Dolisie (Longitude S04°11'33.5"; Latitude E 012°40'14.9").

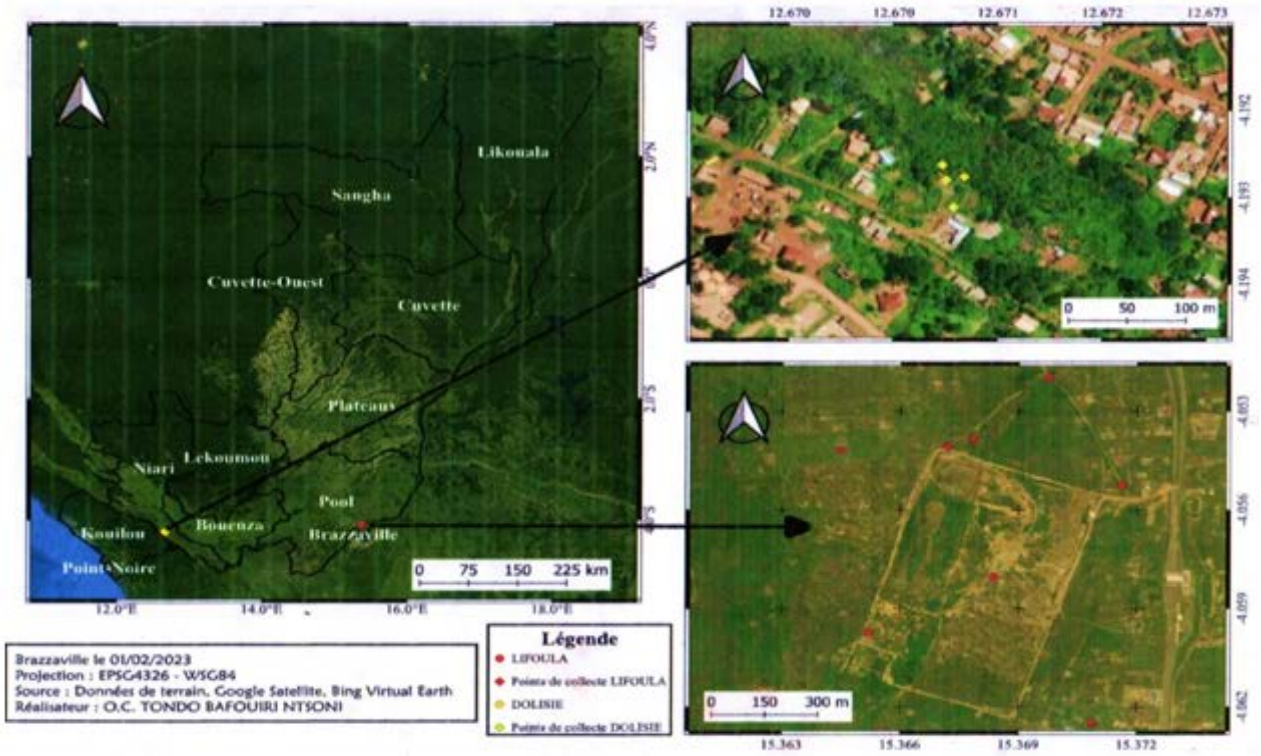


Figure 1. Location of the municipal landfills of Lifoula and Dolisie

2.2.2. Taking Soil Samples

The soil samples were taken using a sterilized spatula at a depth of between 5 and 10 cm and placed in sterile bags then immediately transferred to the refrigerator at 4°C in the laboratory for experiments.

2.2.3. Isolation, Purification and Identification

2.2.4.1. Isolation of Actinomycetes

10g of soil from each sample was placed in an Erlenmeyer flask containing 9mL of sterile distilled water to obtain a stock solution. 1mL of this stock solution was placed in 9mL of saline in a sterile test tube to obtain the dilution of 10-1. From this dilution, a series of dilutions was made up to the 10-5 dilution. 100 µL of each dilution were then inoculated by spreading on the ELAL medium whose composition for 1000 mL is: Yeast extract-Agar (12 g) + soluble starch (5 g) + Skimmed milk (3 mL) + Agar (9 g) . This medium was added to Griséofulvin (25 mg/l) which allows the elimination of certain fungi with its pH adjusted to 7.5. A heat treatment was previously applied to each dilution at 60°C for 60 min before seeding. in order to inhibit the growth of other bacteria [9]. The cultures on petri dishes were incubated at 37°C for 7 days.

