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It is with great pleasure that I write this editorial to welcome you to the IJCBR. This journal provides a platform for publication of original and reviews research articles, short communications, letter to editor, thesis abstract, conference report, and case studies. These types of publication are directed at the interface of the fields of cancer and biomedical research.

The IJCBR relies on a distinguished expert of the Advisory and Editorial Board Members from the top international league covering in depth the related topics. They timely review all manuscripts and maintain highest standards of quality and scientific methodology and ethical concepts. Meanwhile, we take all possible means to keep the time of the publication process as short as possible.

I take this chance to welcome your contributions to the IJCBR and have every expectation that it will soon become one of the most respected journals in both the fields of cancer and biomedical research.

**Mohamed L. Salem,**

Editor in Chief

INTERNATIONAL JOURNAL OF CANCER AND BIOMEDICAL RESEARCH (IJCBR)

RESEARCH ARTICLE

# Comparing productivity and tolerance of melanin-producing *Streptomyces longisporoflavus* and *Aspergillus niger* under biosorption capacity

**Eman H.F. Abd El-Zaher, Nanis G. Allam and Asmaa E. Allam**

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## ABSTRACT

**Background**: Melanin (Mel) is a naturally occurring pigment made by some microorganisms. *Streptomyces* sp and *Aspergillus niger* are capable of producing a high quantity of brown (Mel1) - black (Mel2) intercellular Mel pigments. Aim: To compare between melanin-producing *Streptomyces* sp and *Aspergillus niger* productive efficiency and tolerance against lead and cadmium ions stress was investigated. **Materials and Methods:** The tested strains were identified by molecular characterizations (16S rRNA & 18S rRNA) gene sequence, Mel1 of *S. longisporoflavus* NR043926 and Mel2 of *A. niger* MT355517 were extracted and purified from the dried microbial biomass. Physicochemical characterizations of extracted Mel pigments were compared with standard melanin based on infrared (IR) analysis. The productive efficiency and tolerance under absorption capacity of the selected strains against lead and cadmium ions were identified as the minimum inhibitory concentration (MICs) of each metal ions. **Results**: The production of Mel pigments and growth of two selected strains were decreased gradually by increasing the concentration of cadmium or lead from (10-750 ppm). Transmission electron microscopic examination showed heavy metals binding occurred on the surface of the microorganism cells, uptake or precipitation inside the melanized inner cell wall, finally vacuoles and microbial cells damage. **Conclusion**: *A. niger* can resist cadmium and lead ions more than *S. longisporoflavus*, Mel2 had a high protective function toward the stress of the heavy metal in comparison to Mel1.

**Keywords:** *Aspergillus niger,* Cadmium, Lead, Melanin, and *Streptomyces longisporoflavi*

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## INTRODUCTION

Melanin (Mel) pigments are hydrophobic and negatively charged high molecular weight pigments. They are commonly found in animals, plants, fungi and bacteria. In fungi and bacteria, melanin pigments are found in their cell wall or they exist as extracellular polymers. Mel is a polymeric phenolic compound which is categorized into two major classes based on its chemical structures and molecular precursors: eumelanin (dark brown-black) and pheomelanin (orange-red). All eumelanin and pheomelanin are strongly cross-linking, complex biopolymers (Herrera et al., 2019). Tyrosinase in some microorganisms is the enzyme involved in the key step in the formation of melanin that helps in the conversion of tyrosine to 3, 4-dihydroxy L-phenylalanine (L-DOPA) by a one-step oxidation reaction under batch culture. (Ali and Haq, 2010). The biosynthesis of 1, 3, 6, 8-tetrahydroxy naphthalene (DHN) melanin is converted in succession to scylatone, vermolone, and DHN are well understood. (Martinez et al., 2000).

It has been reported that *Klebsiella sp* bacteria could produce Mel pigment in the presence of L-tyrosine. *Spissiomyces endophytica* strain SDBR-CMU319, was able to produce a brown-black pigment in the mycelia. Mel production and characterization have been reported also from the genus *Spissiomyces* (Shrishailnath *et al.* 2010; Suwannarach et al., 2019). Mel is an amorphous, dark-colored pigment, insoluble in the most solvents, bleaching by oxidizing agents

($H_2O_2$), and soluble in alkali and phenols (Krysciak, 1985). Pihet *et al.* (2009) reported that melanin deposition protects the pigmented cell against biophysical stress of resonance-stabilized cyclic subunits, heavy metals stress, oxidative agents, ultraviolet rays, and skin cancer. Mel could protect microorganisms including fungi and bacteria, from stresses such as sun-borne UV radiation and reactive oxygen species. Mel also protects microorganisms against high temperatures, chemical stresses like heavy metals and oxidizing agents (Hamilton & Gomez, 2002). Mel pigment can bind to heavy metals, which may influence the growth of microorganisms and production of Mel pigment (Pointing et al., 2000). Cadmium and lead removal by microbial biomass are a rapid process by which the bulk of the removal occurs during the first few minutes of contact between the metal solution and the biomass (Panes et al., 2012).

Joo and Hussein (2012) investigated the tolerance of some resistant fungal strains from soils polluted with heavy metals by MIC depending on the fungal species as the MIC of Cd for *Aspergillus niger* KNU3 and *Penicillium funiculosum* KNU4 was 400 ppm for each, while the other tolerant species e.g. *Penicillium* sp. and yeasts recorded MICs ranged from 200-300 ppm of the same heavy metal. Shiguo Chen et al., 2009 investigated the absorption of cadmium (Cd) and lead (Pb) by squid Mel. Different functional groups are responsible for their absorption within Mel. Infra-red evaluation of metal ion-enriched squid melanin showed phenolic hydroxyl, carboxyl, and amine groups to be the likely functional groups responsible for metal binding. Previous studies have clarified the mechanisms responsible for biosorption, which can be one or a combination of ion exchange, teinting, coordination, absorption, electrostatic interaction, chelation and micro precipitation (Vegliò and Beolchini, 1997; Volesky and Schiewer, 1999). Cd and Pb elimination by microbial biomass are a rapid process in which the bulk of the removal of metal solution and biomass occur within the first few minutes of touch (Pardo et al., 2003; Komy et al., 2006). The biosorption capacity of live, autoclaved and dried biomass of *Yarrowia lipolytica* AUMC 9256 for cadmium (Cd(II)) to produce cadmium nanoparticles was determined by UV-Visible spectroscopy measurements and transmission electron microscopy examinations. Moreover, to identify the possible mechanisms of Cd(II) uptake. The potential implementation of dried *Y. lipolytica* biosorbent for heavy metal removal from different water samples was successfully accomplished using the multistage microcolumn technique. Accordingly, *Y. lipolytica* AUMC 9256 can be considered as a very promising potential to bioremediate Cd(II) ions (Manal, 2019).

The current study aimed to compare between *Streptomyces* sp and *Aspergillus niger* in producing high quantity of brown (Mel1) - black (Mel2) intercellular Mel pigments. Detecion the productive efficiency and tolerance under biosorption capacity of these strains against lead acetate and cadmium chloride activity. Identify the location of heavy metal particles within the melanized microbial cells in control and treated cultures under TEM examination.

## MATERIALS AND METHODS

### Microorganisms

*Streptomyces longisporoflavus* NR043926 and *Aspergillus niger* MT355517 were identifed in 2015 by molecular characterizations (16S rRNA & 18S rRNA) gene sequences in GATC Company using ABI 3730xl DNA sequence using forward and reverse primers (Sigma Scientific Services Co., Cairo, Egypt).

### Chemicals and reagents

Dimethyl Sulphoxide (DMSO), was purchased from El-Gomhoria Company, (Tanta, Egypt). Cadmium chloride, lead acetate powder and other chemicals were purchased from Sigma Company (Cairo, Egypt).

