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Loofah immobilized with *Cladosporium cladosporioides* CEL14 is a potential bioremediating agent for hexavalent chromium in tannery wastewater

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ABSTRACT

Aims: Chromium salt possesses unique characteristics that render it useful in numerous applications in several industrial processes, especially tanning of animal hides which act as a major source of hexavalent chromium toxicity in environment. This study aimed to evaluate the efficiency of loofah immobilized *Cladosporium cladosporioides* CEL14 in remediate tannery wastewater which contains hexavalent chromium.
Methodology and results: A total of 18 fungal species were isolated from three different sites of tannery wastewater in Egypt, of which *C. cladosporioides* CEL14 was the most capable species of chromate remediation with 81% after 7 days of incubation as free cells. The experiments were conducted in minimum salt medium supplemented with 200 ppm chromate in the form of potassium dichromate. Different process parameters studies demonstrated that chromate was completely removed at 30 °C, pH 6, 0.1% malt extract and 0.2% glucose after 7 days of incubation with 20% inoculum size. After that, *C. cladosporioides* was immobilized on a natural support material (loofah). The removal ability of chromate was enhanced through permanent viable immobilization on loofah pieces, which showing complete removal of chromate within 3 days. The toxicity assessment of treated tannery effluents revealed that 74% of *Brassica napus* seeds were germinated upon exposure to the treated effluent.
Conclusion, significance and impact of study: This study revealed that *C. cladosporioides* CEL14 isolate has high potential as bioremediating agent against toxic hexavalent chromium. The removal ability of toxic chromate was enhanced through permanent viable immobilization on loofah pieces. This technology is simple, cost effective, efficient and environmentally friendly. The loofah immobilized with *C. cladosporioides* CEL14 has potential to be applied in wastewater treatment of small-scale tanneries after onsite trials.

Keywords: Chromate removal, fungal immobilization, tannery wastewater, bioremediation, toxicity

INTRODUCTION

Industrial tannery wastewater is a major source of chromium contamination in our environment. The leather manufacturing can be divided into three steps, viz., the preparatory stage that includes liming, soaking, unhairing and preservation of hides; the tanning stage that uses trivalent chromium sulfate salts; and finally the crusting stage (Sivakumar *et al.*, 2010; Moretto, 2015). In particularly, tanning stage is the primary step, which the animal hides are turn into leather and several techniques are currently being used such as vegetable tanning, smoke tanning and chromium tanning. However, the use of chromium salts is known to be the most effective technique and it is widely applied in worldwide tanning industry as it enhances several properties of leather to make it better, such as providing higher thermal stability and strength and making it lighter weight (Falkiewicz-Dulík, 2015; Nashy and Eid, 2019).

The amount of chromium salts required for chrome tanning is approximately 2% or more of the weight of the hides and skins, and the chromium fixation is only 60-70%. As a result, about 0.5 kg of chromium may remain in the tanning solution used per 1000 kg of tanned hides. This results in both material loss and disruption of the ecological balance and redox reactions giving rise to toxic hexavalent form (Nur-E-Alam *et al.*, 2020). The use of chromium salts to tan the hides is a completely wet process and it is reported approximately 32 L of water per kg of skin processed is being generated (Bharagava and Mishra, 2018). Each ton of salted hides during tanning process results in an average amount of 50 m^3 of liquid wastes, 600 kg of solid wastes and 240 kg of leather (El-Khateeb *et al.*, 2017). Besides, tannery wastewater is a

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highly polluted effluent as it contains suspended solids, settleable solids, gross solids, biological oxygen demand, chemical oxygen demand, ammonium content, sulfide, neutral salts (sulfates and chlorides), oil, grease, solvents and finally chromium compounds (Bosnic *et al.*, 2000).

In the aquatic environment, chromium is found in either the insoluble form as trivalent chromium (Cr^{3+}) or the hexavalent (Cr^{6+}) soluble form (Kaur *et al.*, 2014; Chang *et al.*, 2016). Trivalent chromium (Cr^{3+}) is least toxic as it does not have the ability to pass through the cell membrane in a direct manner and gets precipitated (Tariq *et al.*, 2019). However, hexavalent chromium (Cr^{6+}) is known as the most poisonous form of chromium being released into the environment by industrial outflowing. The toxicity is due to its strong oxidizing power and being easily transported through the cell membrane (Viti *et al.*, 2014). Some studies report the formation of soluble organochromium (III) complexes after the reduction of hexavalent chromium by bacterial cellular organics (Puzon *et al.*, 2005).

Hexavalent chromium is highly toxic, mutagenic, teratogenic and carcinogenic to humans and animals due to abnormal interactions with intracellular proteins and nucleic acids (Bharagava and Mishra, 2018; Nur-E-Alam *et al.*, 2020). Moreover, plant growth is highly affected with exposure to hexavalent chromium through soil or water resulting in large alterations in the process of germination and the growth of roots, stems and leaves. In addition, Cr^{6+} causes harmful effects on plant physiological activities such as photosynthesis; these metabolic alterations lead to necrosis and chlorosis (Shanker *et al.*, 2005). Consequently, the toxicity caused by hexavalent chromium becomes the most life-threatening pollutant that must be controlled as stated by the US Environmental Protection Agency (US EPA, 1998), which is when chromate level reaches >0.05 mg/L, they will cause severe damage to human health which includes diarrhea, ulcers, eye and skin irritations, kidney malfunction and lung cancer (Bharagava and Mishra, 2018). Besides, it will also induce birth abnormalities and a decline in reproductive health, in addition to being extremely carcinogenic and mutagenic (Mohanty *et al.*, 2005).