2.2.4.2. Purification of Actinomycete Isolates

The purification of the isolates was carried out by successive subcultures on ELAL medium until a homogeneous culture was obtained where all the colonies were identical to each other. The purified isolates are then preserved in a liquid medium (BHIB brain heart infusion broth) supplemented with 1% glycerol and placed at 4°C after 24 hours of incubation at 37°C.

2.2.4.3. Identification of Actinomycete Isolates

The identification of the isolates was carried out based on the appearance of the colonies and microscopic observation which allows the observation of the filamentous appearance of the actinomycetes after Gram staining. The examination of each preparation was carried out using an immersion objective (X 100) [10].

2.2.5. Isolation of Micromycetes

10g of soil from each sample are placed in an Erlenmeyer flask containing 90 mL of sterile distilled water, a stock solution is obtained. A series of dilutions was made from the stock solution ranging from 10-1 to 10-5. A volume of 1 mL of each dilution was spread on the surface of the dishes containing the PDA "potato dextrose agar" medium, suitable for a broader spectrum of fungi. The growth of contaminants is inhibited by the addition of chloramphenicol (5 mg/L) to culture media before sterilization [11].

2.2.5.1. Purification of Micromycetes

After 7 days of incubation, purification of the isolates made it possible to obtain pure cultures from the different colonies. The surface of the Petri dishes containing the sterile PDA medium was divided into four sectors, and each sector was inoculated, using a sterile loop, with a quantity of spores and hyphae from each colony present in the dishes. previous isolation. The plates were incubated at 30°C for 7 days.

2.2.5.2. Identification of Micromycetes

The identification of fungal isolates is based on the observation of morphological criteria by macroscopic and microscopic observation. Macroscopic observation of the colonies makes it possible to carry out an initial characterization (appearance of the colonies and their sides, size and color). Microscopic observation was carried out by placing a quantity of spores as well as mycelia in a drop of distilled water between slide and coverslip. The preparation is then observed under an optical microscope at magnification (X40) then (X100).

2.2.6. Evaluation of the Resistance of Isolates to Heavy Metals

2.2.6.1. Microbial Cultures Used

The isolates obtained on ELAL and PDA media underwent a growth test on these media enriched with heavy metals and their resistance to different heavy metals such as Lead (Pb), copper (Cu), zinc (Zn), nickel (Ni), Chromium (Cr) and Tin (Sn) was evaluated.

2.2.6.2. Preparation of Metallic Solutions

The metallic solutions were prepared from the following heavy metal salts: Pb(NO3)2 (Lead II nitrate); CuSO4.5H2O (Copper II sulfate); ZnSO4.7H2O (Zinc Sulfate heptahydrate); Ni(NO3)2.6H2O (Nickel II nitrate hexahydrate); Cr(NO3)3.9H2O (Chrome III nitrate monohydrate) and SnCl2.2H2O (Tin chloride dihydrate).

2.2.6.3. Resistance Profiles of Actinomycete Isolates to Heavy Metals

From the young colonies obtained, one or two colonies of actinomycetes are taken using a sterile loop and diluted in 1 mL of distilled water, then the turbidity of the whole is measured using a spectrophotometer so as to obtain an optical density ranging from 0.08 to 0.1 at 620 nm which corresponds to 106-107 CFU/mL. The flooding or sheet culture technique is applied for seeding. The entire surface of the solid Muëller Hinton medium is flooded with 100 µL of the inoculum of the actinomycetes isolate then placed in an incubator at 30°C for 15 min in order to promote adhesion of the isolates to the medium. Wells of 8 mm in diameter are prepared on the surface of the Mueller-Hinton medium, previously seeded with the microbial isolates. Each metal solution with a concentration of 20 mM was then placed in the well and the dishes were incubated at 30°C for 24 hours. The activity was evaluated by measuring the inhibition zones after 5 days of incubation for actinomycetes at 30°C. Only cultures showing no inhibition were considered resistant isolates [1].