### Molecular identification of melanin-producing *Streptomyces* sp.

The 16S rDNA of bacteria was amplified and prepared by PCR using universal primer 9F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1541R (5'- GGT TAC CTT GTT ACG ACT -3'). Thermal cycling conditions were as follows: DNA denaturation at 98 °C for 3 min followed by 30 cycles at 94 °C for 1 min, annealing of the primers at 52 °C for 1 min, and then extension at

72 ˚C for 5 min. The reaction mixture was held for 5 min at 72 ˚C and then cooled to 4 ˚C. Sequencing of the PCR product was carried out by using an automated sequencer (Macrogen Inc., Seoul, Korea) and the same primers as above for sequence determination. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI Blast. A phylogenetic tree based on 16S rRNA gene sequence was constructed with the neighbor-joining method in MEGA version 4 software (Singh et al., 2012).

**Molecular identification of *Aspergillus niger* by 18S rRNA gene sequence.**

The mycelium of *Aspergillus niger* was scratched off from the surface of Czapek's dox media. The fresh mycelia (50 mg) was ground in liquid nitrogen using a mortar and pestle. DNA was extracted from the powdered tissue using genomic plant DNA extraction Mini Kit according to the manufacturer's instructions. The eluted DNA was stored at -20 ˚C (White and Burn, 1990).

**Polymerase chain reaction (PCR) condition**

In automated thermal cycler, an amplification of the internal transcribed spacer (ITS) area was carried out (C1000$^{TM}$ Thermal Cycler, Bio-RAD) using ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and ITS5(5`-GGA AGT AAA AGT CGT AACAAGG-3`) primers. 35 cycles of 94 ˚C for 30 s, 51 ˚C for 1 min, 72 ˚C for 1.5 min, and a final extension at 72 ˚C for 3 min. 1 µl of 25 ng nucleic acid, 1 µl of each primer (10 pmol), 12.5 µl of GoTag® colorless Master Mix (Promega Corporation, USA) and 9.5 µl of nuclease-free water (Promega). 15 µl of all PCR products were assessed by using electrophoresis through 1% agarose gel, with ethidium bromide staining and visualization of DNA bands were made by using a UV transilluminator (Sigma Scientific Services Co., Cairo, Egypt).

**Extraction of melanin pigment**

**Extraction of melanin pigment (Mel1)** *Streptomyces* sp was grown on starch- nitrate medium at (pH 7.0), Petri dishes were prepared and inoculated each with a loop of bacteria. The inoculated Petri dishes were incubated for (7 days at 30°C). Disks (10 mm diameters) were cut from the margin of the colonies, boiled for 1min in 5 ml distilled water and centrifuged for 15 min at (5,000 rpm). After washing and centrifugation, the pigment was extracted by autoclaving the pellets for (20 min at 120°C). The pellets were dissolved in (3ml of 1M NaOH). The alkaline extracted melanin was acidified to (pH 2.0) with concentrated HCl for melanin precipitation. The precipitated melanin was washed in 3 changes with distilled water and dried overnight at (20°C) in a dry atmosphere. Then, the extracted pigment was further purified by acid hydrolysis (5 m1 of 7.0 M HCl) in a sealed glass vial for (2h at 100°C). The precipitated melanin was washed three times with distilled water and dried overnight at 20°C in a dry atmosphere. The precipitated melanin was further purified by acid hydrolysis (5 ml of 7 N HC1) in sealed glass vials for (2h at 100°C) according to Gadd (1988).

**Extraction of melanin pigment (Mel2)** *Aspergillus niger* was grown on sabouraud's liquid medium at (pH 7.0 for 10 days at 30°C). Upon centrifugation, at the end of the incubation cycle (for 20 min at 8000 rpm). The precipitate obtained to remove carbohydrates and proteins was purified by acid hydrolysis using 6.0 M HCl at 100 °C for 2h. The precipitate melanin washed distilled $H_2O$, after washing it was dried over anhydrous $Na_2SO_4$, then dissolved in 1M NaOH and centrifugation at 4000 rpm for 15 min for TLC separation, infra-red spectra analysis and using authentic melanin as reference according to (Rowley and Pint, 1972) and (Sava, 2001).

**Preparation of standard melanin** Standard melanin was purchased from Sigma Company (Cairo, Egypt). Standard melanin solution was prepared by dissolving 0.05 g of melanin in 2 ml of 1.0 M NaOH.

**Physicochemical properties of Mel extracted from *Streptomyces longisporoflavus* NR043826 (Mel1) and *Aspergillus niger* MT355517 (Mel2)** According to Thomas (1955) and Ellis and Griffiths (1974), the isolated melanin pigments from each strain were tested for several physical and chemical characteristics including color, solubility, solubility in different organic solvents, polyphenol test, $FeCl_3$ (1% w/v), precipitation in 3.0 N-HC1 and solubility in 1.0 M

KOH (100°C for 2 h), and was compared with the standard melanin.

**Infrared (IR) spectrum of melanin**

IR spectrum of the extracted Mel pigments was obtained using spectrophotometer Perkin-Elmer1430 (Micro Analytical Center, Faculty of Science, Cairo University, Cairo, Egypt). Small discs were made from the mixture of about 1.0 mg of the tested materials and 300 mg of pure KBr, followed by pressing into a disc and used for determination of the infrared spectra. The measurements were carried out at infra-red spectra between 400 and 4000 nm (Sherborok-Cox et al., 1984). *In vitro*, protective efficiency and tolerance under biosorption capacity of *S. longisporoflavus* NR043826 and *A. niger* MT355517 against cadmium and lead ions stress.

**Heavy metal tolerance**

The two tested strains were checked for their tolerance to heavy metal against cadmium chloride and lead acetate. Aqueous metal solutions were prepared in dist. $H_2O$ and added to starch nitrate media for *S. longisporoflavus* NR043826 and Sabouraud's media for *A. niger* MT355517 to obtain a final concentration 10 ppm in each case. Each strain was cultured onto triplicate of their media with 10 ppm of cadmium or lead, streaked on the surface of media then incubated for 24h at 37 °C for the bacterium (Mrvcic et al., 2009), and 7 days at 28 °C for the fungus.

**Determination of MICs of *S. longisporoflavus* NR043826 and *A. niger* MT355517, Mel1 and Mel2 production against cadmium and lead stress**

The MICs of the tested strains on growth and Mel production were detected against cadmium and lead ions, separately. The concentration of each metal solution was gradually increased (10 – 750 ppm). Two microbial strains were inoculated on the surface of tested strain media which supplemented with each concentration of metal solutions. The flasks were incubated at 37 °C for 24 h. The concentration of metal was increased till MICs was achieved as visualized by the cessation of melanin production and organism's growth according to Washington and Sutter (1980); Joo and Hussein (2012).

**Transmission electron microscopy (TEM) examination**

The control and treated cultures were examined by TEM in order to identify the location of lead particles within the microbial cells. Solution of 5 mg/L of lead acetate was prepared. A weighed amount of each tested strain cell pellets was suspended in a lead solution with a final concentration of 1.0 g/L of microbial strains. The pH of the suspension was adjusted to 6.0, and the samples were incubated for 1 h at 28 °C followed by centrifugation at 5,000 rpm for 20 min. Control was conducted in the lead-free distilled water. The location of lead particles (250, 500 and 750 ppm) within each strain cells of treated cultures was identified according to Halttunen *et al.* (2008). After fixation and dehydration, samples were embedded in Araldite 502 resin. The plastic molds were cut at 805 nm thicknesses in Ultra cut ultra-microtome, and then they were stained with 1% toluidine blue. Photography was made using JEOL, JEM-100 SX electron microscope (Japan) in Faculty of Medicine, Tanta University.

**Statistical analysis**

Statistical analysis was carried out If there a significant difference between means, Tukey post hoc comparisons among different groups were performed. For all statistical tests P values ≤ 0.05 was considered to be statistically significant. Data and statistical analysis were performed using Excel 2016 (Microsoft Corporation, USA), SPSS statistical version 23 software package (SPSS Inc., USA) and Minitab version 18 (SAS, 1985).

## RESULTS

**Molecular identification of *Streptomyces* sp.**

Using 16S rDNA sequence, we compared the tested nucleotide sequences with the members of actinomycetes. This clearly showed that the tested *Streptomyces* sp strain revealed 99% similarity with other strains of *Streptomyces* sp accessed in GenBank. The strain identified as *S. longisporoflavus* NRRL ISP-5165 (Accession NO: NR043826) as shown in Figure 1. Other strains belong to section *Streptomyces* sp exhibited slightly similarity (85%) to our tested strain.