In developed countries, various physicochemical processes such as filtration, electrochemical application, ion exchange, evaporation, chemical precipitation, oxidation, reduction, reverse osmosis, adsorption-desorption and photocatalysis have been used for the removal of toxic metals from the environment (Hassan *et al.*, 2017; Jobby *et al.*, 2018). Although certain physical and chemical treatment processes are simple and quick, and may aid metal recovery, the process requires high operational costs, high energy consumption and secondary pollution creation. Furthermore, majority of them only work well in the presence of high metal concentrations and their efficiency can be influenced by the presence of interfering substances (Dhal *et al.*, 2013; Vijayaraj *et al.*, 2018). Most of the industrial wastewater is released to the environment without proper treatment and only 5% is being properly processed before release into environment in developed countries. Waterborne infections continue to affect the majority of the world's population and the quality of water resources has rapidly deteriorated, particularly in poor developing countries. All of these drawbacks must be taken into account when building a metal removal technique that is both sustainable and cost-effective (Ahluwalia and Goyal, 2007).

Bioremediation is an alternative to conventional methods used for the removal of toxic metals. The direct use of living organisms possessing catabolic potential and/or their products such as enzymes and biosurfactants is a novel approach to enhance their remediation efficacy (Schenk *et al.*, 2012; Le *et al.*, 2017). Living species such as bacteria, fungi, yeast, algae and plants have been demonstrated their remediation ability, with bacteria and fungi are proven to be the most effective. Fungi have been reported to exhibit significant tolerance toward heavy metals and become dominant organisms in some polluted habitats. Fungal biomass has been recognized as a highly effective adsorbent for the accumulation of hazardous metals such as chromium, copper, mercury, nickel, cadmium and lead from wastewaters, in addition to extracellular enzyme synthesis. Melanin, the dark pigment located in the fungal cell wall, can reduce the toxic effect of heavy metals due to the presence of various functional groups such as carboxyl, amino and hydroxyl which have metal ion chelating property (Fogarty and Tobin, 1996; Ledin, 2000; Hawley *et al.*, 2004). This property helps them resist and function in highly toxic sites with heavy metals as they have a protective cell wall material with excellent binding properties; fungi also produce sufficient metabolites that can be used in wastewater treatment (Pradhan *et al.*, 2017). Unlike bacteria, fungi can also survive in low pH, grow in low moisture environment and colonize substrates rapidly using their hyphal system (Nigam *et al.*, 2015; Bello *et al.*, 2020).

Mycoremediation studies of toxic hexavalent chromium were previously reported on many studies. A Cr (VI) reduction investigation was conducted using Fusarium isolated from polluted soil from a tannery effluent. The isolated fungal strain removed all the Cr (VI) at temperature of 25 °C, incubation time of 72 h and pH of 5.0 (Guria *et al.*, 2014). In another study, four out of 20 fungal strains (*Penicillium commune*, *Paecilomyces lilacinus*, *Cladosporium perangustum* and *Fusarium equiseti*) were isolated from highly polluted tannery effluents. To treat toxic hexavalent chromium found in industrial tannery wastewater with concentration 9.86 mg/L, consortiums of four fungal strains were inoculated in a 1 L bioreactor with 1% glucose and 0.01% ammonium nitrate supplement. The results showed that 73% of Cr (VI) was removed from the wastewater sample in 12 h (Sharma and Malaviya, 2016).

In the present study, a local fungal isolate which was isolated from tannery wastewater is investigated for its potential to remediate tannery wastewater which contains hexavalent chromium under laboratory conditions at different physical and chemical parameters. The efficiency of bioremediation was also investigated upon

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the immobilization of the fungal isolate on loofah pieces. The effect of the treated water on the viability of *Brassica napus* seeds was further investigated.

MATERIALS AND METHODS

Collection of tannery wastewater samples

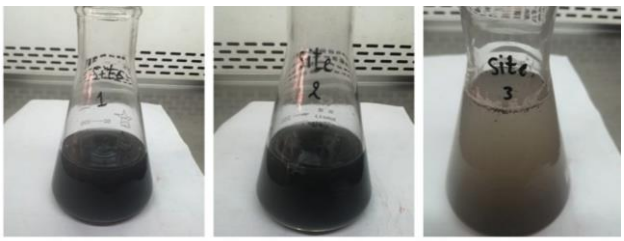
Nine wastewater samples were collected from three different tannery sites located in Suar Majra Aleuyun district in Old Cairo, Egypt (30°01'11.0"N, 31°14'26.0"E). Figure 1 show the water samples were collected in polyethylene containers and stored at 4 °C until further analysis.

Physicochemical analysis of tannery wastewater samples

Act No. 85 of 1983 issued by the Egyptian Ministry of Irrigation, which is concerned with protecting the water of the Nile River and the environment from pollution describes the acceptable range of wastewater physical and chemical characteristics before draining into fresh water. Therefore, the color, odor, turbidity, total suspended solids, chemical oxygen demand and biological oxygen demand of the collected tannery effluent samples were analyzed according to the American Public Health Association methods (Baird *et al.*, 2017). Parameters, including pH, total dissolved solids and electrical conductivity were measured using AD8000 Professional Multi-Parameter Bench Meter (Adwa, Hungary). Heavy metal concentrations were measured using an atomic absorption spectrophotometer after digesting the samples using a triacidic mixture (Bennett *et al.*, 2013).

Isolation and identification of chromium resistant fungal isolates

The wastewater samples were serially diluted to obtain a 5-fold dilution, and then 0.1 mL inoculum of the diluted sample was spread on potato dextrose agar plates supplemented with different concentrations of potassium dichromate ($K_2Cr_2O_7$) ranging from 50 to 1000 ppm. The plates were incubated at 28 °C for 7 days (Sharma and Malaviya, 2016). The fungal colonies that appeared on the plates were purified and identified based on their microscopic and macroscopic properties. The isolates were cultured on potato dextrose agar, malt extract agar, and czapek yeast agar as three-point inoculation. The plates were incubated at 25 °C for 7 days. Pigment production and colony characteristics (color, shape, size and texture of the colony) were recorded. Microscopic properties investigated using a lactophenol cotton blue stained slide culture mounted with a small portion of the fungal mycelium (conidia, hyphae, conidial head, conidiophores, spores, etc.) were observed. Morphological identification result was compared with the reference available (Pitt and Hocking, 2009; Bensch *et al.*, 2012; Visagie *et al.*, 2014).



a b c

Figure 1: (a) Tannery wastewater sample collected from site number 1, (b) Tannery wastewater sample collected from site number 2 and (c) Tannery wastewater sample collected from site number 3.

