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Heavy Metal Tolerant Filamentous Fungi from Municipal Sewage for Bioleaching

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ABSTRACT

Heavy metal contamination in ground water resources and in air is a major problem today which requires appropriate treatment before discharge in to environment. Bioleaching is proposed as an ideal method of removing heavy metals before discharge into environment. In the present study heavy metal tolerant filamentous fungi was isolated from municipal sewage towards their development as ideal biocatalyst for bioleaching of heavy metals from municipal sewage. Among the fungi isolated from municipal sewage sludge two isolates, which were identified as *Aspergillus niger* and *Penicillium chrysogenum*, showed tolerance to copper, cadmium and lead, the main petroleum related heavy metals present in wastes and waste water These strains were exposed to heavy metals up to 800 ppm. Heavy metal concentrations had a direct impact on the cultural and morphological characteristics of fungi during growth. Lead caused least variations in cultural and morphological characteristics, compared to cadmium which was the most toxic, causing significant cultural and morphological variations. Among the two organisms, the *A. niger* showed maximum resistance while *P. chrysogenum* recorded the least tolerance particularly for cadmium. A growth pattern, which was consistent for each fungus under the influence of varying concentrations of heavy metals, suggest that both fungal species are able to adapt to high concentrations of heavy metal and holds potential for use in bioleaching of heavy metals.

Key words: *Aspergillus*, metal resistance, municipal solid waste, *Penicillium*, screening, submerged growth

INTRODUCTION

Once upon a time, water supply was adequate to meet the various demands of human beings. But now a day's throughout the world today, the rise in water demand in urban areas coupled with possible adverse climate scenarios, increasing awareness on environmental issues and lack of additional water resources, pose new challenges to water resource managers (Kodikara *et al.,* 2010). Water-considered the oil of the 21st century has been at the core of every discussion on sustainable development (Joshi and Kapadia, 2010).

Heavy metals, a serious form of pollutants greatly affects water supplies and agriculture lands. Heavy metals and trace elements in subsurface environments come from natural and anthropogenic sources. The intense petrochemical works, rapid industrialization and urbanization during last two decades increase this pollution enormously. Heavy metal pollution of the biosphere has received huge concern due to its toxicity, abundance, persistence and subsequent accumulation in environment. These toxic heavy metals in air, soil and water are global problems that are a growing threat to humanity (Dong *et al.,* 2010). Contamination of surface and ground water sources and soils by these metals is on an increase globally, in Gulf region particularly in Saudi Arabia (Hashem, 1993) due to acid rain and gulf war. Because of their high solubility in the aquatic environments, heavy metals can be absorbed by living organisms. Once they enter the food chain, large concentrations of heavy metals may accumulate in the human body. If the metals are ingested beyond the permitted concentration, they can cause serious health disorders. The fear of polluting the Arabian Gulf has led the regional organization for protection of the marine environment based in Kuwait to adopt a policy of not discharging wastewater into the sea (Banks, 1991). It has been reported that sewage in the Riyadh area contains considerable amounts of heavy metals (Mashhady, 1984).

Saudi Arabia, located in an arid region where water resources are limited, is the third largest consumer of water per capita in the world. In recent years, the demand for potable water has increased due to the growing population. Households are not the only consumers of clean water, but industry and agriculture also need water on a daily basis. It has been estimated that the agricultural sector accounts for 94% by volume of the total water consumption in Saudi Arabia (Dabbagh and Abderrahman, 1997). Further, the municipal sewage and industrial waste water discharged into environment also pollute the available ground water resources and coastal environments.

Typical sewage may contain oxygen-demanding materials, grease, oil, scum, pathogenic bacteria, viruses, pesticides, refractory organic compounds and heavy metals (Giller *et al.,* 2009). Sewage, on treatments and disposal in to open environment results in the accumulation of large amount of sludge, which are disposed off worldwide through land disposal. One of the main concerns in the land disposal of sludge is the presence of toxic heavy metals which gets magnified during various physico-chemical and biological interactions occurring in sludge treatment. Another major concern over the presence of these heavy metals in the environment is their non-biodegradability and consequent persistence. Since, the presence of heavy metals can pose a long-term environmental hazard once accumulated in land with time (Dutta, 2002; Mc-Bride, 1995), reduction or elimination of heavy metals from these sludge before land application is necessary (Dutta, 2002; Mc-Bride, 1995). The commonest way of disposing waste water sludge and solid waste is incineration, which results in heat and electricity production, as well as a significant reduction in the waste volume. However, the produced ash is a problem (Fedje *et al.*, 2010).

