

## Isolation of hexavalent chromium (vi) tolerant fungi from tannery effluent and their growth responses to different concentrations of chromium (vi)

### Aislamiento de hongos tolerantes de cromo hexavalente (vi) de los efluentes de Tannery y sus respuestas de crecimiento a las diferentes concentraciones de cromo (vi)

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#### ABSTRACT

The main objective of this study was to isolate, characterize, and determine chromium (VI) tolerance capacities of fungal strains from tannery effluent of Kolkata Leather Complex area. Chromium (VI) is a heavy metal that is known to be toxic as well as carcinogenic. It is an important heavy metal widely used in various industries of which the tanning industry deserves special mention. The majority of Cr (VI) waste is discharged into the environment annually as a consequence of industrial and manufacturing activities. Kolkata Leather Complex is an industrial complex intended to serve as a central leather-tanning complex for Kolkata located at Bantala, near East Kolkata, India. Samples (tannery effluent) were collected in sterile capped tubes from Kolkata Leather Complex Area and brought to the laboratory for analysis. The samples were serially diluted and were plated on Czapekdox agar plates and incubated at 30°C. After a few days, a number of morphologically different colonies were selected and sequentially subcultured for pure colony isolation on the same medium. They were preserved in the refrigerator at 4°C. Lactophenol cotton blue staining was done to observe and record the fungal morphological characteristics. They were further assessed for their hexavalent chromium tolerance capacities and subsequently the metal tolerance index was derived for each strain respectively. All 16 fungal strains isolated from the Kolkata Leather Complex tannery area were found to be chromium (VI) tolerant starting at 0.1mg/ml Cr (VI) concentration. Maximum chromium (VI) tolerance was displayed by sample S12 (2mg/ml) which was later identified as having 99.82% similarity with *Aspergillus nomius* strain.

Keywords: effluent, fungi, heavy metal, isolation, hexavalent chromium, tannery, tolerance.

## RESUMEN

El objetivo principal de este estudio fue aislar, caracterizar y determinar las capacidades de tolerancia al cromo (VI) de las cepas de hongos del efluente de la curtiduría del área del complejo de cuero de Kolkata. El cromo (VI) es un metal pesado que se sabe que es tóxico y cancerígeno. Es un metal pesado importante ampliamente utilizado en diversas industrias de las cuales la industria del curtido merece una mención especial. La mayoría de los residuos de Cr (VI) se descargan al medio ambiente anualmente como consecuencia de las actividades industriales y de fabricación. Kolkata Leather Complex es un complejo industrial destinado a servir como un complejo central de curtido de cuero para Kolkata ubicado en Bantala, cerca de East Kolkata, India. Se recogieron muestras (efluente de la curtiduría) en tubos tapados estériles del área del complejo de cuero de Kolkata y se llevaron al laboratorio para su análisis. Las muestras se diluyeron en serie y se sembraron en placas de agar Czapekdox y se incubaron a 30° C. Después de unos días, se seleccionaron varias colonias morfológicamente diferentes y se subcultivaron secuencialmente para aislamiento puro de colonias en el mismo medio. Se conservaron en el refrigerador a 40°C. Se realizó una tinción con azul de algodón de lactofenol para observar y registrar las características morfológicas fúngicas. Posteriormente se evaluaron sus capacidades de tolerancia al cromo hexavalente y, posteriormente, se obtuvo el índice de tolerancia al metal para cada cepa respectivamente. Se descubrió que las 16 cepas de hongos aisladas del área de curtiduría del complejo de cuero de Kolkata eran tolerantes al cromo (VI) a partir de una concentración de Cr (VI) de 0.1 mg / ml. La máxima muestra de tolerancia al cromo (VI) se mostró en la muestra S12 (2 mg / ml) que luego se identificó con una similitud del 99.82% con la cepa *Aspergillus nomius*.

Palabras clave: efluentes, hongos, metales pesados, aislamiento, cromo hexavalente, curtiduría, tolerancia.