Selection of the most potent fungal strain

Resistance to hexavalent chromium was determined in terms of minimum inhibitory concentration, which is the concentration of hexavalent chromium that inhibited the visible growth of the fungal isolate. The PDA plates supplemented with 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ppm potassium dichromate were inoculated with 8 mm agar plugs obtained from young fungal colonies and incubated at 28 °C for 7 days (Ezzouhri *et al.*, 2009; Saranya *et al.*, 2020).

The selected fungal isolates were then screened for their chromium removal capabilities in a minimal salt medium containing (g/L) $Na_2HPO_4{\cdot}2H_2O$, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2; ammonium ferric citrate, 0.01; $Ca(NO_3)_2{\cdot}4H_2O$, 0.05 (Thakur, 1995). The pH was adjusted to 5.5 and the medium was supplemented with 200 ppm potassium dichromate in Erlenmeyer flasks inoculated with individual fungal isolates and incubated at 30 °C in a rotary shaker for 7 days. Hexavalent chromium concentration was measured at different time intervals of 1, 3, 5 and 7 days using the spectrophotometric diphenylcarbazide (DPC) method. Briefly, 0.39 mL of 6N sulfuric acid was added to 0.3 mL of sample and diluted with 4.21 mL of distilled water and finally, 0.1 mL of DPC solution was added, mixed thoroughly and kept at room temperature for 10 min. A violet color was produced, and the absorbance was measured at 540 nm wavelength using a double beam Unico UV-2000 UV/Visible Spectrophotometer (Onchoke and Sasu, 2016). The concentration of hexavalent chromium in the sample was calculated based on a standard curve. The percentage of heavy metal removal was calculated using the following equation:

$$\text{Heavy metal removal percentage} = (c^o - c)/c^o \times 100$$

where c^o is initial heavy metal ion concentration and c is final equilibrium concentration of test solution (Waly *et al.*, 2010).

Chromium uptake

The uptake of chromium was determined in the fungal mycelium by acid digestion and the chromium content

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was measured by atomic absorption spectroscopy. For acid digestion, the fungal pellets were transferred into a known weight crucible and dried overnight at 60 °C in an oven. The weight of dried pellets was measured. Then, 1 g of dry ash of fungal mycelium was crushed using pestle and mortar and added to a conical flask which contain 20 mL of an acid mixture (HNO_3:H_2SO_4:$HCLO_4$ 10:1:4) and mixed thoroughly. The flask was then placed on a slow heating hot plate until the production of nitrogen dioxide brown fumes in the digestion chamber and the volume reduced to 3-5 mL. Next, after cooling the flask, 5 mL of 12 N concentrated HCL was added, and the volume was made up to 50 mL using distilled water. The solution was filtered through a Whatman No. 1 filter paper. This solution was further analyzed using Savanta AA Atomic Absorption Spectroscopy (GBC Scientific Equipment, Australia) to determine the chromium in the sample (Srivastava and Thakur, 2006).

Effect of different operating parameters

One factor at a time method was adopted to determine the suitable parameters for hexavalent chromium removal by the selected most potent fungal strain. Each analysis was done in three replicates and the obtained results were averaged. Most of the suitable parameters for hexavalent chromium removal were selected using a minimal salt medium supplemented with 200 ppm potassium dichromate. These parameters included carbon sources (sucrose, dextrose, sodium acetate, sodium citrate, cellulose and starch) at a concentration of 0.2% and nitrogen sources (sodium nitrate, yeast extract, peptone, malt extract, urea and beef extract) at a concentration of 0.1%. The pH was adjusted to 5.5 with an inoculum size of 10% (w/v) of fungal isolate for 7 days at 30 °C in a rotary shaker, and then the samples were collected after 1, 3, 5 and 7 days.

For the optimization of pH, the minimal salt medium was supplemented with 200 ppm potassium dichromate, 0.2% sodium acetate and 0.1% sodium nitrate, and the pH was adjusted to different values of 2, 5, 6 and 8. Next, the medium was inoculated with 10% (w/v) of the fungal isolate and incubated for 7 days at 30 °C in a rotary shaker. For the optimization of inoculum size, Erlenmeyer flasks containing minimal salt medium supplemented with 200 ppm potassium dichromate, 0.2% sodium acetate and 0.1% sodium nitrate, with pH adjusted to 5.5, were inoculated with 5% (w/v), 10% (w/v), 15% (w/v), 20% (w/v) and 30% (w/v) of the fungal isolate respectively and incubated at 30 °C for 7 days in a rotary shaker (Srivastava and Thakur, 2006; Nagendram *et al.*, 2015; Sakthivel *et al.*, 2016; Kumaresan *et al.*, 2020).

The samples were collected at each time interval during the optimization of process parameters were centrifuged at 10,000 rpm for 10 min, and the concentration of hexavalent chromium was determined in the supernatant using the spectrophotometric diphenylcarbazide (DPC) method (Onchoke and Sasu, 2016). The method was similar to previous mentioned.

Molecular identification of the most capable fungal isolate to remediate chromate

The identification of the most potent fungal isolate was confirmed by a molecular technique provided by Sigma Scientific Service Company. The DNA was extracted from the fungal culture and then subjected to PCR and amplified using a universal primer pair forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The resulting PCR product was sequenced (GATC Company, Germany) using ABI 3730xl DNA sequencer a high-throughput long-read Sanger sequencing instrument analyzing the amplified complete gene sequence of internal transcribed spacer 2 and 5.8S ribosomal RNA and the partial sequence of internal transcribed spacer 1 and the large subunit ribosomal RNA gene. The strain has been deposited in the culture collection Ain Shams University with the strain number CCAS-2020-3. A neighbor-joining tree was constructed from the models of Jukes and Cantor (1969).