Heavy metal elements in municipal solid waste are gasified during incineration process and then concentrated in ash particles mainly in the form of metal oxides, which are packed in silicate crystal lattices. Generally, MSWI fly ash may be detoxified by removing or recovering toxic metals before being reused as construction materials (e.g., cement, concrete, ceramics and glass) (Ferreira *et al.,* 2003). It has been suggested that the treatment of the ash may lead to both detoxification as well as potential resource recovery. Conventionally, thermal treatment, chloride evaporation (Tateda *et al.,* 1998) and chemical leaching (Tateda *et al.,* 1998; Hong *et al.,* 2000) are used in the detoxification or decontamination of incineration fly ash. Unfortunately, these techniques suffer from the main disadvantage of high-energy requirement, as well as the liability of hazardous chemical usage during the treatment.

Biohydrometallurgical approaches are generally considered a green technology with low-cost and low-energy requirement. Three main groups of microorganisms have been used for bioleaching process; these are autotrophic bacteria (e.g., *Thiobacilli* sp.), heterotrophic bacteria (e.g., *Pseudomonas* sp., *Bacillus* sp.) and heterotrophic fungi (e.g., *Aspergillus* sp., *Penicillium* sp.) (Schinner and Burgstaller, 1989). According to the general statements made about the differences in sensitivity of different groups of microbes: fungi are more resistant than bacteria (McGrath, 2001). Compared to bacterial leaching, fungal leaching has the following advantages: (1) ability to grow under higher pH and thus is more suitable in bioleaching of alkaline solid waste; (2) a generally faster leaching process with shorter lag phase and (3) ability of excreted metabolites (e.g., organic acids) to form complexes with metal ion, thus reducing its toxicity to the biomass (Burgstaller and Schinner, 1993; Castro *et al.*, 2000). In recent studies, heavy metals in MSWI fly ash have been demonstrated to be extractable by *A. niger* according to four main mechanisms in bioleaching process, including acidolysis, complexolysis, redoxolysis and bioaccumulation. In this context the present study deals with screening of potential fungi that can tolerate heavy metals from municipal sewage solids and their prospects for utilization in bioleaching.

MATERIALS AND METHODS

Isolation of fungi: Potential fungi that could grow in medium containing heavy metals were isolated from sewage sludge collected from municipal waste water treatment plant (North plant), Riyadh, Saudi Arabia during the period between January 2009 to December 2009. Sludge disposed at the collection was aseptically collected ad transferred into sterile polythene bags, transported immediately to laboratory and processed for microbiological analysis. Ten gram of the collected sludge was added into presterilized Ringers solution and homogenized for 30 min in an sterile electric blender and appropriate serial dilutions were prepared and used as inocula for plating on Czapek Dox agar containing sucrose 30.0 g; sodium nitrate 3.0 g; magnesium sulfate 0.5 g; potassium chloride 0.5 g; iron (III) sulfate 0.01 g; di-potassium hydrogen phosphate 1.0 g and agar-agar 13.0 g in 1 L of distilled water was prepared and used for isolation of fungi. After inoculation the plates were incubated at 25°C for 3-7 days (Saxena *et al.*, 2006). Fungal colonies with different morphologies were selected, subcultured, purified by repeated streaking and maintained as stock culture in the same medium. The pure cultures were used for further studies

Screening for metal tolerance: All the fungal isolates were evaluated for their metal tolerance and growth efficiency in the presence of varying concentrations of heavy metals that included copper as $CuSO_4 \cdot 5\,H_2O$, cadmium as $Cd\,(NO_3)_2 \cdot 4\,H_2O$ and lead as $Pb\,(NO_3)_2$ based on the methods of Nazareth and Marbaniang (2008) using Czapek Dox agar. The strategy adopted for screening of metal tolerant fungi was to evaluate growth in agar plates containing increasing concentrations of metals by successive transfer of colonies to fresh plates with increased concentration of metal till there was no growth. Fungal growth was monitored by measuring the spread of the culture from the point of inoculation or centre of the colony. Appropriate controls were also maintained for comparative evaluation. Tolerance was measured from the growth of the fungi in the presence of metal divided by growth in the same period in the absence of metal (Valix *et al.*, 2001). Isolates which showed significant growth and tolerance to high concentration of metal were ranked and two best strains were selected and identified to species level on the basis of their cultural and morphological characteristics (Alexopoulos *et al.*, 1996; Burnett, 1976).

Impact of heavy metal concentration on growth and morphology of fungi: Impact of varying concentrations of heavy metal on growth and physiological response of fungi to metals was assessed by growing the fungi in Sabouraud broth incorporated with desired heavy metals. Two strains, namely *A. niger* and *P.chrysogenum,* selected from screening studies were used in this study. Inoculums for the studies were prepared as spores by consecutive cultivation, three times, on Sabouraud Dextrose Agar (SDA) plates. Five-day-old conidia were harvested from Sabouraud dextrose agar surface using sterile distilled water and appropriate dilutions were made such that the spore suspension contained 3.5×10^6 spores mL^{-1}. The heavy metal tolerant fungi were grown on S-CDA plates in the presence of the metal at a mid-point of the maximum tolerance concentration and at a sub-maximal tolerance level (Saxena *et al.,* 2006). Two sets of flasks were prepared containing medium incorporated with metals and without metal (control). After inoculation of the medium at 1% level (v/v), the flasks with inoculated media were incubated at 25°C for a period of 7 days, one set in a static condition and another set in an environmental shaker (150 rpm). The fungal cultures were periodically examined visually for changes in their cultural characteristics such as growth pattern, colony morphology, sporulation microscopically and pigment production. The pellet formation in the medium and changes in colony morphology during submerged cultivation were also determined using micrometry under trinoccular Nikon microscope. The dry weight of the mycelial mat obtained from the flask culture after incubation was also determined (Saxena *et al.,* 2006) as given below:

$$\text{Percentage of increase in the dry weight of the mycelial mat as compared to its fresh weight} = \left[\frac{y1\text{-}x1}{y\text{-}x}\right]\times 100 \text{ or } \left\{\frac{\text{Dry weight}}{\text{Wet weight}}\right\}\times 100$$

RESULTS

About 100 fungal isolates were obtained from the municipal sewage sludge on plating the sample on Czapek Dox agar medium. All the isolates were initially grown in Czapek Dox broth containing selected heavy metals and checked for growth. Based on their growth and biomass obtained they were ranked and the best two strains which showed maximal biomass alone were selected for further studies. Both the two strains were identified as *Aspergillus niger* KSUF10 and *Penicillium chrysogenum* KSUF65.

Both the strains grew faster in the absence of heavy metals. *A. niger* growth was luxuriant and the colonies were dark brown velvety with black conidial heads. On reverse side the colony was pale yellowish (Fig. 4). When observed under light microscope, the conidia appeared to be well arranged and spore chains were formed after 3 days of incubation. *P. chrysogenum* produced flat colonies, blue green spores and found to produce green yellow pigment at the centre with age. The maximum tolerance levels of the cultures to the heavy metals tested are shown in Fig. 1. Both the cultures showed concentration dependent reduction in their growth response to both copper and cadmium, while recording some tolerance to lead. The cultures, in the presence of metals, showed striking variations in colony morphology as compared to the controls; which were more pronounced (Fig. 4). With increasing metal concentrations, both the cultures showed delayed sporulation along with increase in concentration. Whereas, the pigment production in *P. chrysogenum* increased with concentration of the metal. *A. niger* showed some kind of concentration dependent extracellular compound production in the presence of copper. Further both the isolates exhibited marked changes in their conidial head and size of the conidium. Cultures grown for five days with different concentration of metals showed variation in fungal biomass when compared with control medium. Significant decrease in mycelial mass (Fig. 2, 3) was observed both in static and aerated incubation conditions along with increasing concentration of metal when compared to control (Fig. 5, 6). The mycelial growth over the surface of the medium to form a mat, which is also referred as pad, was observed during the growth of fungi in the metal containing medium.

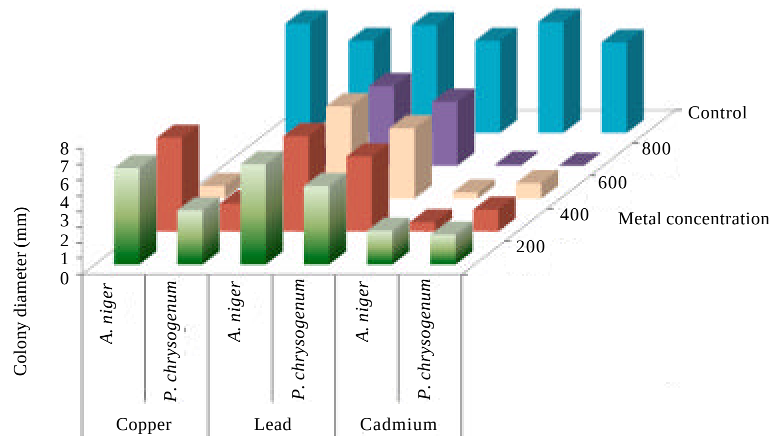

Fig. 1: Plot of municipal sludge fungal tolerance and growth to heavy metals

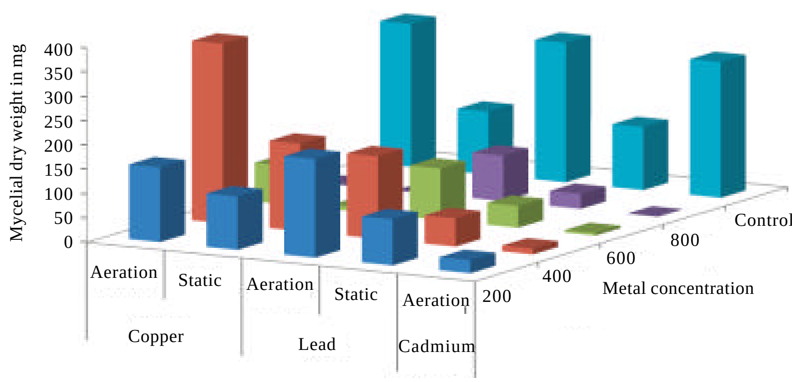

Fig. 2: Plot of municipal sludge fungi *A. niger* and *P. chrysogenum* tolerance and growth response to heavy metals in submerged (static) condition