### Molecular identification of *Aspergillus niger*

Using the genotypic identification 18S rDNA sequence, comparison of the tested nucleotide sequences with the members of *Aspergillus sp* strain in GenBank revealed 99.9% similarity with other strains of *Aspergillus niger* (Accession NO: MT355517) as shown in Figure 2. The fungal strain belongs to section *A. niger* such as *Aspergillus niger* KY825168 and *Aspergillus niger* MH748223 exhibited slightly similarity (99.8 %).

### Physicochemical properties of Mel1 and Mel2

Properties of the extracted Mel pigments were illustrated in Table 1. They were insoluble in water and organic solvents such as (acetone, chloroform and ethanol) or such solvent mixture, were acidified by concentrated HCl to pH 2.2, were decolorated by oxidizing agents ($H_2O_2$ and NaOC1), gave positive reactions to polyphenol compounds and produced 1 percent (w/v) flocculent brown precipitates with $FeC1_3$.

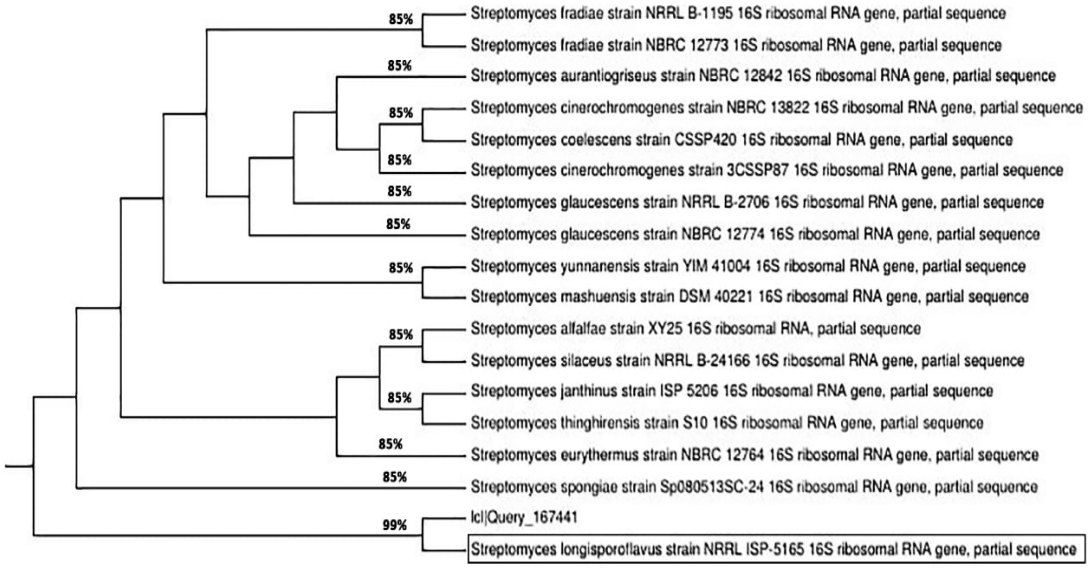



**Figure 1.** Phylogenetic analysis of *S. longisporoflavus* based on complete sequencing of 16S rDNA.

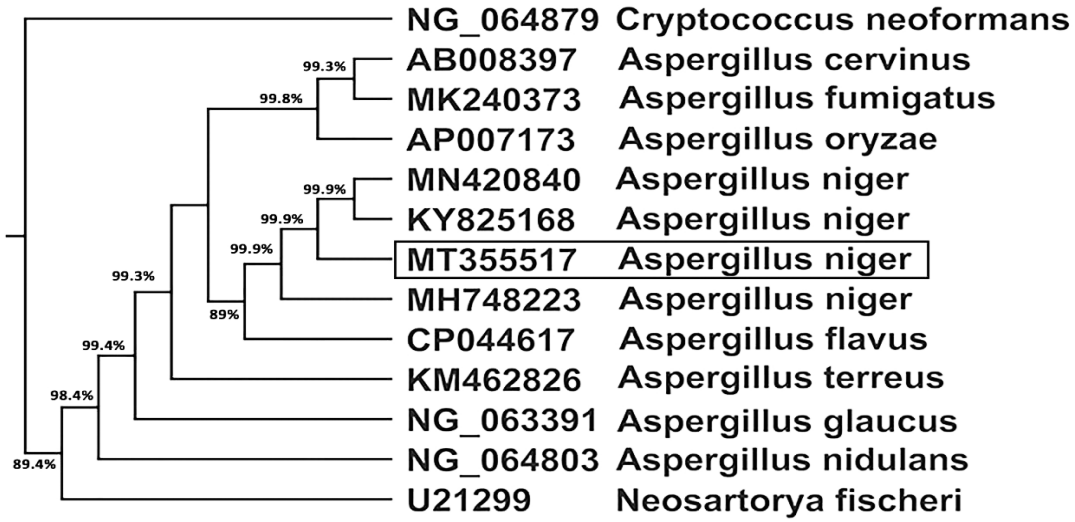



**Figure 2.** Phylogenetic analysis of *A. niger* MT355517 based on complete sequencing of 18S rDNA.

**Infrared (IR) analysis**

The precipitated Mel pigments have peaks with wave numbers appeared at 3412.42 $cm^{-1}$, 2924.1452 $cm^{-1}$, 2860.88 $cm^{-1}$, 2367.19 $cm^{-1}$, 1746.23 $cm^{-1}$,1641.13 $cm^{-1}$, 1455.03 $cm^{-1}$, 866.846 $cm^{-1}$, 708.712$cm^{-1}$ and 606.503 $cm^{-1}$. (Figure 3a) show the extracted Mel1. Whereas the extracted Mel2 recoded peaks at wave numbers 3404.71 $cm^{-1}$, 2924.52$cm^{-1}$, 2859.92 $cm^{-1}$, 2631.39 $cm^{-1}$, 1739.48 $cm^{-1}$, 1635.34 $cm^{-1}$, 1445.39 $cm^{-1}$ , 884.202 $cm^{-1}$, 785.85 $cm^{-1}$ and 606 $cm^{-1}$ (Figure 3b).

The wave number occurred can be ascribed to the following: (3412.42 $cm^{-1}$ and 2924.1452 $cm^{-1}$) and (3404.71 $cm^{-1}$ and 2924.52$cm^{-1}$) attributed to OH and NH bonds. (2860.88 $cm^{-1}$and 2367.19 $cm^{-1}$) and (2859.92 $cm^{-1}$, and 2631.39 $cm^{-1}$) and due to C-H or H-C=O bonds, (1641.13 $cm^{-1}$ and1635.34$cm^{-1}$) due to C=O bonded, (1455.03 and 1445.39 $cm^{-1}$) due to C = N, and (866.846 $cm^{-1}$ and 884.202$cm^{-1}$) due to C. The authentic Mel recoded peaks at wave numbers (Figure 3c), 3285.14$cm^{-1}$, 2925 $cm^{-1}$, 2850 $cm^{-1}$, 1724 $cm^{-1}$, 1631$cm^{-1}$, 1455.99 $cm^{-1}$, 896.737$cm^{-1}$ and 697.623 $cm^{-1}$.

*In vitro,* the protective efficiency and tolerance under biosorption capacity of the tested strains against cadmium and lead ions.

**Determination of minimum inhibitory concentrations (MICs) of *S. longisporoflavus* NR043826 and *A. niger* MT355517 growth, Mel1 and Mel2 production against cadmium and lead ions stress**

The ability of microbes to tolerate and grow in the presence of Cd and Pb ions was evaluated by measuring the MICs of tested strains against Cd and Pb ions stress, and also to investigate the relation between the presence of melanin and its protection against Cd and Pb ions stress. The concentration of each metal solution added was gradually increased from (10–750 ppm) by increasing amounts of metal salts added to the media for 24h at 37°C, separately. The production of melanin and growth of all tested strains were decreased gradually by increasing the concentration of Cd or pb from (10 – 750) ppm. There is a significant negative relationship between different concentrations of cadmium and lead ions (ppm), melanin production, and dry weight of both tested strains was illustrated in (Figures 4 and 5).