Morphological examination of *Cladosporium cladosporioides* mycelia by scanning electron microscopy (SEM) and energy-dispersive X-ray analysis (EDX)

SEM coupled with EDX analysis was applied to observe the morphological alterations that occurred in the mycelium of *Cladosporium cladosporioides* after chromate remediation in comparison with the organism incubated in the absence of chromate served as control. The fungal mycelia were separated from the broth medium, cut into small pieces measuring approximately 5 × 5 mm, and then treated with 2.5% glutaral solution at 4 °C for 2 h to perform an outer fixation. This was followed by washing with phosphate buffer (50 mM, pH 6.8), after which the samples were placed in 0.1 M osmium tetroxide (OsO_4), which was used as a secondary fixative for 2 h. Next, the samples were dehydrated in a series of ethanol (30%, 50%, 70%, 85%, 90%, 95% and 100%) each for 10 min. This was followed by critical point drying (Ban *et al.*, 2012), after which gold spraying of samples was performed using S160A Sputter Coater (BOC Edwards, UK). The samples were examined and photographed using Quanta™ 250 FEG (FEI Company, the Netherlands). Both the treated and untreated samples were also subjected to EDX.

Viable immobilization of the most potent fungal strain to *Luffa aegyptiaca*

Vegetable sponge also known as loofah, which was obtained from the mature dried fruit of *Luffa aegyptiaca,* was used for immobilization and storage of the fungal strain. This plant is highly cultivated in Asia, Africa and tropical America and is commonly known as loofah (Iqbal and Zafar, 1994).

For the preparation of loofah before immobilization, a mature dried fruit of loofah was peeled to obtain the

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placental cushion of the ovary, which is made up of a fibrous network. It was then cut into two halves to remove seeds; these halves were again cut into rectangular pieces (16 × 18 × 3.5 mm), soaked in boiling water for 30 min and washed with tap water. They were soaked in distilled water for 24 h (distilled water was changed 3-4 times). The fibrous pieces were oven dried at 70 °C and then sterilized by autoclaving. Finally, the pieces were soaked in potato dextrose broth (PDB) in aspect condition for 5-10 min (Iqbal and Zafar, 1993).

Immobilization was performed in Erlenmeyer flasks containing 70 mL of PDB medium and two pieces of loofah that were pre-weighed and inoculated with 1 mL of fungal spore suspension and incubated at 28 °C at 100 rpm for 24 h. The pieces were then removed and transferred to 70 mL of fresh PDB medium and incubated at 28 °C at 100 rpm for 8-12 days. Then, the immobilized fungi were placed in 3% sterile saline solution and stored at 10 °C for preservation purpose (Iqbal and Zafar, 1994; Abdullah *et al.*, 1995).

The bioremediation of tannery wastewater using loofah immobilized with *C. cladosporioides* CEL14 was tested in a 5 L bioreactor at 30 °C with a glucose at concentration of 0.2% and malt extract at concentration of 0.1% and pH adjusted to 6, as a simulation of effluent tannery wastewater treatment, which was prepared in the laboratory.

Impact of treated and untreated effluents on the germination behavior of *Brassica napus*

A total of 9 seed groups were used in this experiment. Each set consisting of three replicates and the obtained results were averaged. The first group was the control in which tap water was used in irrigation. Next is the untreated group Ut25, Ut50, Ut75 and Ut100, with different concentrations of untreated tannery wastewater (25%, 50%, 75% and 100%) were used in irrigation. For the treated group T25, T50, T75 and T100 with different concentrations of treated polluted tannery effluents by loofah immobilized *C. cladosporioides* CEL14 in the bioreactor (25%, 50%, 75% and 100%) were used in irrigation.

Brassica napus seeds were surface sterilized by soaking in 0.1% mercuric chloride ($HgCl_2$) solution for 10 min and then washed three times with sterile distilled water, followed by soaking for 1 min in 90% ethanol. Finally, the seeds were cultivated after appropriate washing with sterile distilled water (Bakhsh *et al.*, 2016). For each group, 50 seeds were placed in a Petri dish that was lined with two Whatman No. 1 filter papers. These filter papers were moistened with 5 mL of tap water for the control and the same volume was used for different percentages of treated and/or untreated wastewater effluents and incubated at 28 °C for 30 days. The results of seed germination recorded from the 3rd day onward till the 30th day and several values of germination parameters were calculated, include the following:

1. Germination percentage = Number of germinated seeds/Number of total seeds × 100 (Carpýcý *et al.*, 2009)

2. Mean germination time and mean germination rate (Al-Ansari and Ksiksi, 2016)
Mean germination time = $\sum fx/\sum f$
where F is the number of seeds germinated on day x.
Mean germination rate = 1/Mean germination time

3. Germination index = $\sum(Gt/Dt)$
where Gt is the percentage germination and Dt represents germination days (Siddiqui and Al-Whaibi, 2014).

4. Seedling vigor index = Germination % × Mean of seedling length (shoot + root) (Vashisth and Nagarajan, 2010)

5. Peak value, germination value and mean daily germination percentage (Gairola *et al.*, 2012)
Peak value = Highest of germinated seeds/Number of days
Germination value = Peak value/Mean daily germinated
Mean daily germination percentage = Total number of germinated seeds/Total number of days

6. Speed of germination = $\sum(n/t)$
where n is the number of seeds newly germinating at time t and t is the number of days from sowing (Mitra *et al.*, 2014).

The IBM SPSS 26.0 statistical software for windows was used for analyzing the significance of differences between the values of indices. The Mann-Whitney U test with a significance level of α=0.05 was used to determine the effect of treated and untreated effluents on seed germination.

RESULTS AND DISCUSSION

Tannery wastewater characterization

The physical and chemical characteristics of tannery effluents exceed the limit established by the Egyptian Regulation Law of Wastewater (Law No. 48, 1982) as shown in (Table 1). The effluents were dark bluish in color due to a high load of organic and inorganic compounds such as chromium salts used during tanning (Sundar *et al.*, 2002). They also had a foul odor that could be related to the putrefaction of organic compounds in skin (Dekeirsschieter *et al.*, 2009). The effluents had high electrical conductivity and total dissolved solids, which can be attributed to the presence of high inorganic salt content that causes osmotic stress and increases reactive oxygen species production (Thacker *et al.*, 2006; Sultan and Hasnain, 2007; Rahman *et al.*, 2016). The presence of large amounts of suspended solids in the effluents can cause damage to soil properties by decreasing the rate of water holding capacity (Chowdhury *et al.*, 2013).