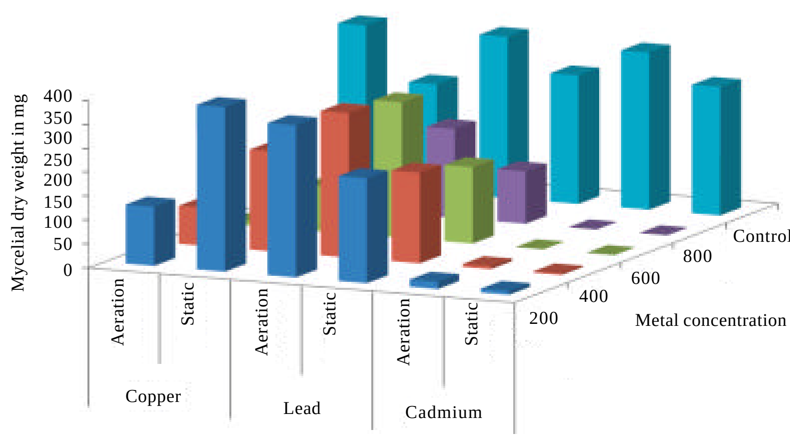

Fig. 3: Plot of municipal sludge fungi *A. niger* and *P. chrysogenum* tolerance and growth response to heavy metals in submerged (aerated) condition