2.2.6.4. Resistance Profiles of Mycelial Isolates to Heavy Metals

For fungi, 100µL of each of the metal solutions were distributed on the surface of the PDA agar on which 100µL of the inoculum of the isolate tested (105 CFU/mL) were inoculated by completely flooding the dish. After incubation at 30°C for 7 days, the test result is translated into the calculation of inhibition percentages [8]. Inhibition percentages were calculated using the following formula:

PI= [(A-B) /A]x100

PI=Percentage of inhibition of the fungus tested; A= Estimated average diameter of mycelial growth of the fungus on the control medium; B= Estimated average diameter of mycelial growth of the fungus in the presence of the metal solution; A and B, expressed in millimeters (mm), can also represent the heights of the colonies when the growing mycelial strain occupies the entire surface of the agar.

2.2.7. Demonstration of Enzymatic Activities

In order to demonstrate enzymatic production, the isolates were cultured on LB medium with shaking at 37°C after 24 hours of incubation. For each culture, 2mL is used to measure the optical density at 600nm using a Zuzi type spectrophotometer. The culture was centrifuged for 5 minutes and the supernatant recovered, for each isolate, was used to carry out tests to demonstrate all enzymatic activities.

2.2.7.1. Demonstration of Proteolytic Activity

In a 250mL Erlenmeyer flask containing 100mL of 0.1N PBS, 1g of agarose was dissolved and then brought

to the boil until completely dissolved. After cooling to 55°C, 10mL of skimmed milk was added and the mixture homogenized then poured into Petri dishes; after solidification, we prepared wells in the gel and placed 50µL of supernatant from the centrifugation of the culture in each well. The boxes are placed in an oven at 37°C for 12 hours. The observation of a clear translucent area indicates that the isolate produces a proteolytic enzyme with a caseinolytic effect [12,13].

2.2.7.2. Demonstration of Amylolytic Activity

1g of starch and 1.5g of agar are dissolved in 100mL of distilled water and sterilized in an autoclave at 121°C for 15 minutes, the whole is poured onto the Petri dishes and each supernatant is placed in the well corresponding previously carried out in the gel. After 48 hours of incubation at 37°C, the revelation takes place with Lugol; the observation of a translucent area indicates that the isolate produces an amylase enzyme and the diameter of each translucent halo is measured [14].

2.2.7.3. Demonstration of Lipolytic Activity

6g of agar was dissolved in 400mL of distilled water and then mixed. After homogenization, the medium is autoclaved at 121°C for 15 minutes. After cooling the medium to 45°C, 20 drops of Tween 80, previously sterilized, were added using a sterile Pasteur pipette. The medium is poured into the petri dishes and the wells are made on the agar medium. Each well is filled with the supernatant then the boxes are incubated in an oven at 37°C for 24 hours. After incubation, the positive test results in the presence of a clear zone around the well. The clear zone characterizes the hydrolysis of the ester by the isolate studied and the diameter of each clear zone is measured [15].

2.2.7.4. Demonstration of Cellulolytic Activity

In order to demonstrate the cellulolytic activity, a mixture of 4g of cellulose and 6g of agar were dissolved in 400mL of distilled water, sterilized for 15 minutes in an autoclave at 121°C then poured into the petri dishes. The wells were made on the gel and the supernatant placed in each well, the petri dishes were incubated in the oven for 48 hours at 37°C. The revelation takes place with Lugol; the observation of a translucent zone indicates that the isolate produces a cellulase type enzyme and the diameter of each translucent halo is measured [15].

3. Results

3.1. Isolation of Actinomycete Isolates

Figure 2 shows the actinomycete colonies in the soils of the Lifoula and Dolisie landfills. We respectively selected 2 soil isolates from the Lifoula landfill and 3 soil isolates from the Dolisie landfill on the basis of the shape, size and color of the colonies by isolating only one colony among those which present the same characteristics.