## INTRODUCTION

The soil and water are frequently contaminated with toxic heavy metals and organic pollutants as an out-turn of human activities (Siddiquee *et al.* 2015). Heavy metal like Chromium (Cr) is used in an array of different industrial processes like in steel production, wood preservation, leather tanning, aluminum productions, nuclear power production, metal processing, electroplating, iron sheet cleaning, chrome plating, wood preservation, water cooling, inorganic chemical production, galvanometric, electric, mining industries, battery manufacturing, mine drainage, metal corrosion inhibition, paints and pigments, metal plating, etc. and as a result it is being discharged into the environment through the waste water generated from these processes (Papp 1985, Mishara and Doble 2008, Ranjan *et al.* 2009, Gupta and Rastogi 2009). Chromium can exist in hexavalent as well as in trivalent forms. However, in its hexavalent form it is very toxic, carcinogenic and mutagenic in humans as well as in animals whereas the trivalent form is less toxic, less soluble and thus a lesser hazard (Philip *et al.* 1998). Exposure to hexavalent chromium may lead to various adverse health effects including growth inhibition, cancer, organ damage, nervous system damage, and in extreme cases, death (Akpor and Muchiem 2010). The removal of heavy metals is a primary consideration for all environmental protection agencies so the development and application of cost-effective processes for the removal of heavy-metals and the detoxification of metal-contaminated wastewaters is essential to improve the competitiveness of industrial processes and simultaneously minimize the environmental hazards of toxic metal-containing effluents (Volesky and Naja 2007). However, the economic consideration of treatment based

on volume, concentration of metals, and salinity and characteristics of wastewaters makes it impossible to use conventional technologies such as precipitation and sludge separation, chemical oxidation or reduction, ion exchange, reverse osmosis, cementation, coagulation, flocculation, electrochemical treatment, membrane processes, and evaporation (Ahn *et al.* 2009, Patrón-Prado *et al.* 2010). Using microorganisms as biosorbents for heavy metals is an effective alternative to existing methods for toxicity reduction and recovery of valuable metals from industrial effluents, because of its good performance and low cost of biosorbent material (Volesky 1987, Brierley 1990 and Gadd *et al.* 1988).

Despite the fact that heavy metals are acutely toxic to most microbes, there are metal tolerant strains. Long term exposure to metals favors proliferation of microbes that are tolerant to metals and this has been investigated by assaying habitats exposed to anthropogenic or natural metal contamination over extended period (Hutchinson and Symington 1997). Many fungi have been observed to tolerate and detoxify metals by various mechanisms including extra and intracellular precipitations, valence transformations and active uptake. They usually have a high surface to volume ratio and possess the ability to detoxify metals. These are the reasons for which they are considered as potential alternative to chemical compounds for various remediation purposes of dilute solutions of metals and solid wastes (Magyarosy *et al.* 2002).

Among the use of chromium in various industries leather or tanning industry deserves special mention where chromium salts are used to convert hides into leather and the waste water generated during this process contains chromium often above the permissible limit of 0.1 mg/L (Das *et al.* 2011).

India holds 10<sup>th</sup> position in the world for its foot-wear production and is one of the major producers of raw-skins and hides. There are over 2500 tanneries in the country and about 80% of them are engaged in the chrome tanning process (Rajmani *et al.* 1995). There are nearly 500 tanneries in Bantala and they carry out 22-25% of all the tanning done in India. It is situated about 14 km from the central business district of Kolkata and spans an area of about 4.5 square kilometers. The complex is meant to serve as a leather-tanning hub for Kolkata (WBIDC annual report 2009-2010). There are pumping stations which carry the effluent from the different tanneries to a Common Effluent Treatment Plant (CETP). But, many problems and disputes concerning completion of the common effluent treatment plant have caused serious difficulties, with toxic waste polluting adjoining areas and the construction activities of some companies and so there is an enormous pressure on the government from various pollution control bodies to regulate and minimize the amount of chromium released by these tanneries (Das 2008).

The main objective of the present study was to isolate chromium tolerant fungal strains and to determine the metal tolerance capacities of the selected strains and derive their respective metal tolerance index so that in the future their biomass can be used for the biosorption of hexavalent chromium.

## MATERIALS & METHODS

Sample collection and handling: Samples (tannery effluent) from common discharge canal was collected from Kolkata Leather Complex area. The samples were collected in a sterile capped tube (50 ml capacity). The tubes were then brought to the laboratory for analysis. The samples were shaken vigorously (approx. 20 times) to obtain a uniform distribution of the organisms before plating.

Isolation of fungal strains from the effluent: The sample was serially diluted in 5 test tubes and marked as stock, ( $10^{-1}$ ), ( $10^{-2}$ ), ( $10^{-3}$ ), ( $10^{-4}$ ), ( $10^{-5}$ ). All the samples were plated on Czapekdox agar plates and incubated at 30°C. After a few days a number of morphologically different colonies were selected and sequentially subcultured for pure colony isolation on the same medium. They were tagged as S1 - S16 and preserved in the refrigerator at 4°C for further analyses.