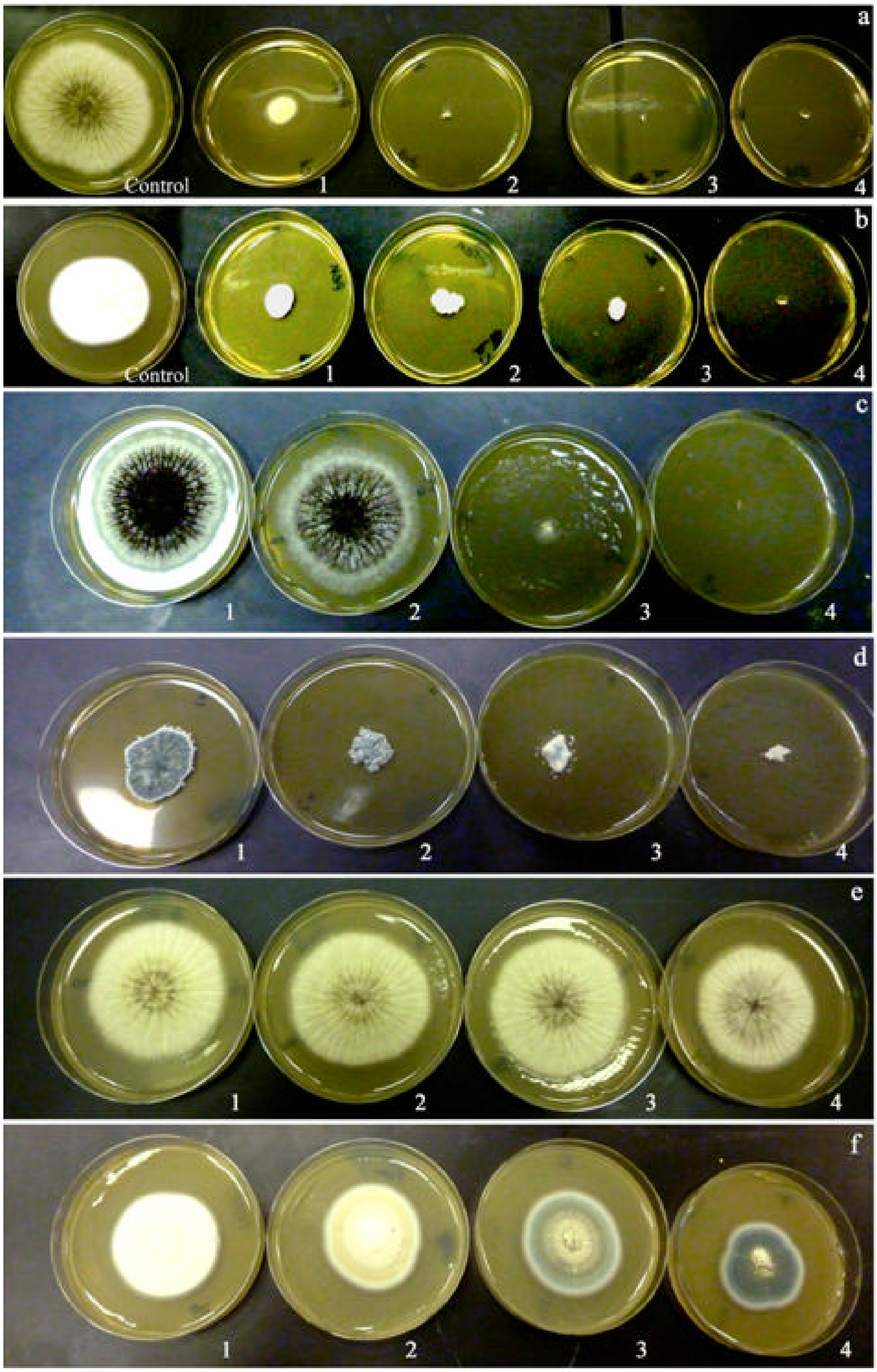

Fig. 4: Cultural and morphological characters of municipal waste isolate *A. niger* (a, c, e) and *P. chrysogenum* (b, d, f) on CDA with metal copper (a, b), lead (c, d) as well as cadmium (e, f) in the conc. 200 (1), 400 (2), 600 (3) and 800 (4)

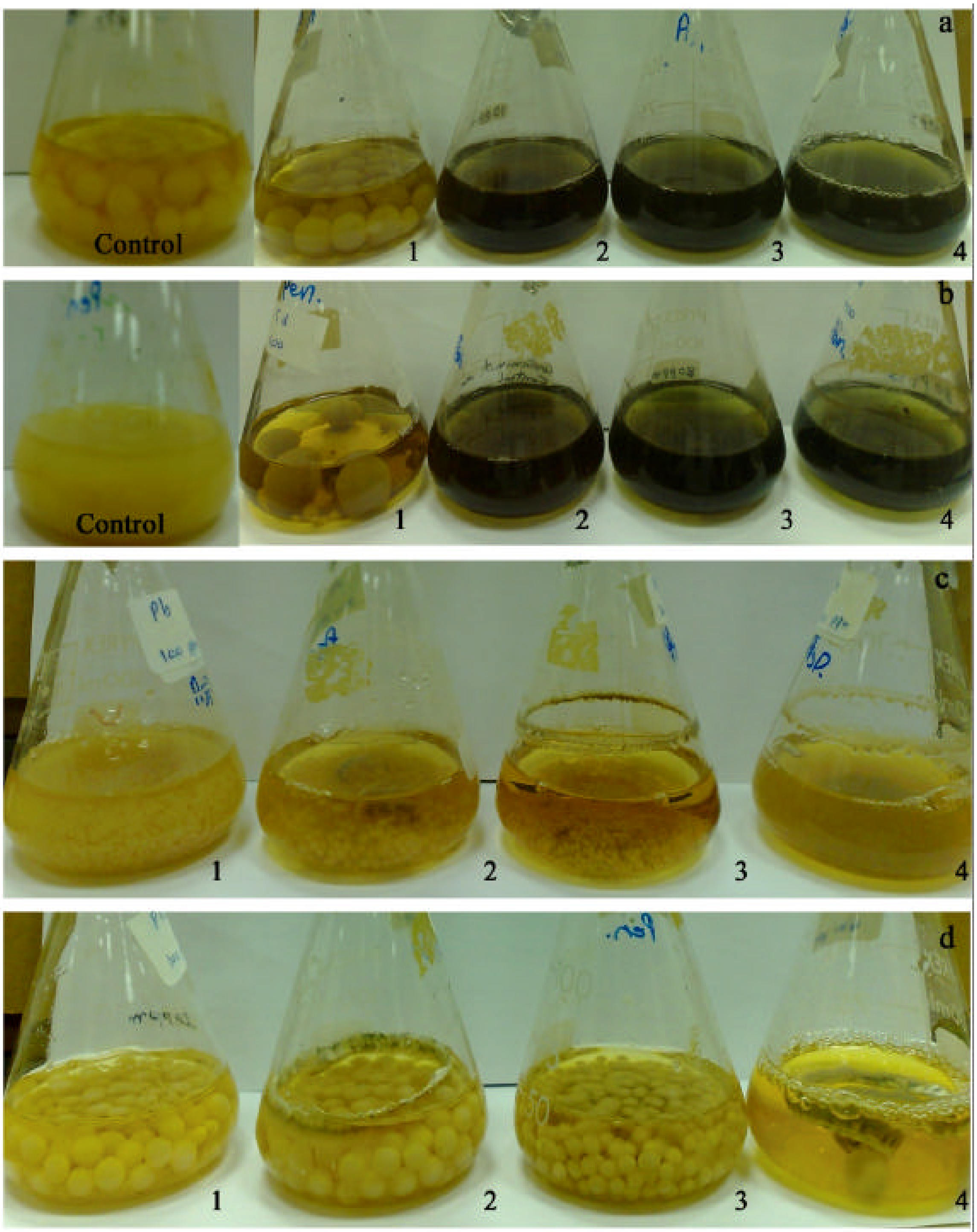

Fig. 5: Submerged growth (static) cultural and morphological characters of municipal waste isolate *A. niger* (a, c, e) and *P. chrysogenum* (b, d, f) on CDA with metal copper (a, b), lead (c, d) as well as cadmium (e, f) in the conc. 200 (1), 400 (2), 600 (3) and 800 (4)