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Table 1: Physical and chemical characteristics of collected wastewater effluents.

	Site 1	Site 2	Site 3	Acceptable range before draining into fresh water US EPA (2018)
Physical characteristics				
Color	Dark bluish green	Dark bluish green	Light bluish green	Colorless
Odor	Foul odor	Foul odor	Foul odor	Odorless
TSS mg/L	1800.00 ± 19.13	1972.00 ± 20.59	928.00 ± 16.01	30
EC s/cm	55.61 ± 2.21	46.31 ± 1.01	39.61 ± 1.12	<400 µs/cm
Chemical characteristics				
COD mg/L	4638.00 ± 28.75	5628.00 ± 25.53	3538.00 ± 36.25	30-40
BOD mg/L	140.00 ± 1.01	146.00 ± 4.16	129.00 ± 1.21	20-30
pH	3.50 ± 0.10	3.00 ± 0.06	4.20 ± 0.02	9-6
Cr (VI) mg/L	145.71 ± 2.32	180.00 ± 2.16	19.20 ± 0.57	0.05
Zn mg/L	1.26 ± 0.02	1.23 ± 0.02	0.10 ± 0.01	1
Cu mg/L	0.21 ± 0.01	0.28 ± 0.03	0.26 ± 0.01	1
Cd mg/L	0.17 ± 0.01	0.19 ± 0.01	0.15 ± 0.01	0.01

BOD = biological oxygen demand, TSS = total suspended solids, EC = electrical conductivity, COD = chemical oxygen demand.
Data represents mean value ± SD of three replicate samples.

Identification of isolated fungal strains and their ability to reduce chromate

Among microorganisms, fungal biomass has the advantage of possessing high proportion of cell wall material with excellent metal binding abilities (Kapoor and Viraraghavan, 1998; Gupta *et al.*, 2000). In the present study, a total of 18 fungal species belonging to seven genera, *Aspergillus*, *Alternaria*, *Penicillium*, *Cladosporium*, *Rhodotorula*, *Candida* and *Acremonium* were isolated and identified based on microscopic and macroscopic characteristics (Table 2). These species exhibited different degrees of chromate reduction and resistance, which could be related to the entry of all metal ions into the plasma membrane and cytoplasm through the cell wall (Hastuty and Hidayat, 2019), which consists of a variety of polysaccharides, proteins, lipids, inorganic salts and pigments. These contents of the cell wall have several functional groups such as amino, carboxyl, thiol, sulfhydryl and phosphate groups that are ionized to negatively charged sites that bind metal ions via electrostatic attraction, providing a number of active sites for metal binding. Differences in the cell wall composition among different species of microorganisms and intraspecies differences can thus cause significant differences in the type and amount of metal binding to those species (Tobin *et al.*, 1984; Latha *et al.*, 2012). Of the 18 fungal species, *C. cladosporioides* CEL14 was found to be the most resistant isolate to chromium up to 800 ppm as according to minimum inhibitory concentration no visible growth was detected when the concentration of chromate higher than 800 ppm. Besides, it shows good removal capabilities for toxic chromate with 81% reduction was recorded after 7 days of incubation, followed by *Aspergillus fumigatus* (79% chromate reduction), *Penicillium notatum* and *P. paradoxum* (65% chromate reduction) (Table 2).

The biomass of *Cladosporium* is an efficient biosorbent of copper, nickel, cyanide, cadmium, gold, silver, pesticides and several organic compounds, including aromatic hydrocarbons and ketones has been reported by several researchers (Pethkar *et al.*, 2001; Juhasz *et al.*, 2002; Qi *et al.*, 2002; Buszman *et al.*, 2006). Similarly, a chromium resistant strain of *C. cladosporioides* was also isolated from a highly polluted site (Sharaf and Alharbi, 2015), which demonstrated the ability to resist elevated concentrations of toxic chromate of up to 800 ppm. Garza-González *et al.* (2017) also found that *C. cladosporioides* represents an efficient system to remove Cr (VI) from aqueous solutions. The highest removal capacity of *C. cladosporioides* biomass was 492.85 mg/g after 288 h of experimentation.

Molecular identification of the most potent fungal isolate

Identification of the most potent fungal strain in chromate reduction was further confirmed by molecular techniques by analyzing the amplified complete gene sequence using Sanger sequencing technology. The phylogenetic tree confirmed that the selected fungal strain was closely related to *C. cladosporioides* (Figure 2). The sequence data that support the findings of this study have been deposited in GenBank with the accession code (MW866630). Nucleotide sequence similarity was analyzed using the basic local alignment search tool (BLAST) of National Center for Biotechnology Information (NCBI) databases. It is used to align and sequence the product with the 20 closely related species, and it shows 99.79% of identity to *C. cladosporioides* isolates CZCU-8 and BAB-6501.

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Table 2: Morphological identification and percentage of chromate removal by locally isolated fungal strains from tannery wastewater.