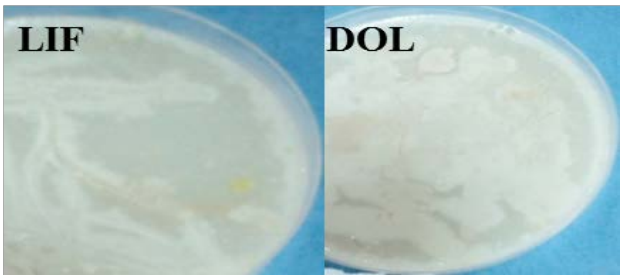
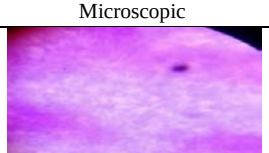
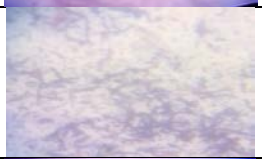


Figure 2. Actinomycete colonies in the soils of the Lifoula and Dolisie landfills

3.1.2. Macroscopic and Microscopic Aspects of Actinomycete Isolates

Table 1 presents the macroscopic and microscopic appearances of the actinomycete isolates from the soils of the Dolisie and Lifoula landfills after 7 days of incubation at 30ºC. The results showed that all colonies were powdery, round, yellow or purple in color, filamentous and Gram+ suggesting the genus *Streptomyces*.

Table 1. Macroscopic and microscopic aspects of actinomycete isolates from soils of Dolisie and Lifoula landfills

Isolates		Microscopic	Macroscopic	Morphology	Genus
Dolisie	A_1L		Dark yellow round powdery colonies of 3mm	Gram+ filaments	*Streptomyces*
	A_2L		Round purple powdery colonies of 5 mm	Gram+ filaments	*Streptomyces*
Lifoula	A_1D		Round purple powdery colonies of 3mm	Gram+ filaments	*Streptomyces*
	A_2D		Yellow round powdery colonies of 2 mm	Gram+ filaments	*Streptomyces*

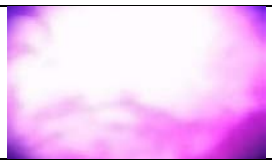
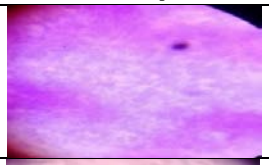
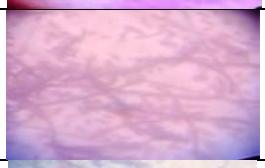
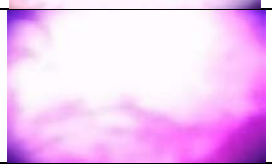
	A_3D		Round purple powdery colonies of 5 mm	Gram+ filaments	*Streptomyces*

Table 2. Phenotypic identification of mycelial isolates from the soils of Lifoula and Dolisie landfill

Isolates		Microscopic	Macroscopic	Morphology	Genus
Dolisie	A_1L		Dark yellow round powdery colonies of 3mm	Gram+ filaments	*Streptomyces*
	A_2L		Round purple powdery colonies of 5 mm	Gram+ filaments	*Streptomyces*
Lifoula	A_1D		Round purple powdery colonies of 3mm	Gram+ filaments	*Streptomyces*
	A_2D		Yellow round powdery colonies of 2 mm	Gram+ filaments	*Streptomyces*
	A_3D		Round purple powdery colonies of 5 mm	Gram+ filaments	*Streptomyces*