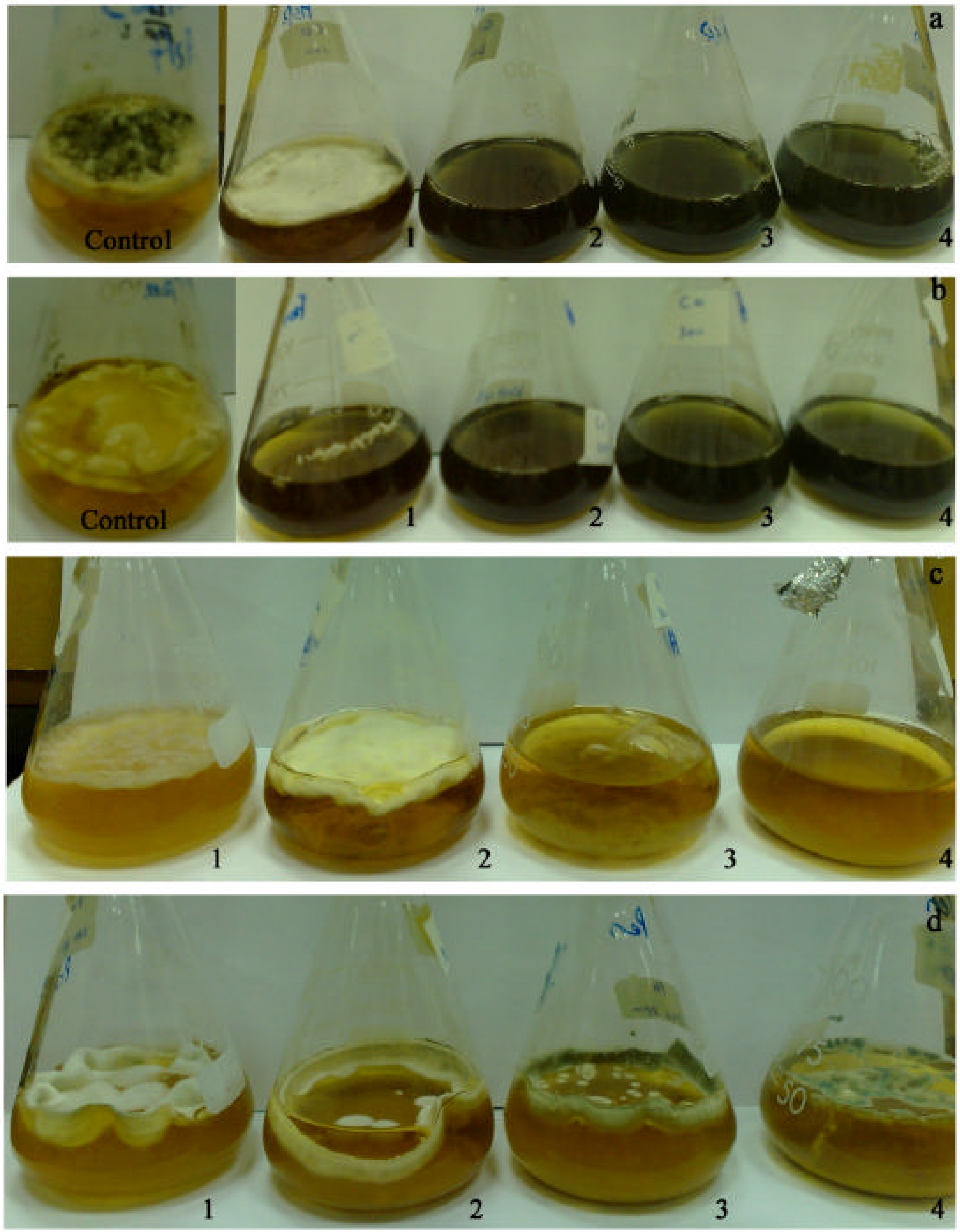

Fig. 6: Submerged growth (aerated) cultural and morphological characters of municipal waste isolate *A. niger* (a, c, e) and *P. chrysogenum* (b, d, f) on CDA with metal copper (a, b), lead (c, d) as well as cadmium (e, f) in the conc. 200 (1), 400 (2), 600 (3) and 800 (4)