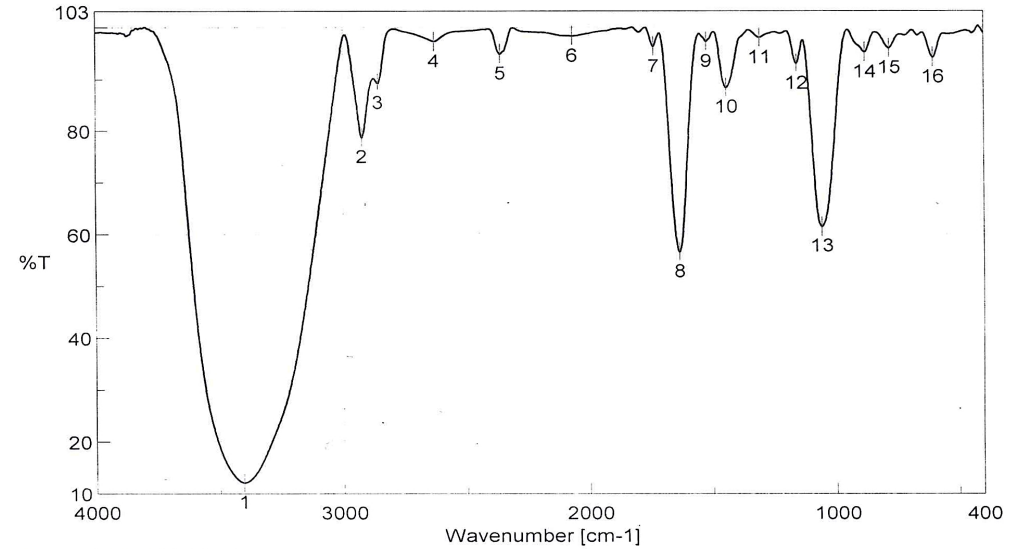
The results revealed to the MICs of *S. longisporoflavus* NR043826 and Mel1 production was at 250 ppm. The MICs of *A. niger* MT355517 growth and Mel2 production was at 500 ppm against lead acetate and cadmium chloride stress. The results detected in (Table2 and 3) demonstrated that Pb is more toxic than Cd. *S. longisporoflavus* NR043826 showed less resistance to Cd and Pb in comparison to *A. niger* MT355517 which can tolerate cadmium more than lead. Mel2 had a high degree of defence and protective action against the heavy metal stress.

**Transmission electron microscopy (TEM) examination**

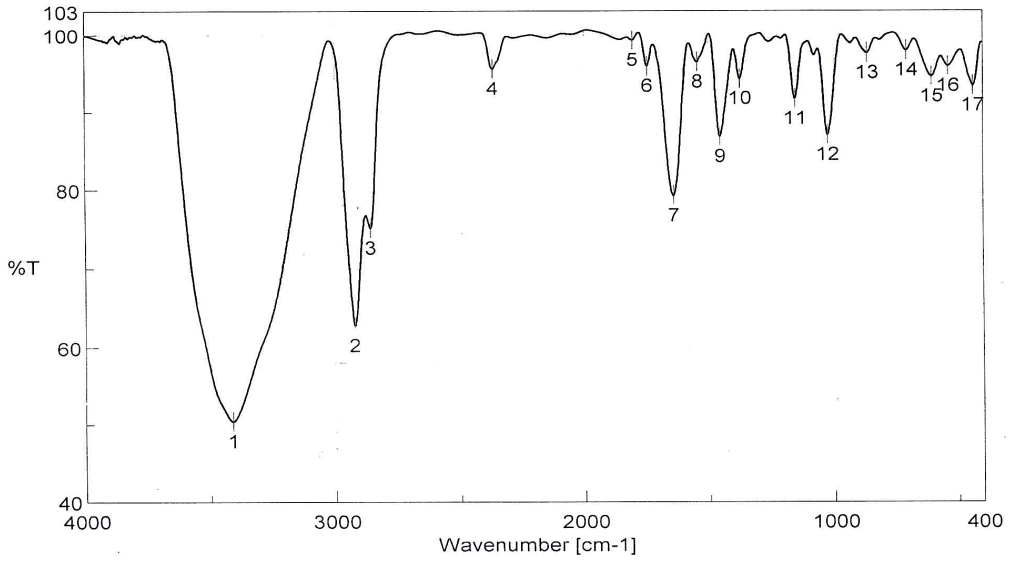
Transmission electron microscopy micrographs of *S. longisporoflavus* NR043826 and *A. niger* MT355517 without, and with addition of lead were represented in Figures (6-8), Particles of lead were visible on the surface of the selected strain cells, whereas no Pb was visible on control micrographs. The TEM micrographs revealed the binding occurred on the surface of the microorganism cells whereas there was uptake or precipitation of Pb inside the melinized inner cell wall as shown in Figures (7a-9a) which treated with lead particles (250, 500ppm). Figures (7b-9b) showed vacuolation and damages of the microorganism's cells which treated with lead particles (500 and 750ppm).

## DISCUSSION

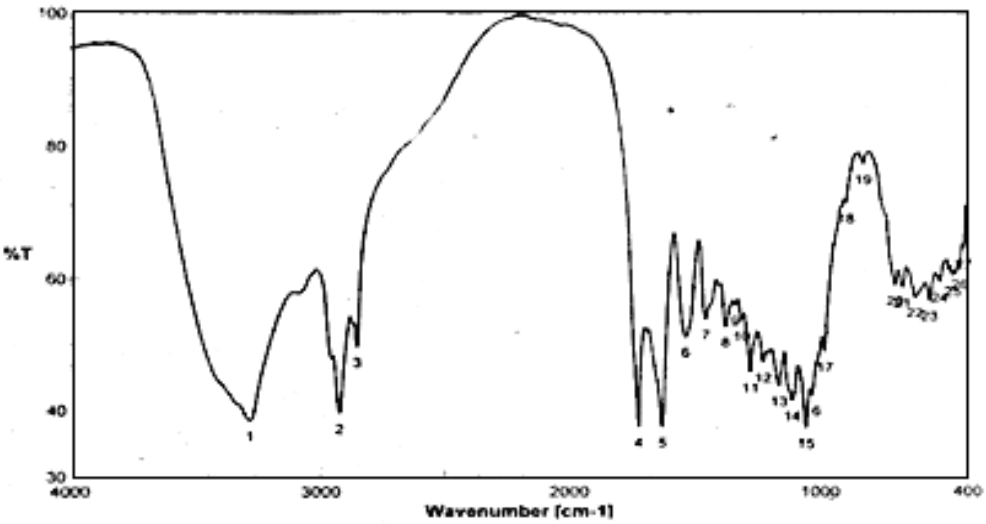
The molecular identification of *Streptomyces* sp revealed 99% similarity with other strains of *Streptomyces* sp accessed from GenBank to *S. longisporoflavus* NRRL ISP-5165 (Accession NO: NR043826). Almost similar results obtained by *Coelho and Linhares (1993)* who isolated melanogenic actinomycetes from soil. They found that 52% of pigment-producing colonies as possible Mel producers, which are primarily anaerobic, filamentous, Gram-positive, opportunistic bacteria that colonize human mucous membranes.




a)




b)




c)

**Figure 3** The infra-red analysss of Mel1 (a), Mel2 (b) and standard Mel (c).

There are over 40 currently recognized species of *Streptomyces* that are morphologically similar and resist identification by traditional phenotypic tests, fast partial sequencing of the 16S rRNA gene demonstrated complete molecular identity between our strain and other *Streptomyces* strains (Wong *et al* 2011), *Aspergillus niger* recorded the highest multi heavy metals resistance (Altschul et al., 1990) was identified and revealed 99.9% similarity with other strains of *A. niger* as accessed from GenBank (Accession NO: MT355517). *Aspergillus niger* is one of the *Aspergillus* genus' most common species and the best-characterized fungus-forming conidiospore. Henry et al., (2000) detected amplification fragments ranging from 565-613bp for different *Aspergillus* species, which was 595 bp in *A. niger*. In other fungal groups the ITS region was very useful in resolving taxonomic difficulties, as demonstrated by Driver et al., (2000) in the taxanomic revision of Inglis and reclassify entomopathogenic species of *Aspergillus*, previously misidentified by classical methods.