Lactophenol cotton blue staining: To visualize and record various morphological characteristics of the fungal isolates, Lactophenol cotton blue staining was carried out and observed under low to higher magnification of optical microscope.

Growth responses of the isolated fungal strains against different concentrations of Cr (VI): To assess and derive chromium tolerance of the isolated fungal strains, agar plug transfer method was followed. In this, agar plug from previously cultured plate was inoculated at the center of a freshly chromium (VI) amended Czapekdox agar plates and incubated at 30°C. Using standardization formula ( $V_1S_1=V_2S_2$ ), increasing concentrations of Cr (VI) (0.1-2 mg/ml) was prepared aseptically by mixing appropriate amounts of Potassium Dichromate solution (stock) with molten agar. After solidification of the plates they were inoculated with the agar plugs and incubated at 30°C. The growth of the isolates were recorded regularly after 24 hours (Santhi and Guru 2014). The diameter of the growth and time taken to grow and spread is a measure of the chromium (VI) tolerance by the different isolates. As such the isolates which grew quickly or/and spread far from the center can be inferred as more tolerant to chromium (VI) at the defined conditions.

Heavy metal tolerance index: The Metal Tolerance Index (Ti) was determined as the ratio of the extended radius of the treated colony to that of the untreated colony in the plates grown.  $Ti = Dt/Du$ , where Dt is the radial extension (cm) of treated colony and Du is the radial extension (cm) of untreated colony (Santhi and Guru 2014). The Metal Tolerance Index gives a more clear comparative idea of the heavy metal tolerances displayed by the fungal isolates. All of the experiments were performed in triplicates and the data from it were expressed as mean value  $\pm$  SD.

## RESULTS & DISCUSSION

Colony and morphological characteristics of the isolated fungal strains: Different fungal colonies were observed in all the spread plates at different dilutions, from the sample acquired from Kolkata leather complex tannery area. 16 fungal strains were isolated (presented as S1-S16) employing streak plate method. The pure fungal colonies displayed distinct morphological characteristics. Some isolates were observed to give pigmentations (see Table 1 and Fig.1). The morphological characteristics of the isolated fungal strains were observed and recorded. For this, lactophenol cotton blue staining was carried out and the samples were observed under 400x magnification power of the microscope (see Fig. 2). The various recorded observations are depicted in details in Table 1.

Growth responses of the isolated fungal strains against different concentrations of Cr (VI): The growth responses of the different isolates at different concentrations of chromium were studied in this experiment. The responses of the fungi were dependent on the time of incubation and Cr (VI) concentrations used. They all were incubated for a maximum of 2 weeks at 30°C and the concentrations of Cr (VI) used were from 0.1 mg/ml to 2 mg/ml. The analysis of the results inferred that the isolate S12 was much

more adapted to grow in the presence of Cr (VI) than the other isolates at high Cr (VI) concentration of 2mg/ml (see Table 2).

Table 1. Colony and Morphological characteristics of the isolated fungal strains (S1 – S16).