DISCUSSION

Organisms under the stress of the environment develop various mechanisms in order to cope with the adverse conditions. In fungi, severe stresses may be tolerated by inherent physiological characteristics, or by adaptation through a temporary alteration in their developmental pattern (Nazareth and Marbaniang, 2008) which is highly evidenced in this study. Both the tested fungi showed marked morphological changes during their development in this study. Microbes interact with metals and minerals in natural and synthetic environments, altering their physical and chemical state, with metals and minerals also able to affect microbial growth, activity and survival (Gadd, 2010). All the three tested metals toxic nature is already known one which significantly influenced the growth of the fungi in this study also. Number of earlier studies indicated that metal resistant fungi have the ability to grow in an environment containing heavy metals. The fungal survival in presence of toxic metals mainly depends on intrinsic biochemical and structural properties, physiological and/or genetic adaptation, including morphological changes and environmental modification of metal speciation, their availability and toxicity (Gadd, 1993), the relative importance of each factor often being difficult to determine. Physiological changes induced in response to metal stress in various microorganisms have been recorded (Nazareth and Marbaniang, 2008). Metals are directly and/or indirectly involved in all aspects of microbial growth, metabolism and differentiation (Gadd, 1992). The mycelial development, sporulation and extracellular secretion were found to be significantly affected by heavy metals as observed in this study corroborates with earlier observation.

Mycelial growth of certain fungi occurs in form of pellets under submerged conditions in liquid medium with agitation (Tucker and Thomas, 1992). This usually results from aggregation of spores before germination, entrapment of spores by germ tubes or less commonly, aggregation of young mycelia. It depends on the physico-chemical and physiological characteristics of spores and hyphae (Prosser, 1994). Bioleaching of fly ash by the fungi *A. niger* was already established and first reported by Bossahard *et al.* (1996). Productivity of filamentous fungi, like *A. niger*, is closely connected with the occurring morphological development based on macroscopic growth (Papagianni, 2004). Each product has its own optimum fungal morphology, as pelleted or freely dispersed mycelia. Depending on the product targeted, *A. niger* is grown either as disperse mycelia (e.g., pectic enzyme production) (Calam, 1976) or as pellets (e.g., citric acid production) (Metz *et al.*, 1979) in submerged cultivations. The biomass dry weight and pellet size were used to follow growth characteristics of filamentous fungi during submerged fermentations (Kelly *et al.*, 2006). Advantages of pellet cultivations are the significant, decrease of the viscosity and the easier separation of the biomass from the cultivation broth. The initial values of the A. *niger* pellet diameter were of a similar size of approximately 0.9 mm, relatively unstructured and irregular at the periphery and had overall a more homogeneous structure. When the concentration of the heavy metals increased, the size of the pellets decreased and become more compact with more or less circular morphology. In *P. chrysogenum* surface of pellets is loosely held together at lower concentration of heavy metals with thin hyphae whereas hyphae on the inner side remain closely packed and dense, the intensity of which increases with metal concentration. The metal toxicity, have been reported to have a tendency to increase with molecular weight (Avakyan, 1994).

The relative toxicity of the heavy metals for each strain becomes obvious at the higher concentrations. These heavy metals are expected to be as major pollutants in the municipal solid waste of Saudi Arabia due to its ever increasing petrochemical and e-waste. The growth pattern, for each of the fungi in the presence of heavy metals, as shown in kigures show predictable patterns, which is similar for each fungus. These results indicated the possible adaptation of the fungi to heavy metal stress conditions and their potential for use in extracting metal from wastes. In conclusion, the results of the present study suggest that native fungi isolated from municipal sewage sludge of Riyadh city holds immense potential for use as a bioleaching agent towards bioremediation of heavy metals in municipal.

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REFERENCES

Alexopoulos, C.J., C.W. Mims and M. Blackwell, 1996. Introductory Mycology. John Wiley and Sons, New York.

Avakyan, Z.A., 1994. The toxicity of heavy metals to microorganism. Qatar. Univ. Sci. J., 14: 3-65.

Banks, P.A., 1991. Wastewater reuse case studies in the Middle East. J. Water Sci. Technol., 23: 2141-2148.

Bossahard, P.P., R. Bachofen and H. Braudl, 1996. Metal leaching of fly ash from municipal water incineration by *A. niger*. Sci. Technol., 30: 3066-3070.

Burgstaller, W. and F. Schinner, 1993. Leaching of metals with fungi. J. Biotechnol., 27: 91-116.

Burnett, J.H., 1976. Fundermentals of Mycology. 2nd Edn., Edward Arnold Publishers Ltd., London, ISBN: 0-7131-2617-5.

Calam, C.T., 1976. Starting investigation and production cultures. Process Biochem., 11: 7-12.

Castro, I.M., J.L.R. Fietto, R.X. Vieira, M.J.M. Tropia, L.M.M. Campos, E.B. Paniago and R.L. Brandao, 2000. Bioleaching of zinc and nickel from silicates using *Aspergillus niger* cultures. Hydrometallurg, 57: 39-49.

