Tropical Journal of Applied Natural Sciences
Vol. 2 Issue 2 (2024)

Tropical Journal of Applied Natural Science
ISSN: 2449-2043

Isolation, Screening and Identification Of Multi – Metal Resistant Fungi Isolated From Biogas Slurry Sample

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Abstract

The potentials of filamentous fungi as bioaccumulating agents of heavy metal toxicants in soil or aquatic ecosystems have been widely documented as better and efficient option for bioremediation technique. This study was undertaken to isolate, screen and identify multi – metal resistant fungi isolated from biogas slurry samples. Water samples were collected, fungi strains isolated, screened for multi - resistant abilities and finally identified using standard microbiological and molecular procedures, respectively. The results revealed that there were increase in the growth diameters of the fungal strains as the concentrations (10 - 200 pm) of all heavy metals from day 0 to day 20. Some of the metals were inhibitory while some were stimulatory effects at certain levels to the fungal biomass. The growth of the fungi at increasing concentrations revealed that these strains especially strains S6 and S9 possess multi – resistant genes and thereby making adaptable to these inorganic pollutants. The strains S6 and S9 were later identified as *Aspergillus niger* and *Colletotrichum* sp., respectively and their heavy metal bioremediation applicabilities to heavy metal contaminated soil or aquatic environment are therefore recommended.

Keywords: *Aspergillus niger*, Bioaccumulation, *Colletotrichum* sp., Heavy metal, Multi - Resistant

INTRODUCTION

The poisoning of soil, water, and air by harmful chemicals is one of the main environmental issues that the world is currently facing. The widespread usage of pesticides in agriculture and industrialization has led to a significant issue with environmental contamination from artificial substances. Furthermore, because toxic metal ions are non-biodegradable and can build up in living tissues to become concentrated throughout the food chain, which can result in a variety of diseases and ailments, the rising contamination of groundwater by these ions offers a serious environmental risk (Madhavi *et al*., 2013).

Heavy metals are considered one of the most dangerous environmental pollutants created as a result of human economic activities, both agricultural and non-agricultural (Tkaczuk *et al*., 2019). In the soil they react with other chemicals, accumulating in different forms with differentiated bioavailability to plants (Singh and Kalamdhad, 2011). The solubility of heavy metals in soil, and hence their biological effects, decreases with increasing content of organic matter or clay minerals, and increases under higher acidity (Tkaczuk *et al*., 2019). Metals in natural concentrations are often essential in the functioning of living organisms, but often have toxic effects when present in excessive amounts (Singh and Kalamdhad, 2011). Additionally, their presence in soil has a major impact on cellular structures, growth and development, and biological activity of soil microorganisms (Hassn *et al*., 2014; Tkaczuk *et al*., 2019).

The abiotic and biotic methods have been employed for the elimination of heavy metals from aqueous medium. Frequently applied abiotic methods include chemical precipitation, chemical reduction, carbon adsorption, ion exchange, solvent extraction, reverse osmosis, membrane

process, evaporation and electrochemical processes. These methods are considered as in effective due to some technical restrictions such as sludge production, operational intricacies or economic constraints. Therefore, the research has been focused towards biotic methods due to its potential applications in environmental protection and recovery of toxic or strategic heavy metals (Hansda *et al*., 2015). Among the different methods, bioaccumulation and biosorption have been demonstrated to possess good potentials for the removal of heavy metals. Bioaccumulation is the active uptake of toxicants by living cells however; biosorption mechanism is the passive uptake of toxicants by dead/inactive biological materials.