Samples (Bantala)	Colony Characteristics	Morphological Characteristics
S1	The colony surface is woolly in appearance and off-white in color. Reverse is off-white.	Septate hyphae,with sterigmata, conidiophore and conidiospores.
S2	The colony surface texture is powdery, light brown in color. Reverse is light brownish.	Septate hyphae, conidiophores are branched with brush like heads bearing spores, cluster of sterigmata usually in one place.
S3	The colony surface texture is powdery light green in color initially, turns deep green with conidial production and with white borders. The reverse is off-white.	Septate hyphae,with sterigmata, conidiophore and conidiospores.
S4	The colony surface is powdery, light brown in color. Reverse is light brownish.	Septate hyphae, thin mycelium, with sterigmata, conidiophore and conidiospores.
S5	The colony surface is powdery, brown in color. Reverse is light brownish.	Septate hyphae,with sterigmata, conidiophore and conidiospores.
S6	The colony surface is smooth then turns powdery, green in color with conidial production. Reverse is orange.	Septate hyphae,with sterigmata, conidiophore and conidiospores.
S7	The colony surface texture is powdery green in color initially, turns deep green with conidial production and with white borders. The reverse is off-white.	Septate hyphae, conidiophores are branched with brush like heads bearing spores, cluster of sterigmata usually in one place.
S8	The colony surface is powdery, green in color. Reverse is dark.	Septate hyphae,with sterigmata, conidiophore and conidiospores.
S9	The colony surface is powdery with patch like growth, green in color. Reverse is white.	Septate hyphae, thin mycelium, conidiophores are branched with brush like heads bearing spores, cluster of sterigmata usually in one place.
S10	The colony surface is powdery, green in color. Reverse is off-white.	Septate hyphae,with sterigmata, conidiophore and conidiospores.
S11	The colony surface is woolly in appearance and white in color. Reverse is off-white.	Septate hyphae with thin thread-like mycelium was only observed.
S12	The colony surface texture is powdery, white in color initially, turns deep green with conidial production and with white borders. The reverse is off-white.	Septate hyphae,with sterigmata, conidiophore and conidiospores.
S13	The colony surface is woolly in appearance and white in color. Reverse is white.	Septate hyphae with thread-like mycelium was only observed.
S14	The colony surface is powdery, light brown in color. Reverse is light brownish.	Septate hyphae,with sterigmata, conidiophore and conidiospores.
S15	The colony surface texture is powdery, white in color initially, turns deep green with conidial production and with white borders. The reverse is off-white.	Septate hyphae, thin mycelium, conidiophores are branched with brush like heads bearing spores, cluster of sterigmata usually in one place.
S16	The colony surface is powdery with patch like growth, raised center and green in color. Reverse is white.	Septate hyphae, conidiophores are branched with brush like heads bearing spores, cluster of sterigmata usually in one place.