Mel1 of *S. longisporoflavus* NR043826 and Mel2 of *A. niger* MT355517 gave dark brown and black solutions, respectively. The two extracted Mel pigments were insoluble in water and organic solvents, were soluble completely after 2 hours in KOH at 100 ˚C, were decolorized by an oxidizing agent, were precipitated when their solutions were acidified by concentrated HCl to pH 2.2, gave a positive reaction for polyphenols and produced flocculent brown precipitates with $FeC1_3$ 1% (w/v). These results are almost similar to the results of Shrishailnath et al., (2010) who studied chemical characterization of the Mel pigment particles produced by *Klebsiella* sp. indicating that melanin pigment was acid resistant, alkali-soluble, insoluble in most of the organic solvents and water, got bleached when subjected to the action of oxidants as well as reductions, and was precipitated with ferric chloride and potassium ferricyanide. In our present study, the Infrared (IR) absorption spectra of the extracted Mel pigments were described in Figures 3a and 3b that revealed the absorption bands expected from aromatic compounds and their wave number as the same as the wavenumber of the standard Mel pigment.

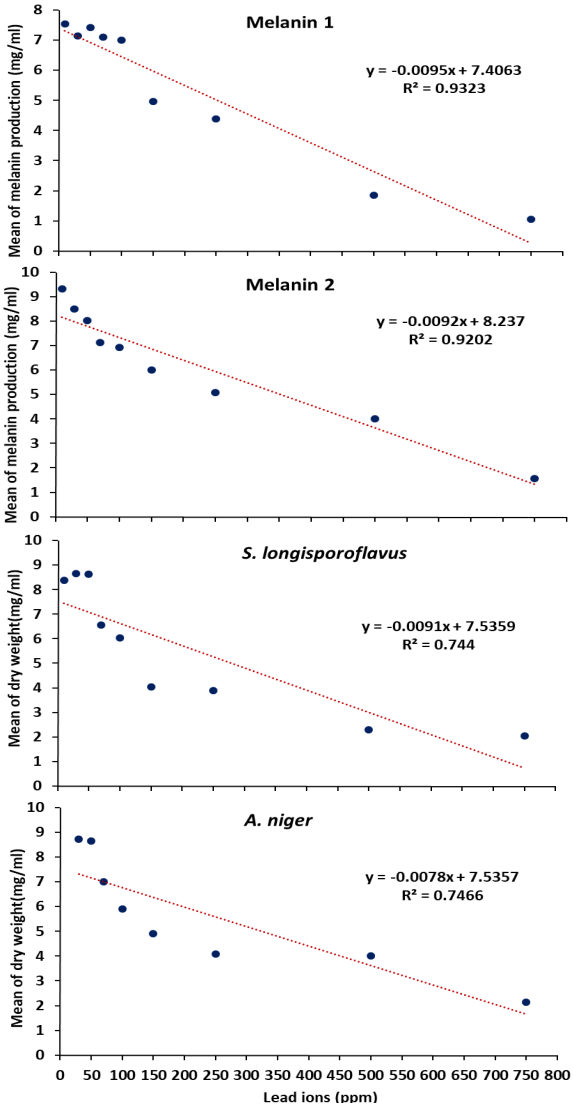



**Figure 4.** Regression analysis between different concentrations of Pb salts, Mel1, Mel2 production, *S. longisporoflavus* NR043826 and *A. niger* MT355517 growth.

These data were in consistent with the previous results provided by Bonner and Duncan (1962); Ellis and Griffiths (1974). The present study showed that *S. longisporoflavus* NR043826 and *A. niger* MT355517 were possible to tolerate, grow and producing melanin in the presence of Cd and Pb as detected in (Table 2 and 3), the production of melanin and growth of the microbial strains were decreased gradually by increasing the concentration of cadmium or lead; so, there is a significant negative relationship between different concentrations of cadmium and lead ions (ppm), reducing of melanin production, and dry weight of both tested strains was demonstrated in regression analysis (Figures 4 and 5).

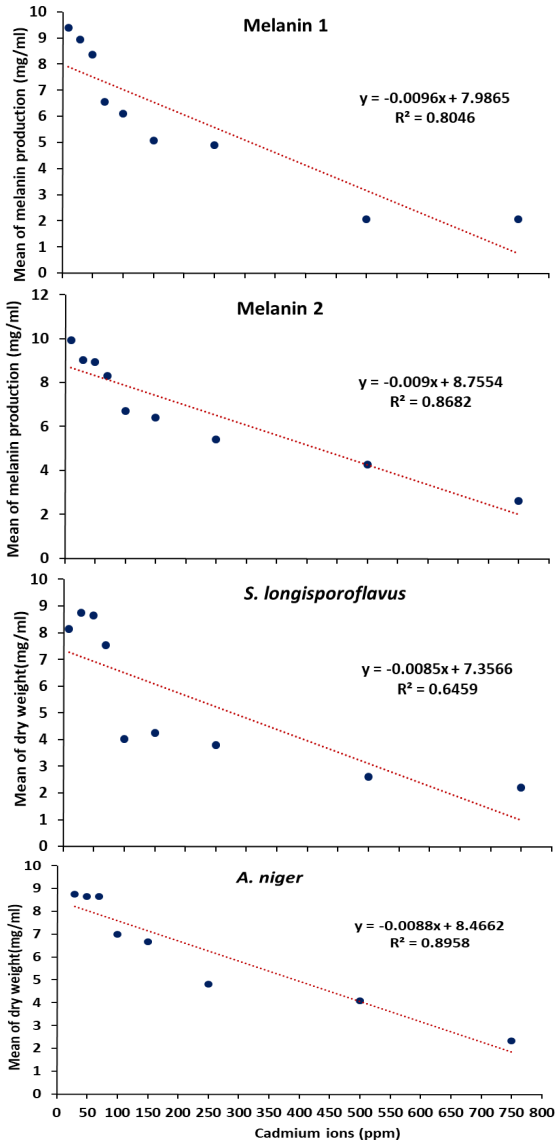



**Figure 5.** Regression analysis between different concentrations of Cd salts, Mel1, Mel2 production, *S. longisporoflavus* NR043826 and *A. niger* MT355517growth.

The MICs of *S. longisporoflavus* NR043826 growth and Mel1 production was at 250 ppm and the MICs of *A. niger* MT355517 growth and Mel2 was at 500 ppm, against lead acetate Pb $(CH_3CO)_2$ and cadmium chloride $(CdCl_2)$. According to the results investigated in (Table 2 and 3) it was clearly that Pb is more toxic than Cd and *S. longisporoflavus* NR043826 showed less resistance to cadmium and lead in comparison to *A. niger* MT355517, which can tolerate cadmium more than lead. Results indicated that Mel2 had a high degree of protectivity and defence in comparison to Mel1 against lead acetate and cadmium chloride stress.