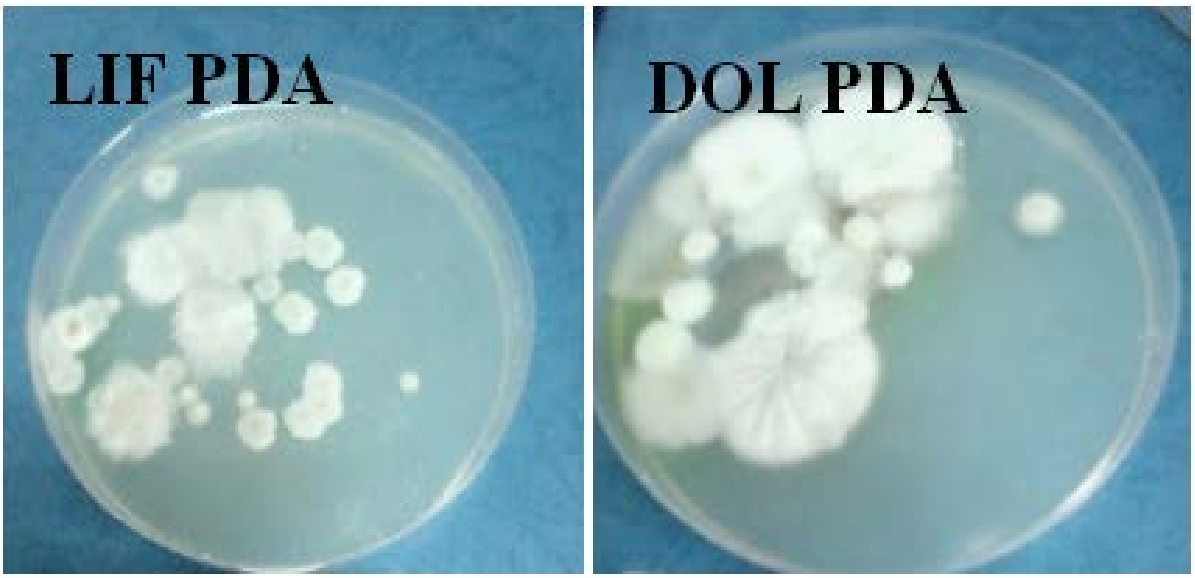


Figure 3. colonies of micromycetes in the soils of the Lifoula and Dolisie landfills

3.2. Phenotypic Identification of Micromycete Isolates

3.2.1. Isolation of Micromycete Isolates

Figure 3 shows the colonies of micromycetes in the soils of the Lifoula and Dolisie landfills. Observation of the shape of the colonies and microscopic aspects made it possible to select two soil isolates from the Lifoula landfill and four (4) soil isolates from the Dolisie landfill.

3.2.2. Macroscopic and Microscopic Aspects of Micromycete Isolates

Table 2 shows the phenotypic identification of mycelial isolates from the soils of the Lifoula and Dolisie landfills. The micromycetes isolated from the soils of the Lifoula

and Dolisie landfill were identified using their macroscopic and microscopic characteristics. It was observed that all the colonies were powdery, round, blackish white in color in the center or black and, filamentous which orients towards the genera *Penicillium, Aspergillus, Rhizopus and Rhizomucor.*

3.3. Heavy Metal Resistance Profiles of Heavy Metal Isolates

Figure 6 illustrates the effect of heavy metals on the soil isolate from the Lifoula landfill.

3.3.1. Heavy Metal Resistance Profile of Actinomycetes Isolates

Table 3. Heavy metal resistance profiles of actinomycete isolates from soils of Lifoula and Dolisie landfills

Isolates	Average diameters (mm)					
	Pb	Cu	Zn	Ni	Cr	Sn
A_1L	0	0	12	11	13	0
A_2L	10.6	10	10	10	11	10
A_1D	0	11.6	11	0	11	10
A_2D	11	10.3	12	11.2	10	12
A_3D	0	10	16.6	0	10	0

Table 3 presents the heavy metal resistance profile of actinomycete isolates from the Lifoula landfill soils. The results indicated variable resistance depending on the isolate. Resistance to Pb, Cu and Tin (Sn) was observed in isolate A1L compared to sensitivity to Zn, Ni and Cr. The A3D isolate is resistant to Pb, Ni and Tin (Sn) while it is more sensitive to Zn with 16.6 mm in diameter. Isolate A1D was resistant to Pb and Ni. Isolates A2L and A2D were sensitive to all heavy metals.