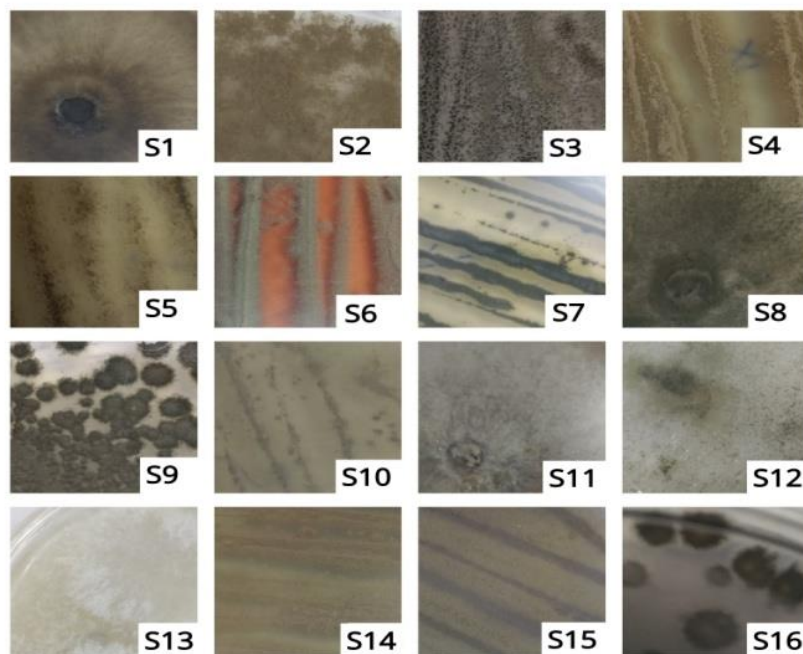


Figure 1. Colony characteristics of the isolated fungal strains

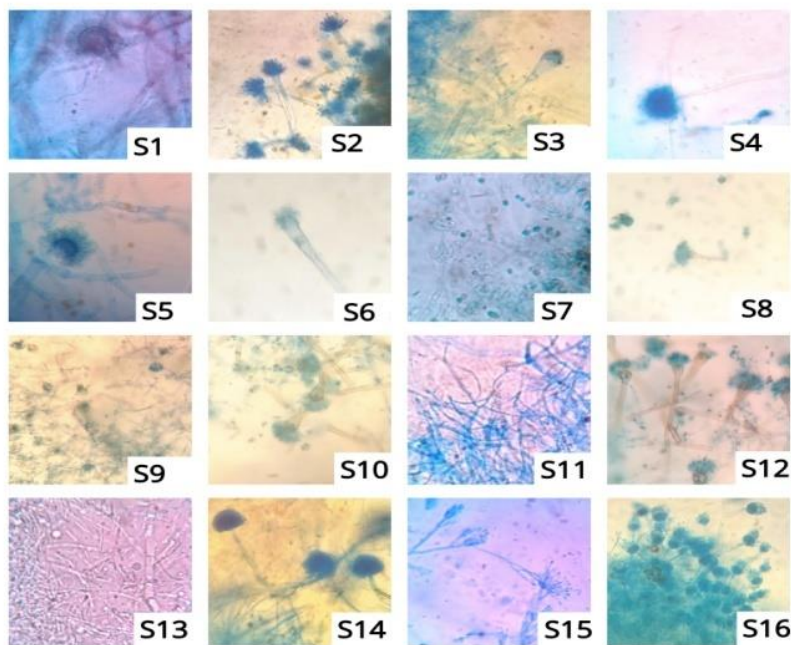


Figure 2. Morphological characteristics of the isolated fungal strains by Lactophenol cotton blue staining – (as observed under 400x magnification power of the microscope)

Table 2. Growth responses of the isolated fungal strains against different concentrations of chromium (VI)

Samples (Bantala)	Maximum Chromium (VI) Tolerant Capacity- (mg/ml)
S1	0.3
S2	0.3
S3	0.3
S4	0.3
S5	0.2
S6	0.3
S7	0.3
S8	1.8
S9	0.5
S10	0.5
S11	0.2
<b>S12</b>	<b>2</b>
S13	0.3
S14	1.8
S15	1.8
S16	0.5

Heavy metal tolerance index: Overall maximum Cr (VI) tolerant capacity at 2 mg/ml concentration was shown by the S12 isolate with a tolerance index value of 0.12 at the same concentration. While some isolates failed to survive at higher concentrations of Cr (VI) used, others thrived and displayed variable metal tolerance index with time. It was observed that as time progressed, the values of the index increased with maximum being at the last (14<sup>th</sup>) day of observation. A comparison of the maximum metal tolerance index values by the various fungal isolates against exposure to different concentrations of hexavalent chromium is provided (see Figs. 3 - 6). It was seen that all the fungal isolates S1 - S4 in Fig. 3 had same trends of maximum tolerance index with maximum value of 1 and managed to survive upto 0.5 mg/ml concentration of Cr (VI), while among S5 - S8 in Fig. 4, S5 was sensitive above concentration of 0.2 mg/ml Cr (VI) with maximum Ti value of 1, followed by S6 and S7 at 0.3 mg/ml having Ti values 0.65 and 0.6 respectively and both having maximum Ti value of 1 at 0.2 mg/ml concentration respectively and S8 had a very high tolerance capacity of 1.8 mg/ml with Ti value of 0.075 with maximum Ti value of 1 at 0.1mg/ml concentration. The fungal isolate S11 (see Fig. 5) was able to survive only upto 0.2 mg/ml of Cr (VI), with maximum Ti value of 1 whereas S9 and S10 survived upto 0.5 mg/ml

concentration with Ti values of 1 and 0.82 and maximum Ti value of 1 at 0.5 and 0.3 mg/ml concentration respectively and S12 with maximum Ti value of 1 at 0.5 mg/ml concentration and Ti value of 0.12, surviving at overall highest concentration of 2 mg/ml; while S13 (see Fig. 6) survived only upto 0.3 mg/ml concentration of Cr (VI) with Ti value of 0.31 and maximum Ti value of 1 at 0.2mg/ml concentration, followed by S16 at 0.5 mg/ml concentration with maximum Ti value of 1 and finally S14 and S15 survived upto 1.8 mg/ml concentration, each with Ti value of 0.062 and maximum Ti value of 1 at 0.3 and 0.5 mg/ml concentration respectively.