Isolate number	Identification of isolate	Chromate concentration after bioremediation (ppm)	Uptake of chromate in mycelium (mg/g)	Removal of Cr (VI) (%)
1	*Acremonium* sp.	86.51 ± 2.25	2.93 ± 0.13	57.00 ± 1.25
2	*Alternaria brassicicola*	81.65 ± 1.25	2.98 ± 0.07	54.00 ± 2.08
3	*Alternaria cheiranthi*	101.34 ± 2.15	2.79 ± 0.51	43.00 ± 2.10
4	*Alternaria infectoria*	74.46 ± 2.35	3.05 ± 0.02	59.00 ± 3.05
5	*Aspergillus flavus*	116.12 ± 1.17	2.63 ± 0.01	35.00 ± 1.01
6	*Aspergillus fumigatus*	42.00 ± 1.52	3.58 ± 0.21	79.00 ± 2.64
7	*Aspergillus niger*	112.95 ± 2.03	2.67 ± 0.13	37.00 ± 1.15
8	*Candida albicans*	83.97 ± 3.52	3.16 ± 0.05	53.00 ± 1.82
9	*Candida glabrata*	90.53 ± 2.12	2.89 ± 0.12	51.00± 2.03
10	*Candida krusei*	72.21 ± 2.64	3.08 ± 0.30	60.00 ± 1.22
11	*Candida* sp.	72.21 ± 1.21	3.08 ± 0.01	62.00 ± 1.17
12	*Cladosporium cladosporioides*	31.95 ± 1.45	4.68 ± 0.36	81.00 ± 3.52
13	*Cladosporium limoniformae*	97.29 ± 3.25	2.83 ± 0.22	46.00 ± 1.52
14	*Penicillium chrysogenum*	76.99 ± 2.12	3.03 ± 0.42	60.00 ± 2.35
15	*Penicillium lagena*	116.12 ± 3.04	2.64 ± 0.03	35.00 ± 1.12
16	*Penicillium notatum*	62.19 ± 1.22	3.18 ± 0.12	65.00 ± 1.25
17	*Penicillium paradoxum*	62.42 ± 1.17	3.17 ± 0.34	65.00 ± 1.17
18	*Rhodotorula* sp.	115.06 ± 2.07	2.65 ± 0.03	36.00 ± 1.12

Data represents mean value ± SD of three replicate samples.

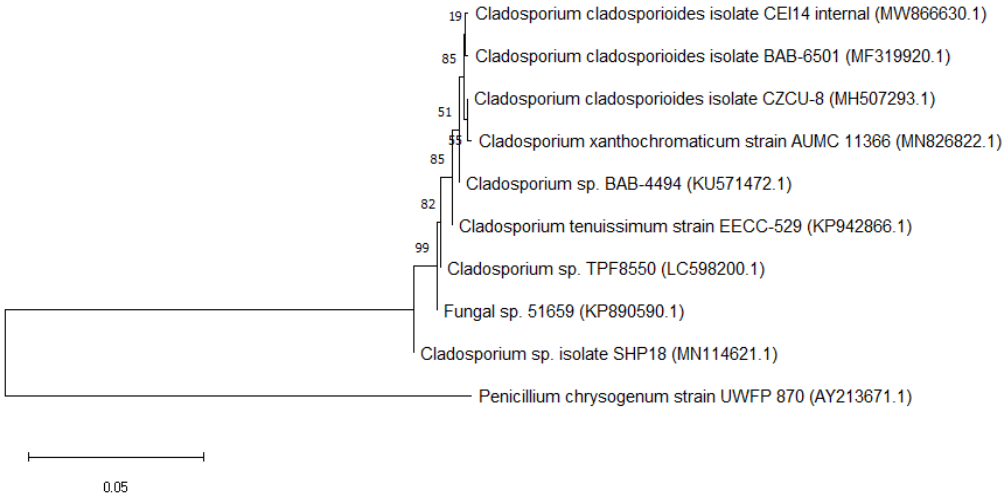

Figure 2: Neighbor-joining tree from ITS sequences showing the relationship between isolated *C. cladosporioides* isolate CEI14 and other closely related cladosporium species retrieved from the Genbank (accession number). Scale bar = 5 nucleotide substitutions per 100 nucleotides.

The effect of different operating parameters towards efficiency of bioremediation

Several factors are known to influence and limit the efficiency of bioremediation, including temperature, pH, redox potential, nutritional status, moisture and chemical composition of heavy metals (Farhan and Khadom, 2015). The composition of medium is considered as an important factor for remediation capacity because during the microbial growth which is associated to mineralization. The microbial cells utilize a quantity of energy from the organic substrate for multiplication that highly increases the population of microbes and its metabolic activity (Sharma and Adholeya, 2011). In a minimal salt medium supplemented with potassium dichromate, *C. cladosporioides* exhibited the maximum percentage of chromate removal, when glucose and sucrose were used as the carbon source and complete bioremediation was observed after 7 days of incubation and mycelium chromate uptake values were 7.43 and 6.58 mg/g, respectively. The percentage of chromate removal when starch, sodium acetate, sodium citrate and cellulose are used as carbon source were 100%, 92%, 94% and 74% after 7 days of incubation, respectively (Figure 3).

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Malays. J. Microbiol. Vol 17(5) 2021, pp. 560-575
DOI: http://dx.doi.org/10.21161/mjm.211192

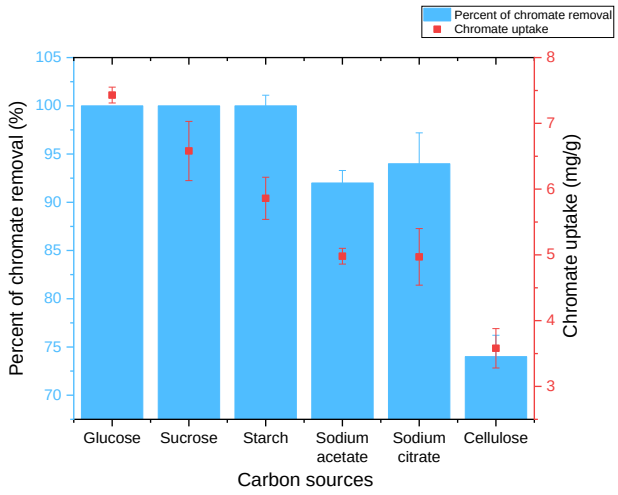

Figure 3: The effect of different carbon sources on the removal percentages of chromate in potassium dichromate by *C. cladosporioides* and the amount of chromate uptake in the mycelium after 7 days of incubation. The data is represented as mean values from three replicates.