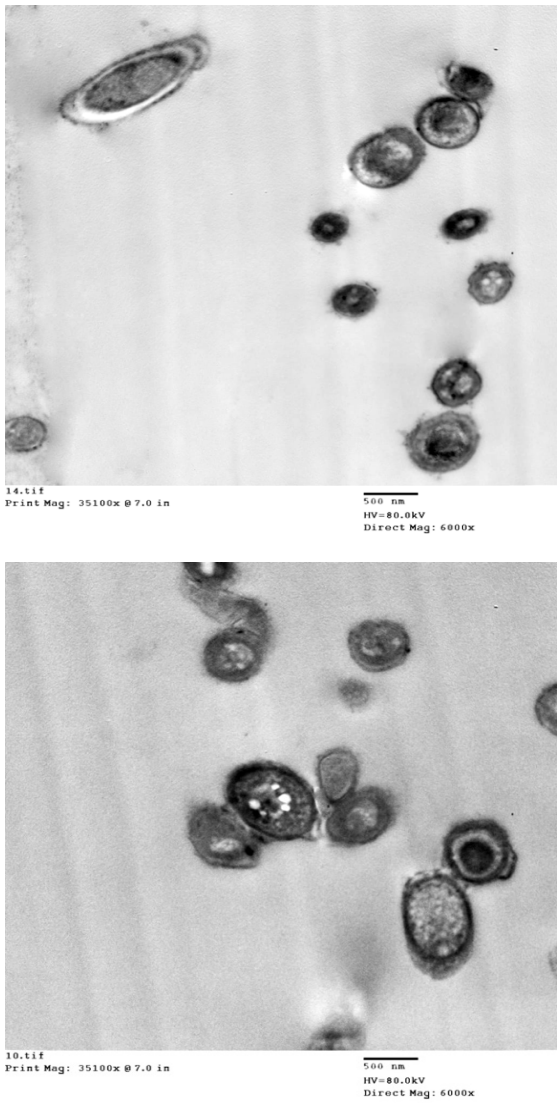
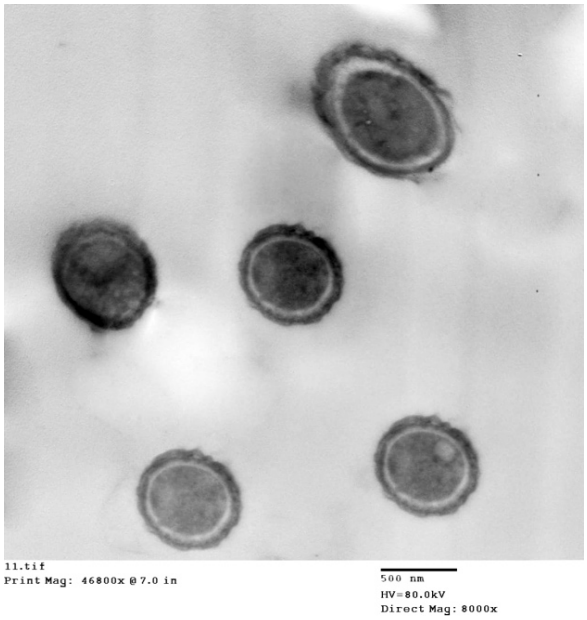
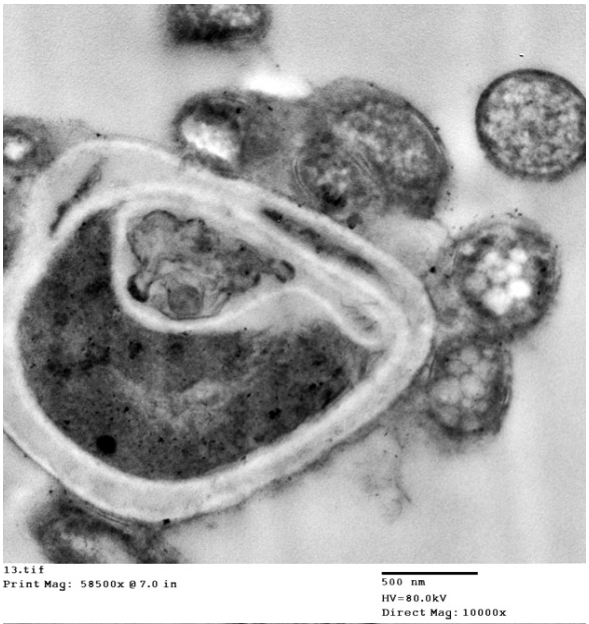
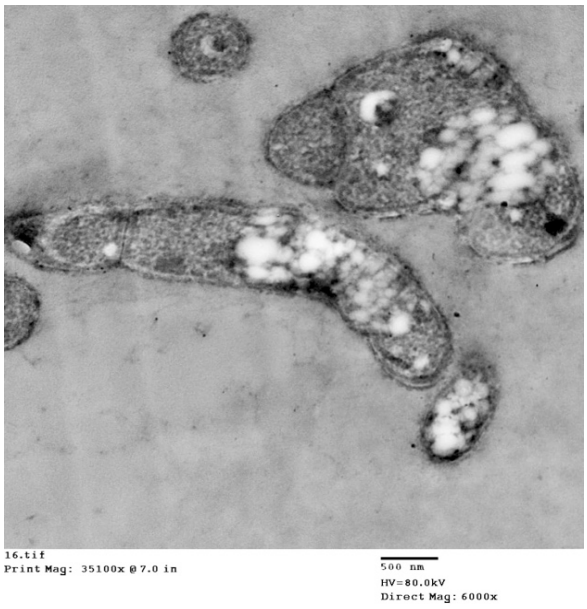


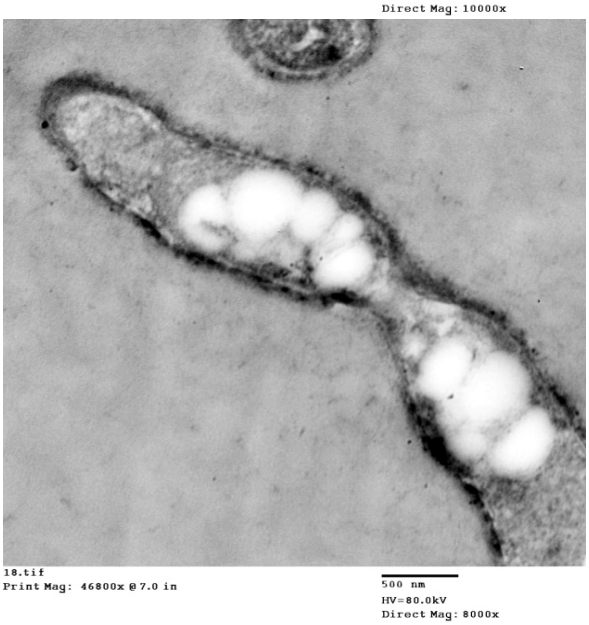

**Figure 6.** Transmission electron micrographs of *S. longisporoflavus* strain NR043826 without the addition of lead (control) Direct Magnification: 6000x and 8000x showed the cells have well-defined outer cell, dense inner cell walls and intact plasma cell membranes.

The obtained results were comparable to those of another study carried out by (Mamdouh et al., 2017) recorded novel fungal species that have tolerant potential to tolerate a high concentration of heavy metals especially, (Cd) and their potent efficacy to uptake it. The results showed a significant distribution of fungal isolates, as 64 fungal isolates were isolated at cadmium concentration of 100 ppm, and screened for further other high concentrations, (200, 300, 400, 500, 600, 700, 800, 900 and 1000 ppm), results showed that the growth of fungal isolates recorded zero at 600 ppm while at 400 ppm, growth was observed for 23 fungal isolates.




a)

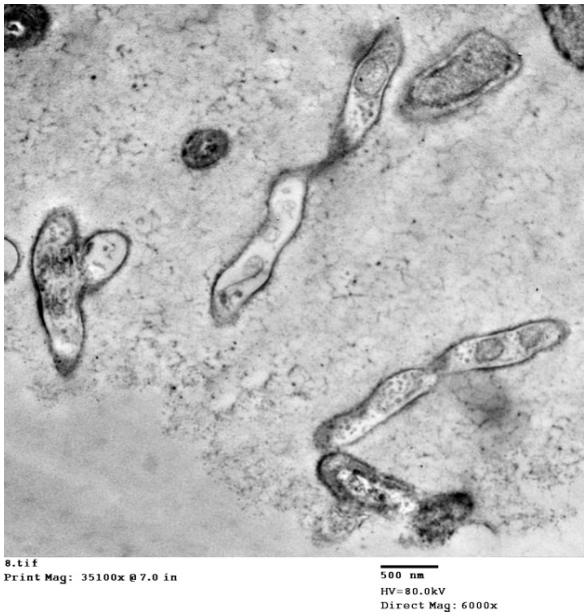



Figure 7. (a)Transmission electron micrographs of *S. longisporoflavus* strain NR043826 treated with lead particles (250 ppm). Direct Magnification: 6000 and 10000 x showed particles of lead were visible on the surface of the actinomycetes cell and uptake or precipitation inside the cell. (b)Transmission electron micrographs of *S. longisporoflavus* strain NR043826 treated with lead particles (500 ppm). Direct Magnification: 6000 and 8000 x showed the cell with vacuoles and ruptured.