3.3.2 Heavy Metal Resistance Profiles of Mycelial Isolates

Table 4 shows the effect of different heavy metals on the growth of micromycetes in the two soil samples. Inhibition of growth of isolated micromycetes was observed to varying degrees. All isolates were sensitive to different heavy metals.

Table 4. Heavy metal resistance profiles of micromycetes isolated from the soil of the Lifoula and Dolisie landfill

Isolates	Inhibition percentages (%)					
	Pb	Cu	Zn	Ni	Cr	Sn
C_1L	33.33	36.66	36.66	33.33	36.66	33.33
C_2L	66.66	57.56	63.33	36.36	46.66	56.67
C_1D	66.66	32.62	72.72	78.78	66.66	57.57
C_2D	32.62	16.66	33.33	16.66	16.66	16.66
C_3D	16.66	33.33	16.66	34.66	32.62	16.66
C_4D	43.33	46.66	56.66	66.66	63.33	53.33

3.3.1. Demonstration of Enzymatic Activities

Figure 4 shows translucent halos indicating enzymatic digestion of different isolates. These halos indicate the production of proteases, amylases, lipases and cellulases.

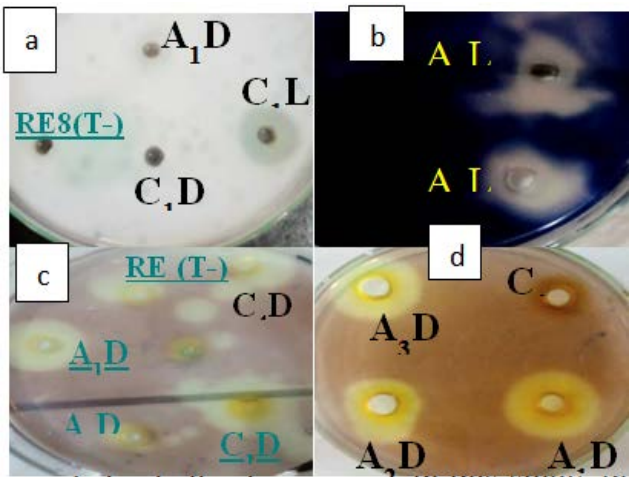


Figure 4. Translucent halos indicating enzymatic digestion by certain isolates: a = skimmed milk; b = starch; c= tween 80 starch and d = cellulose

3.3.2. Enzyme Production

Figure 5 shows the enzymatic production of actinomycetes and micromycetes isolates. The production of proteae was observed in isolates A1L and A2L of actinomycetes with respective diameters of 2 and 1.5 cm and, with micromycetes, isolates C1L, C2L, C2D, C3D and C4D or, isolate C1L presents a diameter of 3cm.

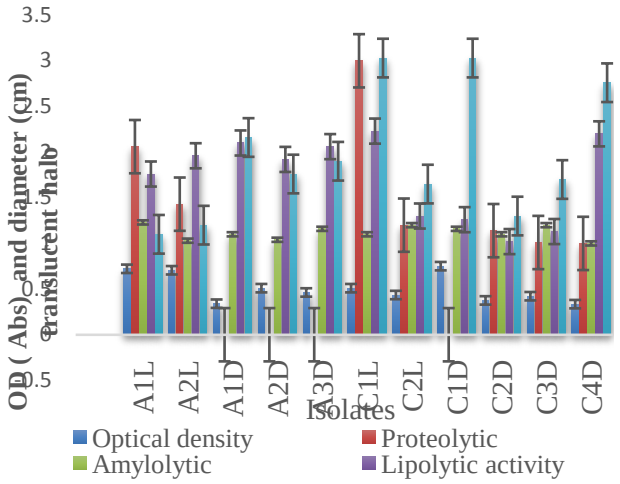


Figure 5. Variation in OD and enzyme production by differents isolates

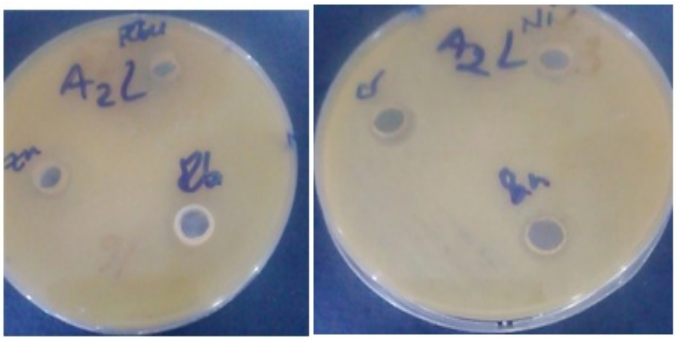


Figure 6. Effect of heavy metals on the A2L isolate from the soil of the Lifoula landfill