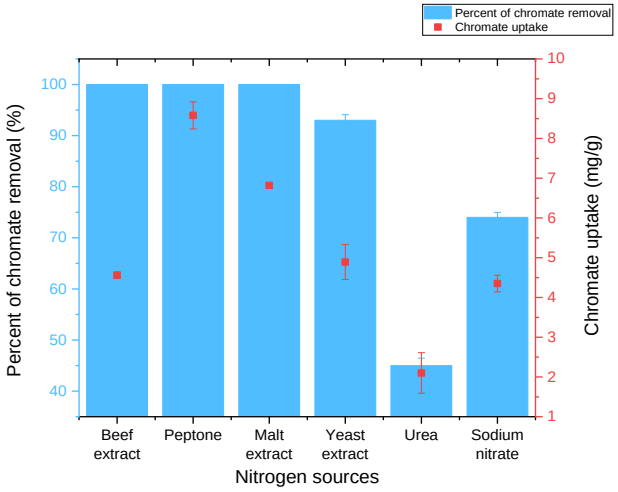

Figure 4: The effect of different nitrogen sources on the removal percentages of chromate in potassium dichromate by *C. cladosporioides* and the amount of chromate uptake in the mycelium after 7 days of incubation. The data is represented as mean values from three replicates.

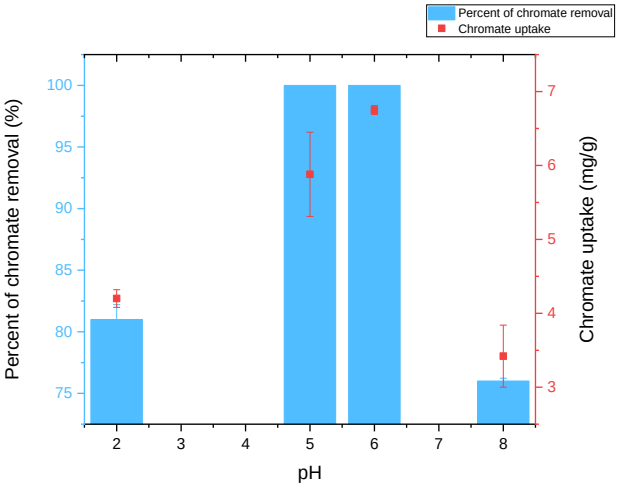

Figure 5: The effect of different pH values on the removal percentages of chromate in potassium dichromate by *C. cladosporioides* and the amount of chromate uptake in the mycelium after 7 days of incubation. The data is represented as mean values from three replicates.

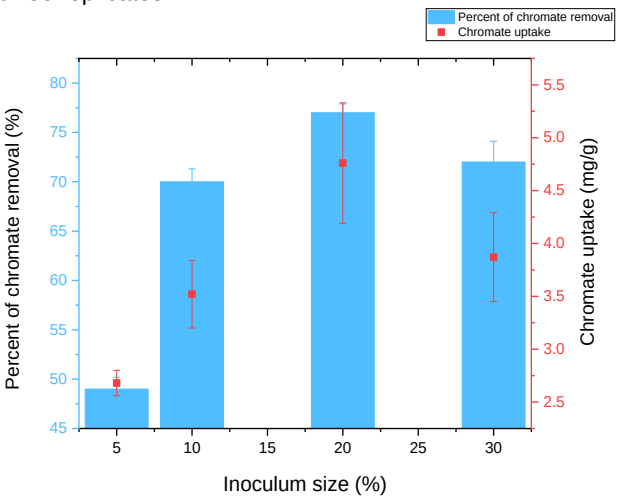

Figure 6: The effect of different inoculum sizes on the removal percentages of chromate in potassium dichromate by *C. cladosporioides* and the amount of chromate uptake in the mycelium after 7 days of incubation. The data is represented as mean values from three replicates.

Among the various nitrogen sources, malt extract and peptone were the best with which the organism completely remediated chromate after 7 days of incubation, with the chromate uptake values being 8.58 and 6.82 mg/g in the mycelium, respectively. The other nitrogen sources, such as sodium nitrate, urea, yeast extract and beef extract exhibited 74%, 45%, 93% and 100% removal ability after 7 days of incubation (Figure 4).

The pH of medium is an important parameter for biosorption process. It was observed that *C. cladosporioides* removed all the supplied chromate at pH 6 at 7 days of incubation with 6.75 mg/g chromate uptake in the mycelium (Figure 5). This finding can be related to the fact that at acidic pH levels, heavy metals tend to form free ionic species with more protons available to saturate metal binding sites. This indicates that at higher proton concentrations, the adsorbent surface is positively charged and hence the attraction between the adsorbent and metal cations is reduced. Therefore, heavy metals become more bioavailable, thereby increasing their

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toxicity to plants and microorganisms. Under basic conditions, metal ions replace protons to form other species such as hydroxometal complexes (Benguella and Benaissa, 2002; Sari *et al.*, 2008).

The inoculum size of *C. cladosporioides* (5, 10, 20 and 30 g/100mL) was tested for the removal of chromate and it was observed that the chromium removal percentage increased with inoculum size until reaching the optimum size after which there was no change. Results showed that 20% inoculum size of biomass exhibited the best chromate removal ability of 77% after 7 days of incubation with 4.76 mg/g chromate uptake in the mycelium with the increase in biomass (Figure 6). This could be related to as the inoculum size increased the number of binding sites and the surface area of the biosorbent also increased, resulting in an increase in the amount of metals ions biosorbed until reaching the optimum size after which there was no change (Sharma and Forster, 1993; Pagnanelli *et al.*, 2012).

Scanning electron microscopy (SEM) and energy-dispersive X-ray analysis (EDX)

The morphological changes in response to chromium accumulation in *C. cladosporioides* and the quantification of chromium within fungal strains were analyzed by SEM and EDX respectively. SEM analysis of the fungus after 48 h of incubation without chromate exposure (Figure 7a) showed that the hyphae were smooth and branched, and there was no peak of chromate at 5.411 keV as determined by EDX (Figure 8a). Conversely, when chromate (400 ppm) containing mycelium was subjected to, a significant peak of chromate at 5.411 keV was detected in EDX, indicating the adsorption of chromate by the fungal (Figure 8b). The presence of chromate particles on the rough mycelium of *C. cladosporioides* was observed by SEM (Figure 7b).