b)

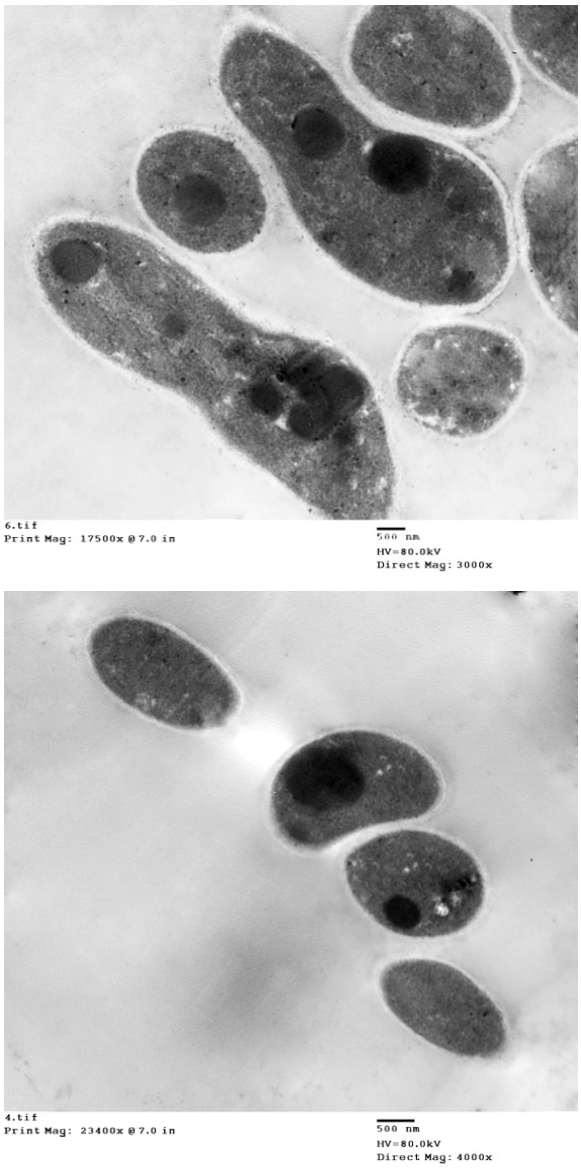



**Figure 8.** Transmission electron micrographs of *A. niger* strain MT355517 without the addition of lead (control). Direct Mag: 3000 and 4000x showed the cells have well-defined outer cell, dense inner cell walls and intact plasma cell membranes.

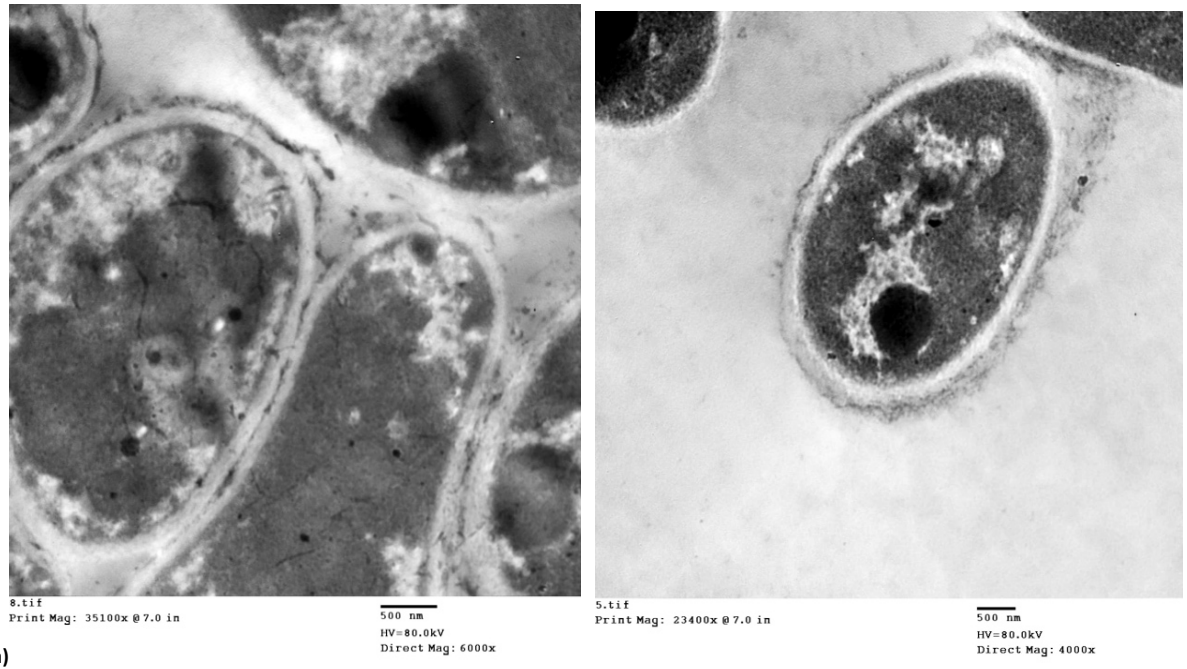
Amazingly, the minimum inhibitory concentration (MIC) was recorded using agar well method and the result showed that 5 fungal isolates were tolerated up to 480 ppm. By using macroscopic, microscopic and molecular techniques, the most potent fungal isolates were identified as *A. niger.* Natural melanin's metal ion chelating activity is used to eliminate heavy metals from the polluted water. We engineered marine bacterial melanin biosynthesis using various growth media, medium components and operating conditions (Harsha et al., 2018).

The growth behavior of *Aspergillus awamori*, *Aspergillus flavus* and *Aspergillus niger* with increasing heavy metals concentration was examined as tolerance index. The synergistic and inhibitory effect on the growth of both heavy metals with a lower and higher concentration. Our finding suggests that the resistant strains isolated from their polluted environment have greater relevance and application in bioremediation of heavy metals (Pawan, 2018).

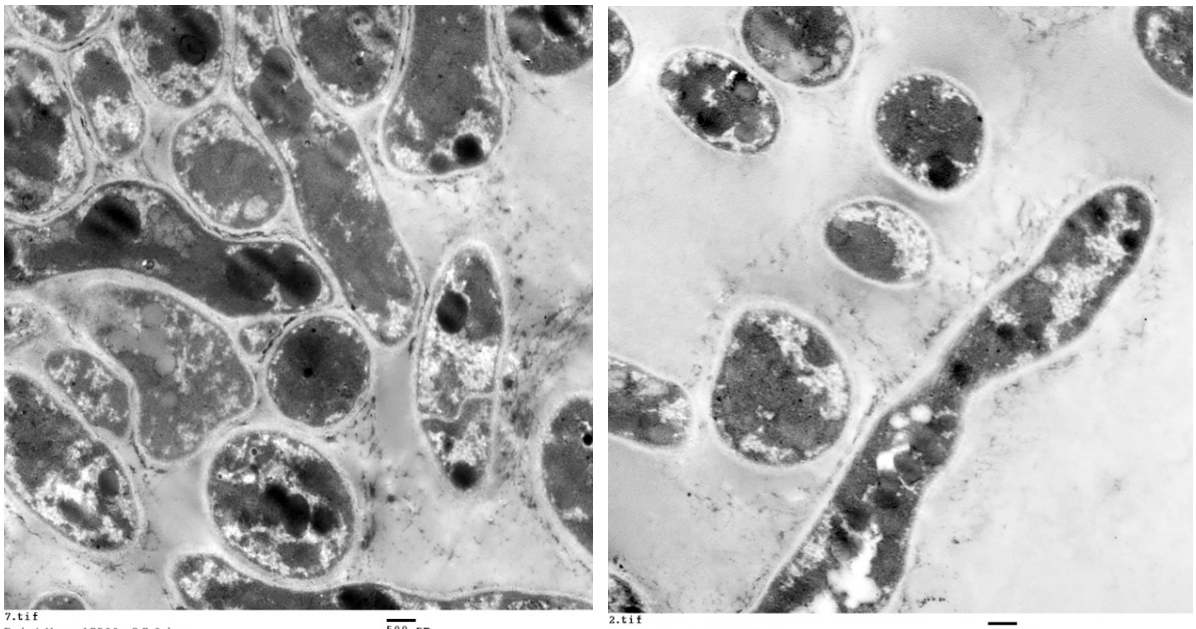
Another study investigating, heavy metal ion tolerance that determined wastewater bacteria collected in Makkah City, Saudi Arabia for isolation (Alaa et al., 2016). Bacterial isolates were tolerant to cupper, cadmium, zinc and cobalt although the tolerance levels for the different concentrations of metal ions were unique for each isolate whereas *Pseudomonas aeruginosa* was shown in the presence of cupper ≈ 130 ppm, followed by zinc and cadmium ≈ 180 and 200 ppm, respectively and finally to cobalt ≈ 220 ppm. *Lactobacillus plantarum* MF04201 had maximum uptake capacity of both $Cd^{2+}$ and $Pb^{2+}$ which was recorded at pH 2.0 and a temperature of 22 °C after 1 hr, and exhibited a high degree of resistance up to 300 and 100 ppm; Furthermore, the results revealed that the use of *L. plantarum* MF042018 is an effective tool for the treatment of hazardous metal-polluted battery-manufacturing effluent (Ameen et al., 2020).