4. Discussion

In this study, the aim was to evaluate the growth and enzymatic production capacities of actinomycetes and micromycetes isolated from the soils of the Lifoula and Dolisie landfills in the presence of heavy metals. Heavy metals, in fact, because of their potential negative effects on soils, raise a lot of concerns about soil fertility and groundwater quality. From our experiment, it appears that all the actinomycete isolates obtained after identification were oriented towards the genus Streptomyces while the mycelial isolates were oriented towards four different genera: Penicillium for isolates C1L and C1D; Aspergillus for isolates C2L and C3D; Rhisopus for isolate C2D and the genus Rhizomucor for isolate C4D. The study of resistance to heavy metals was carried out using a 20 mM concentration solution since the MICs of most heavy metals on Cupriavidus metallidurans CH34, a model bacterium of resistance to heavy metals, hardly exceed 15 mM [16]. The results of this analysis showed that isolate A1L was resistant to Pb, Cu and Sn, isolate A1D to Pb and Ni and isolate A3D to Pb, Ni and Sn while isolates C1L, C2D and C3D were resistant to all heavy metals with inhibition percentages lower than 40%. The results of our study are similar to those of previous studies carried out by other researchers. Indeed, the genus Streptomyces has been reported to be very useful in the bioremediation of soils contaminated by heavy metals. This genus is able to resist in the presence of high concentration of heavy metals such as Cadmium, Mercury, lead, nickel and iron and Copper [17,18]. The results of our experiment showed that out of the 11 isolates tested, 7 isolates (A1L, A2L, C1L, C2L, C1D, C2D, C3D) produced proteases; the largest diameter was observed with isolate C1L: 3cm. All isolates degraded Tween 80 and produced amylase and cellulase with 3cm diameter for isolates C1D and C1L and lipases with 2cm for isolate C4D. These results suggest that our isolates can be used in waste management and bioremediation of environments polluted by agri-food effluents and heavy metals. This degradation results in a transformation of large insoluble organic molecules into simple molecules serving as substrates for other microorganisms [19]. Also, all these enzymes (protease, amylase, lipase and cellulase) have several industrial applications [20,21,22].

5. Conclusion

This study aimed to show how actinomycetes and micromycetes adapt to heavy metal polluted soils in the soils of public landfills of Lifoula and Dolisie in the Republic of Congo. From the results, it appears that all actinomycete isolates are of the genus Streptomyces while those of micromycetes were of the genera Penicillium (C1L and C1D), Aspergillus (C2L and C3D), Rhisopus (C2D) and Rhizomucor (C4D). The sensitivity was a function of the isolate and the type of heavy metals: isolate A1L (Streptomyces sp.) was resistant to Pb, Cu and Sn; isolate A1D (Streptomyces sp.) was resistant to Pb and Ni; isolate A3D (Streptomyces sp.) to Pb, Ni and Sn. Micromycete isolates C1L (Penicillium sp.), C2D (Rhizopus sp.) and C3D (Aspergillus sp.) were the most resistant to all heavy metals with inhibition percentages lower than 40%. Out of 11 isolates tested, 7 isolates (A1L, A2L, C1L, C2L, C1D, C2D, C3D) produced proteases : 3 cm in diameter for isolate C1L. All isolates degraded Tween 80; produced amylase and cellulase: 3 cm in diameter for C1D and C1L; lipases with 2 cm for C4D. These partial results show that the soils of public landfills contain microorganisms with valuable potential in the bioremediation of polluted soils. This study should be continued with a specific identification by biomolecular technics.

References

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