One of the identified reducing agents for heavy metals is the use of microorganisms like fungi. Fungi can tolerate and detoxify metals in many ways. It could be through valence transformation, active uptake, precipitation inside or outside their cells, and biosorption (Tualla and Bitacura, 2016). The high surface-volume ratio of microorganisms and their ability to detoxify metals are among the reasons that they are considered as a potential alternative to synthetic resins for remediation of dilute solutions of metals and solid wastes (Manguilimotan and Bitacura, 2018). The use of fungi, for instance, gained importance because it is eco-friendly, economical, and effective. The cell wall of fungi consists of polysaccharides and proteins that over multiple active sites for binding of metals. The polysaccharides found in the cell walls of fungi are chitin and chitosan, which have been shown to sequester metal ions (Manguilimotan and Bitacura, 2018). Considering the mechanisms of metal resistance by fungi, it is expected that screening of metal tolerant fungi may provide strains with improved metal accumulation. Only limited studies have been conducted in the Nigeria to systematically screen filamentous fungi from biogas slurry for

their multi - metal tolerance. Therefore, the present study was undertaken to isolate, screen and identify multi – metal tolerant fungi isolated from biogas slurry samples.

MATERIALS AND METHODS

Material and Reagent

Metallic salts and other chemicals of analytical grade that will be used in this study will obtained from Loba Chem Company, India.

Slurry Sample Collection

Prior to the experiments and when the slurry was fully made, two samples (100 mL each) from two biogas production plants and drum composter both located at Uli Ihiala Local Government Area Anambra State, Nigeria were collected aseptically using 100 mL sterile 1 L plastic container and properly labelled with sample name, source, time and date. The two 1 L plastic containers were sealed with cotton wool, placed inside polyethene bag and then transported to the Postgraduate Microbiology Laboratory, COOU Uli for analysis as described by Uba *et al*. (2018).

Isolation of Fungi

By adopting the modified method of Uchendu and Mbonu (2020), 10 mL of the spent slurry from biogas plants were aseptically pipetted and placed into 90 mL of sterile physiological saline to form aliquot. A tenfold serial dilution of the slurry suspension was carried out by transferring 1 mL each of the aliquot into test tubes containing 9 mL of sterile physiological saline arranged serially in the order 10^{-1} – 10^{-4}. The amount of 0.1 mL at 10^{-4} dilution was spread over culture plates containing sterile Potato Dextrose Agar (PDA), supplemented with 100 mg ml^{-1} chloramphenicol and 15 mg ml^{-1} of penicillin to inhibit bacterial growth. The samples were uniformly spread on the surface of the medium with a sterile glass rod. All the plates were incubated at 28 ± 2 °C for up to 4 - 7 days. The emerging fungi were transferred to fresh PDA

plates, incubated at conditions above and periodically checked for purity. The predominant forms of fungal growth were tentatively selected and given a laboratory isolated number after purification.

Screening for the Heavy Metal Resistant Potential Strain

The isolates were screened for their potentials to tolerate multi - metals by adopting the modified methods of Rani *et al*. (2014) and Tkaczuk *et al*. (2019). Initially, the fungal strains were grown on culture plates pre-filled with Potato Dextrose Agar (PDA) and incubated at 28 ± 2 °C for 7 days. Following incubation, mycelial agar plugs (6 mm^2) were cut approximately 5 mm from the colony margin and centrally inoculated on the surfaces of prepared sterile potato dextrose agar (PDA) plates containing increasing metal mixture concentrations of 25 ppm, 50 ppm, 75 ppm and 100 ppm. The plates were incubated as previously described above and the colony diameter was measured at 10 and 20 days after inoculation. Each metal experiment and the control with no metals were replicated two times. The two colonies with the most outstanding growths on the highest metal mixture concentration were considered as heavy metal tolerant fungal strains (Manguilimotan and Bitacura, 2018).