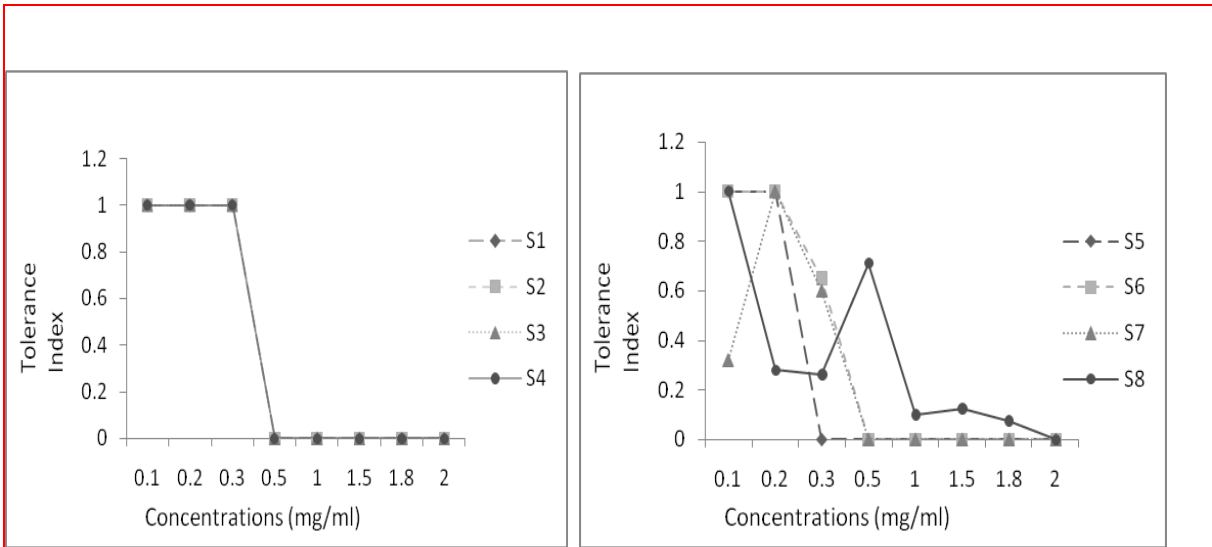


Figure 3. Comparisons of maximum tolerance index among isolates S1 - S4

Figure 4. Comparisons of maximum tolerance index among isolates S5 - S8

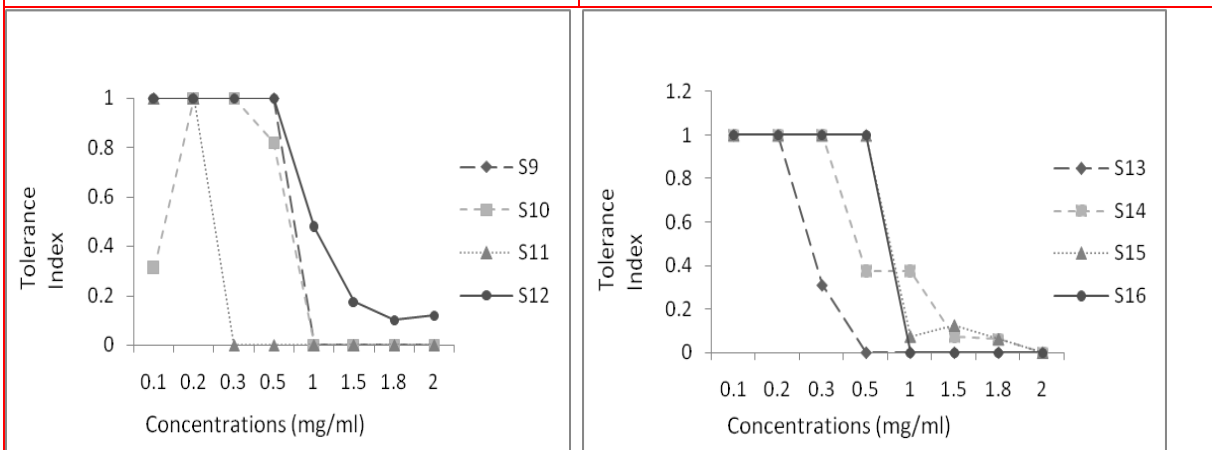
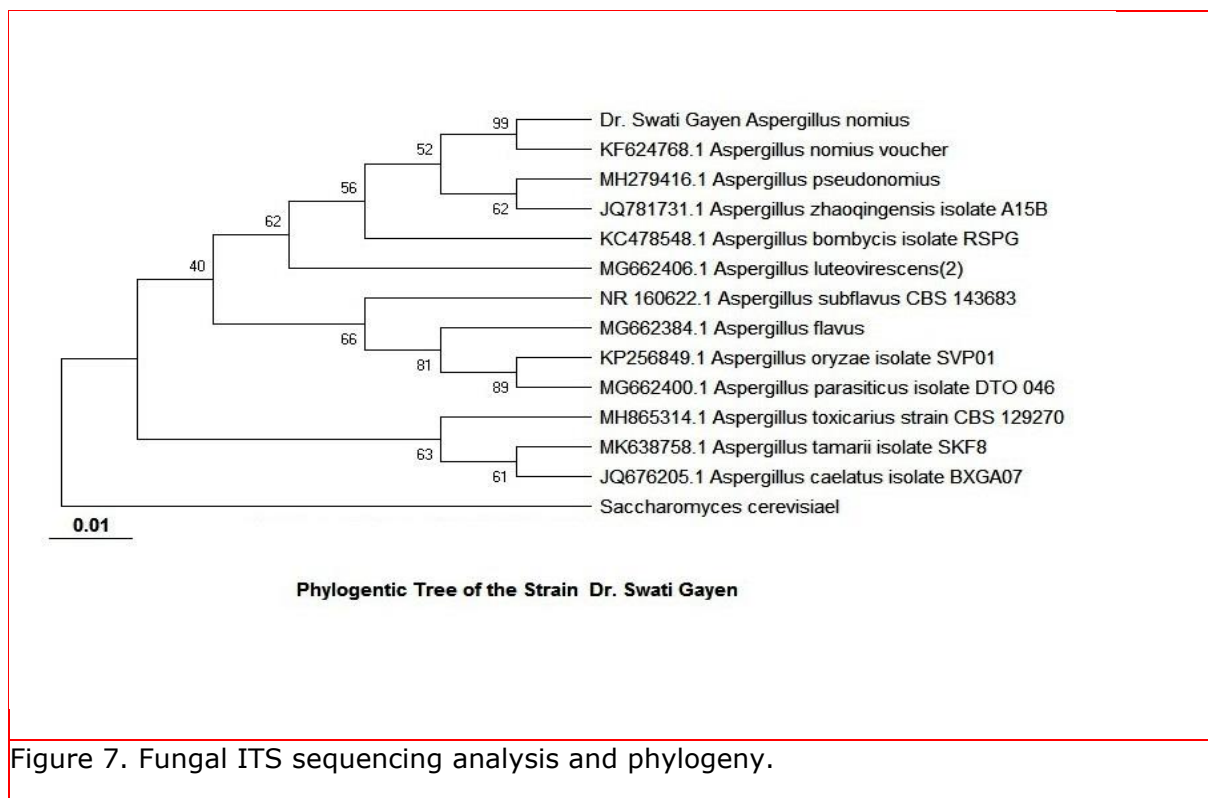