Dabbagh, A.E. and W.A. Abderrahman, 1997. Management of groundwater resources under various irrigation water use scenarios in Saudi Arabia. Arab J. Sci. Eng., 22: 47-64.

Dong, X., C. Li, J. Li, J. Wang, S. Liu and B. Ye, 2010. A novel approach for soil contamination assessment from heavy metal pollution: A linkage between discharge and adsorption. J. Hazard. Mater., 175: 1022-1030.

Dutta, S., 2002. Environmental Treatment Technologies for Hazardous and Medical Wastes. McGraw-Hill, New Delhi, India.

Fedje, K.K., C.C. Ekberg, G. Skarnemark and B.M. Steenari, 2010. Removal of hazardous metals from MSW fly ash: An evaluation of ash. J. Hazard. Mat., 173: 310-317.

Ferreira, C., A. Ribeiro and L. Ottosen, 2003. Possible applications for municipal solid waste fly ash. J. Hazard. Mater., 96: 201-216.

Gadd, G.M., 1992. Microbial Control of Heavy Metal Pollution. In: Microbial Control of Pollution, Fry, J.C., G.M. Gadd, R.A. Herbert, C.W. Jones and Watson-Craiki (Eds.). Cambridge University Press, Cambridge, pp: 59–88.

Gadd, G.M., 1993. Interactions of fungi with toxic metals. New Phytol., 124: 25-60.

Gadd, G.M., 2010. Metals, minerals and microbes: Geomicrobiology and bioremediation. Microbiology, 156: 609-643.

Giller, K.E., E. Witter and S.P. McGrath, 2009. Heavy metals and soil microbes. Soil Biol. Biochem., 41: 2031-2037.

Hashem, A.R., 1993. Heavy metal analysis of water and soil from Saudi Arabia. J. King Saud Univ. Sci., 5: 39-46.

Hong, K.J., S. Tokunaga, Y. Ishigami and T. Kajuichi, 2000. Extraction of heavy metals from MSW incinerator fly ash using saponins. Chemosphere, 41: 345-352.

Joshi, M.B. and V.P. Kapadia, 2010. Sharing water in the 21st century: Rethinking the rationale. Irrigation Drainage, 59: 92-101.

Kelly, S., L.H. Grimm, C. Bendig, D.C. Hempel and R. Krull, 2006. Effects of fluid dynamic induced shear stress on fungal growth and morphology. Process Biochem., 41: 2113-2117.

Kodikara, P.N., B.J.C. Perera and M.D.U.P. Kularathna, 2010. Stakeholder preference elicitation and modelling in multi-criteria decision analysis – A case study on urban water supply. Eur. J. Operat. Res., 206: 209-220.

Mashhady, A.S., 1984. Heavy metals extractable from a calcarious soil treated with sewage sludge. Environ. Pollut. (Series B), 8: 51-62.

Mc-Bride, M.B., 1995. Toxic metal accumulation from agricultural use of sludge: Are USEPA regulations protective. J. Environ. Qual., 24: 5-18.

McGrath, S.P., 2001. How Organisms Live with Heavy Metals in the Environment. A Fact Sheet on Environmental Risk Assessment. ICME, Turkey.

Metz, B., N.W.F. Kossen and J.C. van Suijdam, 1979. The rheology of mould suspensions. Adv. Biochem. Eng., 11: 104-156.

Nazareth, S. and T. Marbaniang, 2008. Effect of heavy metals on cultural and morphological growth characteristics of halotolerant *Penicillium* morphotypes. J. Basic Microbiol., 48: 363-369.

Papagianni, M., 2004. Fungal morphology and metabolite production in submerged mycelia processes. Biotech. Adv., 22: 189-259.

Prosser, J.I., 1994. Kinetics of Filamentous Growth and Branching. In: The Growing Fungus, Gow, N.A.R. and G.M. Gadd (Eds.). Chapman & Hall, London, UK., pp: 301–318.

Saxena, P., A. Bhattacharyya and N. Mathur, 2006. Nickel tolerance and accumulation by filamentous fungi from sludge of metal finishing industry. Geomicrobiol. J., 23: 333-340.

Schinner, F. and W. Burgstaller, 1989. Extraction of zinc from industrial waste by *Penicillium* sp. Applied Environ. Microbiol., 55: 1153-1156.

Tateda, M., M. Ike and M. Fujita, 1998. Comparative evaluation of processes for heavy metal removal from municipal solid waste incineration fly ash. J. Environ. Sci., 10: 458-465.

Tucker, K.G. and C.R. Thomas, 1992. Mycelial morphology: The effect of spore inoculum level. Biotechnol. Lett., 14: 1071-1074.

Valix, M., J.Y. Tang and R. Malik, 2001. Heavy metal tolerance of fungi. Minerals Eng., 14: 499-505.