Characterization and Identification of the Most Tolerant Fungal Strain

The selected dominant and multi - metal resistant fungal strains were preliminary identified according to its macroscopic and microscopic characteristics (Uba *et al*., 2019). It was later identified molecularly to species levels for 16S rRNA genes identification using DNA extraction, gel electrophoresis, polymerase reaction, sequencing and blasting techniques (Uba *et al*., 2018)

RESULTS AND DISCUSSION

The result of the colony growth diameters of different fungal isolates on mercury metal at 20 days is presented in Table 1. From the Table 1, isolate S9 had the highest colony growth diameter of

7.80±0.08 mm at 10 ppm while isolate S9 had the lowest colony growth diameter of 0.86±0.17 mm at 100 ppm. The result of the colony growth diameters of different fungal isolates on lead metal at 20 days is presented in Table 2. From the Table 2, isolates S2 and S9 had the highest colony growth diameter of 8.40±0.08 mm at 50 and 200 ppm while isolate S5 had the lowest colony growth diameter of 2.13±0.05 mm at 10 ppm. The result of the colony growth diameters of different fungal isolates on cobalt metal at 20 days is presented in Table 3. From the Table 3, isolate S9 had the highest colony growth diameter of 8.40±0.08 mm at 20 ppm while isolate S6 had the lowest colony growth diameter of 0.73±0.05 mm at 200 ppm. The result of the colony growth diameters of different fungal isolates on copper metal at 20 days is presented in Table 4. From the Table 4, isolate S7 had the highest colony growth diameter of 6.90±0.08 mm at 10 ppm while isolates S5 and S6 had the lowest colony growth diameter of 0.73±0.05 mm at 200 and 20 ppm. The result of the colony growth diameters of different fungal isolates on nickel metal at 10 and 20 days is presented in Table 5. From the Table 5, isolate S6 had the highest colony growth diameter of 8.53±0.09 mm at 50 ppm while isolate S4 had the lowest colony growth diameter of 0.37±0.05 mm at 100 ppm. The result of the colony growth diameters of different fungal isolates on zinc metal at 20 days is presented in Table 6. From the Table 6, isolate S1 had the highest colony growth diameter of 7.30±0.08 mm at 10 ppm while isolates S4, S6 and S9 had the lowest colony growth diameter of 0.73±0.05 mm at 10 and 200 ppm. The result of the colony growth diameters of different fungal isolates on chromium metal at 20 days is presented in Table 7. From the Table 7, isolate S9 had the highest colony growth diameter of 8.23±0.12 mm at 50 ppm while isolate S8 had the lowest colony growth diameter of 2.30±0.16 mm at 200 ppm. The result of the colony growth diameters of different fungal isolates on arsenic metal at 20 days are presented in

Table 8. From the Table 8, isolate S2 had the highest colony growth diameter of 8.20±0.08 mm at 50 ppm while isolate S9 had the lowest colony growth diameter of 0.60±0.08 mm at 50 ppm.

Metal ions of copper, chromium, lead, zinc, mercury, cadmium and arsenic of the waste water samples affected the growth of the fungal isolates in various ways with the results depending on the concentration of the waste water samples (10 - 200 ppm) and days of growth (10 and 20). Heavy metals are highly toxic to living organisms. They contribute to the deterioration of soil and water chemical properties and limit the population of these ecological microorganisms (Lenart and Wolny-Koładka, 2013). After the preliminary isolation on PDA plates, 9 fungal colonies from the two samples, and whenever possible with different morphologies, were selected for isolation and purification. They were exposed to successive enrichment culture procedure on PDB medium amended with low to high concentration (10 – 200 ppm) of eight heavy metal ions (Cr, Co, Pb, Ni, As, Zn, Hg and Cu) to increase the possibility of obtaining heavy metal multi-resistant fungal strains isolated from the 2 sampling sites previously described above (Darwesh *et al*., 2022). The results in Tables 1 – 8 revealed that there were increase in the growth diameters of the fungal strains as the concentrations (10 - 200 pm) of all heavy metals from day 1 to day 20. Some of the metals were inhibitory while some were stimulatory effects at certain levels to the fungal biomass. The growth of the fungi at increasing concentrations revealed that these strains especially strains S6 and S9 possess multi – resistant genes and thereby making adaptable to these inorganic pollutants. Hassn *et al*. (2014) reported that heavy metals in high concentrations, can significantly limit growth, spore germination, and pathogenicity of fungi for in vitro studies. Lead has a great capacity to accumulate in the environment can be particularly toxic to fungus cells. Tkaczuk *et al*. (2019) reported that heavy metal ions in concentration 1× did not significantly restrict the growth