Figure 5. Comparisons of maximum tolerance index among isolates S9 - S12

Figure 6. Comparisons of maximum tolerance index among isolates S13 - S16





Fungal ITS sequencing analysis and phylogeny: In our current progress of study we were successful in isolating a fungal strain (S12) with a maximum Cr (VI) tolerant ability at 2mg/ml from Kolkata Leather Complex tannery area. Fungal ITS region sequencing and analysis done by MTCC, CSIR-IMTECH revealed that, the most Cr (VI) tolerant strain (S12) we are working with has 99.82% similarity with and is identified as an *Aspergillus nomius* strain (see Fig. 7).

As far our best knowledge, this is an exclusive study where it has been observed that an *Aspergillus nomius* strain is tolerant to hexavalent chromium as high as upto 2 mg/ml concentration, although there have been other studies on various *Aspergillus* strains including *Aspergillus nomius* where it has been seen to be tolerant to other heavy metals like Cu (II), Ni (II), Pb (II), Cd (II), As (II) etc. (Congeevaram *et al.* 2007, Oladipo *et al.* 2016, Chatterjee *et al.* 2019).

This study involves the isolation of hexavalent chromium tolerant fungal strains from KLC tannery effluent and derivation of the metal tolerance capacities in the form of their respective metal tolerance index. We conclude that, this data would be further used in our future studies where their biomass can be employed for the biosorption of hexavalent chromium.

As conclusions, all of the 16 fungal isolates were found to be chromium (VI) tolerant. Maximum chromium(VI) tolerance was displayed by the sample S12 at concentration 2mg/ml. The sample was identified as having 99.82% similarity with *Aspergillus nomius* strain.

## ACKNOWLEDGEMENTS

Authors wish to acknowledge their sincere thanks to the Department Of Science and Technology, Government Of West Bengal for the financial support in this research work.

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Received: 02th May 2019; Accepted: 17<sup>th</sup> February 2020; Published 07<sup>th</sup> January 2021.