Saad et al., (2015), also isolated and purified 20 bacterial resistant strains in the respective of heavy metals from Helwan and 10th of Ramadan city areas and enriched in culture broth containing arsenic (As), lead (Pb), and cadmium (Cd) as $AsHNa_2O_4H_2O$, $Pb(NO_3)_2$, and $CdSO_4$, respectively. The growth parameters were optimized, and the maximum tolerable concentration of the respective bacterial strains growth was determined at 200, 100, and 10 ppm against arsenic (As), lead (Pb), and cadmium (Cd) respectively.

The TEM micrographs of the selected strains shown in (Figures 7a, 7b, 9a, 9b) indicated that the binding of heavy metals occurred on the surface of the microbe's cells whereas there was




**a)**




**b)**

**Figure 9. (a)** Transmission electron micrographs of *A. niger* strain MT355517 treated with lead particles (500 ppm). Direct Mag: 3000 and 10000 x showed particles of lead were visible on the surface of the fungus cell and uptake or precipitation inside the cell. (**B**) Transmission electron micrographs of *A. niger* strain MT355517 treated with lead particles (750 ppm). Direct Mag: 3000 x showed the cell with vacuoles and ruptured

uptake or precipitation of Pb inside the melanized inner cell wall and finally microbial cells vacuolation and damage were appeared too. *A. niger* MT355517 cells showed less vacuolation and rupture in comparison to *S. longisporoflavus* NR043826 cells, Mel1 showed also less resistance and productivity against lead binding capacity. Cadmium was used in earlier experiments then lead was chosen in most experiments through binding capacity, because of Pb is more toxic than Cd and studies on Cd and Pb were often conducted together as the elements seem to react with microorganism's species in similar ways**.** The absorption of the metals from solution indicates cell surface binding, therefore TEM was

performed on ultra-thin sections of *S. thermophilus* exposed to cadmium. TEM was preferable to scanning electron microscopy (SEM) as SEM was not able to determine if heavy metal particles were on the cell surface or in the interior of the cell (Monachese et al., 2012). On the same context, Chakravarty and Banerjee (2012) studied the mechanism of the cadmium binding on the cell wall of an acidophilic bacterium, TEM images of their study revealed the electron-dense region/layer throughout the cell wall only, with no intracellular accumulation. In contrast, other studies reported that Cd interaction with probiotic bacteria occurred through the binding on both the cell wall binding and intracellular accumulation.

## CONCLUSION

Physocochemical characterizations of Mel1 and Mel2 were revealed insolubility in water, decolorization by an oxidizing agent, a positive reaction for polyphenols, and a similarity between their wave number of the absorption bands and the wave number of standard Mel pigment. *S. longisporoflavus* NR043826 showed less resistance against cadmium and lead ions in comparison to *A. niger* MT355517, which can tolerate cadmium more than lead. Also, Mel2 had a high degree of protective efficiency in comparison to Mel1 against lead acetate and cadmium chloride stress. *A. niger* MT355517 cells showed less vacuolation and rupture in comparison to *S. longisporoflavus* NR043826 cells under TEM examination..

## CONFLICTS OF INTEREST

All authors declare no conflict of interest.

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**Statistical analysis of results*:*** The statistical design and the models of statistical analysis must be described, as well as each of the statistical methods used. Sufficient statistical details must be given to allow replication of the statistical analysis. The experimental unit should be defined (e.g., individual or group of animals).

**Results*:*** Data are presented as tables and figures. Brief description of the results for each table and figure should be presented. Unpublished data can be mentioned when necessary.

**Discussion*:*** Should be separate from the Results section and should focus only on intra- and inter-data discussion (the data in the results section) as well as with the relative data in the literature. Don't repeat information already presented in the Introduction section. Start the first paragraph in the Discussion with a paragraph stating the rationale behind the study, the objectives, and the main findings. End Discussion with a short conclusion.

**Acknowledgements*:*** In this section, the authors may acknowledge (briefly) their support staff.

**Conflict of interest*:*** All papers with a potential conflict of interest must include a description/explanation in a separate heading.

**Funding details:** The authors should state the source of findings of the study (with research funder and/or grant number). If no fund, the authors should state that the study is self-funded.

**References**

*Citation of references:* In the text, references should be cited by the author(s) surname(s) and the year of publication (e.g. Salem, 2020). References with two authors should be cited with both surnames (e.g. Salem and Meshrif, 2021). References with three or more authors should be cited with the first author followed by et al. (in italics; e.g. Salem et al., 2021). Names of organizations used as authors (e.g. Food and Drug Administration) should be written out in full in the list of references and on the first mention in the text. Subsequent mentions may be abbreviated (e.g. FDA).

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- References from journal articles are formatted in APA as this example: Al-Amoudi WM (2018). Toxic effects of Lambda-cyhalothrin on the rat thyroid. Involvement of oxidative stress and ameliorative effect of ginger extract. Toxicology Reports, 5: 728-736.
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- References from chapters or parts of books are formatted as this example. Nozière P, & Hoch T (2006). Modelling fluxes of volatile fatty acids from rumen to portal blood. In: Nutrient digestion and utilization in farm animals (Kebreab E, Dijkstra ANM, Bannink A, Gerrits WJJ & France J, eds.), pp. 40–47. CABI Publishing. Wallingford, UK.

**Tables**: The data should be presented in tables or in graphs, not both.

- Each table should be placed on a separate page at the end of the main text.
- Tables are numbered consecutively using Arabic numbering. They are referred to as Table 1, Table 2, etc., with capital 'T', no italics
- Each table has its explanatory caption. The caption is sufficient to permit the table to be understood without reference to the text.
- Abbreviations used in tables/figures have to be defined either as footnotes or in the caption.

**Figures**

- Package the figures in a single PowerPoint file. Each figure in a separate slide.
- Figure size should be readable in a width of approximately 8-175 mm (i.e. the maximum size of printing over two columns).
- Ensure that the font size is large enough to be readable at the final print size, use Calibri font to ensure that they are consistent throughout the figures.
- The figures should preferably be provided as TIFF or EPS files.
- The resolutions of figures must be at least 300 dpi.
- Preparation of images for a manuscript: For guidance, we refer to the Journal of Cell Biology's instructions to authors (http://jcb.rupress.org/site/misc/ifora.xhtml#image_aquisition).
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- Supplementary material is submitted along with the main manuscript in a separate file and identified at uploading as "Supplementary File – for Online Publication Only" The title of the article is included at the top of the supplementary material.

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