of fungus colonies, and in some cases caused a slight growth stimulation. The greatest toxic effect of heavy metal ions on the entomopathogenic fungi was observed when their concentration was 100 times higher than the natural content. Nickel had the greatest inhibitory effect on the growth of fungal colonies, while lead showed the least effects. The fungal species most sensitive to the presence of heavy metals in the medium was *I. tenuipes*, while *I. fumosorosea* had the highest tolerance.

Table 1: Colony growth diameter of different fungal isolate on mercury metal at day 20

Test Isolate \ Concentration (ppm)	200	100	50	20	10
S1	6.30±0.08	6.30±0.08	6.90±0.08	6.80±0.08	7.00±0.08
S2	3.10±0.16	1.03±0.05	4.40±0.08	4.57±0.12	7.20±0.08
S3	1.50±0.08	1.40±0.08	3.00±0.08	5.30±0.08	7.80±0.08
S4	2.40±0.05	2.50±0.08	5.20±0.08	5.37±0.17	5.30±0.08
S5	6.70±0.16	6.90±0.08	7.30±0.08	4.93±0.17	5.37±0.26
S6	6.10±0.16	2.00±0.08	2.97±0.12	2.43±0.09	4.03±0.12

S7	6.70±0.08	2.40±0.08	4.40±0.08	6.00±0.08	5.57±0.12
S8	5.27±0.12	6.07±0.17	8.00±0.16	6.40±0.08	4.15±0.25
S9	1.17±0.12	0.86±0.17	7.00±0.08	6.63±0.26	3.33±0.12

Table 2: Colony growth diameter of different fungal isolate on lead metal at day 20

Test Isolate / Concentration (ppm)	200	100	50	20	10
S1	7.60±0.008	7.30±0.08	8.10±0.16	5.70±0.08	7.80±0.08
S2	7.90±0.16	7.73±0.12	8.40±0.08	8.20±0.08	8.30±0.08
S3	8.00±0.08	7.40±0.08	8.30±0.08	8.000±0.08	4.40±0.16
S4	7.80±0.08	5.73±0.33	7.37±0.05	7.13±0.08	7.57±0.05
S5	7.50±0.08	8.02±0.08	7.90±0.16	3.40±0.14	2.13±0.05
S6	8.30±0.08	6.00±0.08	4.70±0.08	4.60±0.16	2.90±0.16

S7	5.80±0.16	3.80±0.08	5.80±0.16	4.60±0.16	2.90±0.16
S8	3.55±0.26	5.33±0.12	2.77±0.12	5.13±0.12	3.90±0.16
S9	8.40±0.08	3.07±0.05	3.60±0.16	5.40±0.08	3.30±0.08

Table 3: Colony growth diameter of different fungal isolate on cobalt metal at day 20

Test Isolate	Concentration (ppm)				
	200	100	50	20	10
S1	4.17±0.09	4.80±0.08	4.50±0.08	5.40±0.08	3.97±0.12
S2	7.10±0.08	8.00±0.08	8.23±0.12	8.13±0.12	8.30±0.08
S3	0.90±0.08	2.30±0.16	6.90±0.08	3.17±0.17	3.70±0.16
S4	1.70±0.08	2.33±0.05	2.47±0.12	2.40±0.08	6.87±0.12
S5	4.40±0.08	7.50±0.08	7.37±0.12	8.33±0.12	8.30±0.08

S6	0.73±0.05	1.57±0.05	3.90±0.08	5.00±0.08	4.30±0.16
S7	1.00±0.08	6.80±0.08	6.60±0.08	8.20±0.08	4.50±0.08
S8	4.73±0.17	7.50±0.08	8.30±0.08	5.03±0.09	3.70±0.08
S9	1.33±0.08	2.36±0.21	8.30±0.08	**8.40±0.08**	3.73±0.05

Table 4: Colony growth diameter of different fungal isolate on copper metal at day 20

Test Isolate	Concentration (ppm)				
	200	100	50	20	10
S1	6.50±0.08	6.27±0.12	5.77±0.59	5.40±0.08	4.80±0.21
S2	6.30±0.05	5.70±0.16	4.80±0.16	6.37±0.12	1.13±0.12
S3	5.37±0.12	2.63±0.17	3.70±0.16	2.70±0.16	3.67±0.12
S4	4.10±0.08	4.77±0.12	2.70±0.16	2.47±0.17	5.90±0.16
S5	0.73±0.05	3.67±0.16	4.90±0.14	4.90±0.16	6.70±0.16
S6	3.70±0.08	1.03±0.08	1.00±0.08	0.73±0.05	4.03±0.12
S7	1.97±0.12	5.40±0.08	4.90±0.16	4.87±0.12	6.90±0.08
S8	6.80±0.08	6.20±0.57	4.83±0.12	5.93±0.68	2.53±0.12
S9	6.03±0.08	1.90±0.16	3.70±0.16	3.97±0.12	2.37±0.21

Table 5: Colony growth diameter of different fungal isolate on nickel metal at day 20

Test Isolate	Concentration (ppm) 200	100	50	20	10
S1	7.77±0.12	5.33±0.12	7.27±0.05	6.60±0.08	7.80±0.16
S2	0.73±0.05	2.47±0.12	5.80±0.08	7.00±0.08	3.50±0.08
S3	5.40±0.08	5.17±0.12	2.60±0.08	4.53±0.25	1.27±0.12
S4	0.80±0.08	0.37±0.05	3.87±0.12	6.00±0.08	5.90±0.09
S5	0.86±0.12	4.40±0.08	7.50±0.08	7.57±0.12	8.10±0.08
S6	0.80±0.08	7.00±0.08	**8.53±0.09**	2.73±0.05	8.07±0.12
S7	0.73±0.05	0.63±0.08	0.93±0.17	4.93±0.12	4.30±0.16
S8	4.77±0.56	5.20±0.08	5.00±0.22	3.60±0.16	7.73±0.05
S9	0.73±0.05	8.10±0.08	6.23±0.22	5.70±0.16	4.77±0.34

Table 6: Colony growth diameter of different fungal isolate on zinc metal at day 20

Test Isolate	Concentration (ppm) 200	100	50	20	10
S1	4.00±0.08	3.17±0.17	4.37±0.19	3.90±0.16	7.30±0.08
S2	1.00±0.08	2.37±0.12	2.40±0.08	6.90±0.08	4.70±0.16
S3	0.83±0.09	2.73±0.05	7.10±0.08	2.47±0.03	4.57±0.12
S4	0.87±0.17	0.87±0.17	1.53±0.05	0.80±0.08	0.73±0.05
S5	0.80±0.08	4.50±0.08	2.80±0.08	4.87±0.05	4.47±0.05
S6	0.73±0.05	0.90±0.08	2.43±0.12	3.00±0.08	2.97±0.05
S7	3.17±0.05	1.30±0.08	1.13±0.12	4.17±0.17	6.80±0.08
S8	1.40±0.08	3.10±0.16	3.63±0.12	3.53±0.12	7.20±0.08
S9	0.73±0.05	2.40±0.16	2.00±0.08	6.50±0.08	3.37±0.21

Table 7: Colony growth diameter of different fungal isolate on chromium metal at day 20

Test Isolate \ Concentration (ppm)	200	100	50	20	10
S1	4.30±0.08	4.17±0.09	3.57±0.12	3.80±0.08	4.40±0.08
S2	7.87±0.08	7.80±0.08	2.70±0.16	5.63±0.17	4.80±0.29
S3	6.50±0.08	7.03±0.05	6.40±0.22	4.70±0.16	4.70±0.16
S4	4.80±0.08	3.30±0.16	7.50±0.08	4.57±0.12	5.80±0.16
S5	3.30±0.09	3.37±0.12	4.50±0.08	3.80±0.08	4.80±0.08
S6	4.40±0.08	4.73±0.12	3.97±0.16	4.40±0.16	4.60±0.08
S7	4.30±0.16	4.87±0.17	5.60±0.17	4.40±0.08	4.50±0.08
S8	2.30±0.16	3.62±0.21	3.73±0.17	4.43±0.17	4.67±0.21
S9	6.63±0.09	4.73±0.12	8.23±0.12	5.20±0.08	5.10±0.27

Table 8: Colony growth diameter of different fungal isolate on arsenic metal at day 20

Test Isolate	Concentration (ppm) 200	100	50	20	10
S1	3.50±0.16	6.10±0.16	5.30±0.08	7.73±0.08	7.00±0.08
S2	5.27±0.12	4.80±0.08	8.20±0.08	8.00±0.08	7.85±0.08
S3	5.63±0.08	1.50±0.08	7.10±0.16	7.27±0.17	4.57±0.05
S4	3.80±0.08	6.83±0.16	5.90±0.16	6.70±0.16	5.37±0.05
S5	5.277±0.12	6.80±0.16	5.00±0.08	7.10±0.08	6.50±0.16
S6	O.73±0.05	1.43±0.05	3.00±0.08	6.40±0.08	1.87±0.17
S7	0.73±0.05	3.87±0.05	5.70±0.08	6.30±0.08	7.10±0.08
S8	0.70±0.80	0.90±0.08	3.77±0.12	3.67±0.05	1.87±0.05
S9	7.50±0.08	0.70±0.08	0.60±0.08	1.10±0.08	2.20±0.08

The result of the morphological features of the selected strains of heavy metal resistant mould is presented in Table 9. The result showed that isolate S9 had a compact and flat elevated colony which was white at first and later black in colour. The isolate S9 possesses smooth-walled and hyaline conidiophore. It has dark rough – walled, septate and globose to sub-globose conidia with diameter of 4.25 µm. Also, isolate S6 had a darkly pigmented colony with white aerial mycelium, light brown conidial masses and dark brown reverse side. The isolate possesses conidia that are cylindrical with obtuse ends in shape. The conidia length and width is 14 x 5.14 µm. On the basis of possessing first two highest multi – resistant to different heavy metals, two strains coded **S9** and **S6** were selected for identification. The result in Table 9 suggested that they were identified as *Aspergillus niger* and *Colletotrichum* sp., respectively and these strains have been implicated by previous studies carried out by Abera *et al*. (2016) and Manguilimotan and Bitacura (2018).

Table 9: Morphological features of the selected strains of heavy metal resistant mould

Isolate code	Colonial morphology	Microscopic morphology	Identity
S9	It has a compact and flat elevated colony which was white at first and later black in colour	It possesses smooth-walled and hyaline conidiophore. It has dark rough – walled, septate and globose to sub-globose	*Aspergillus niger*

		conidia with diameter of 4.25 µm.	
S6	It has a darkly pigmented colony with white aerial mycelium, light brown conidial masses and dark brown reverse side.	The conidia are cylindrical with obtuse ends. The conidia length and width is 14 x 5.14 µm.	*Colletotrichum* sp.

CONCLUSION

The whole study demonstrated that higher concentrations (100 – 200 ppm) of these metals (Cr, Co, Pb, Ni, As, Zn, Hg and Cu) had significant growth inhibitory on the fungi growth than lower concentration (< 100 ppm) which had stimulatory effects. The strains *Aspergillus niger* S9 and *Colletotrichum* sp. S6 were found to possess highest growth diameters on cobalt and nickel metals exposure and hence suggested as heavy metal bioremediation options.

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