Chromate bioaccumulation can occur via two mechanisms. The first mechanism involves direct physical adsorption, coordination, chelation, microprecipitation, and/or exchanging of ions on the cell outer surface (Wang *et al.*, 2012; Pakade *et al.*, 2019; Tariq *et al.*, 2019). The second mechanism is slower and involves active metabolism-dependent transportation (sulfate transporter) of chromate into fungal cells that is later incorporated into specific organelles mostly mitochondria and lysosome (Shukla *et al.*, 2009; Chandra *et al.*, 2011). Back to the results obtained in this study, the rough outer surface of mycelium shown in SEM image and the results of EDX analysis prove that the first direct physical adsorption mechanism is the dominant.

Evaluation of immobilization matrices to support the most potential strain in chromate removal

The technique of immobilization of fungal isolate has several benefits to fungal bioremediation, such as a higher efficiency of pollutant degradation, multiple uses of the biocatalyst, reduced costs due to elimination of the cell filtration stage, ensuring a stable microenvironment for cells and enzymes, reduced risk for genetic mutations, resistance to shear forces present in bioreactors, increased biocatalyst survival during storage and increased tolerance to high pollutant concentrations and adverse environmental conditions (Xiangliang *et al.*, 2005; Dzionek *et al.*, 2016).

In the present study, this technique used for immobilization was a promising method to achieve the immobilization of *C. cladosporioides* on an appropriate solid carrier (Figure 9). Results showed that loofah is a highly promising matrix as the loofah pieces were completely covered with immobilized hyphae within 4-5 days and appeared as a bulk of fungal mass, with no free hyphae being found under the microscope upon examination of the liquid medium, thereby indicating that immobilization was surviving and permanent. The use of loofah to immobilize *C. cladosporioides* for chromium biosorption was highly effective, as it increased the rate

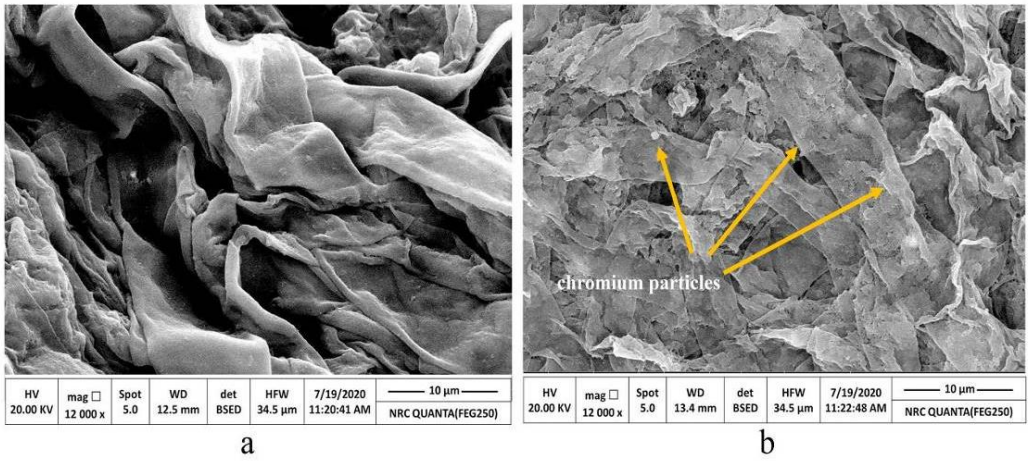

Figure 7: (a) SEM image of *C. cladosporioides* incubated without chromate shows smooth mycelium and (b) SEM image of *C. cladosporioides* after incubation in the presence of chromate shows rough mycelium with chromium particles adsorbed on it (magnification: 12000×, scale bars: 10 µm).

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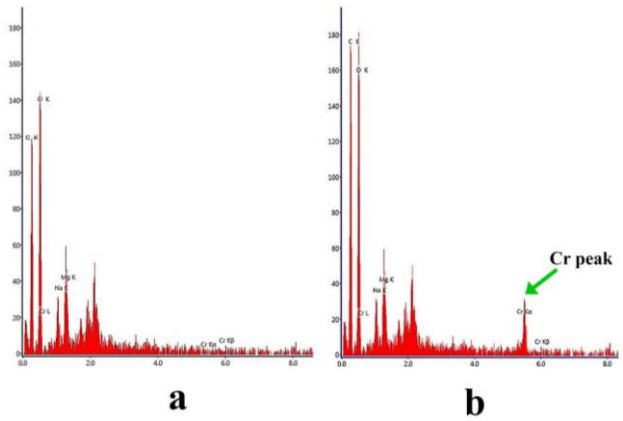

Figure 8: (a) EDX analysis of *C. cladosporioides* after 48 h of incubation without chromate exposure and (b) EDX analysis of *C. cladosporioides* after 48 h of incubation with chromate exposure showing a chromate peak at 5.411 keV.

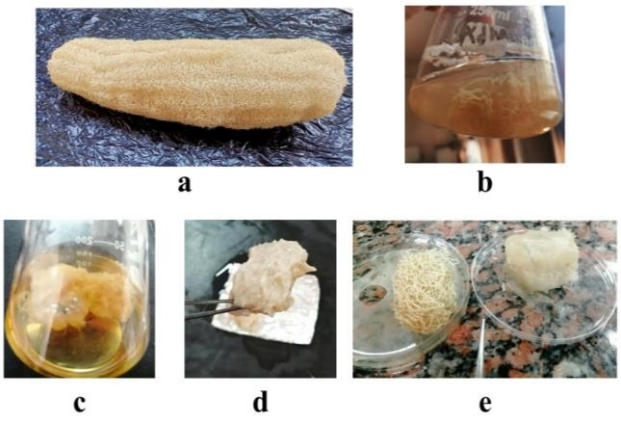

Figure 9: (a) Vegetable sponge of *Luffa aegyptiaca* after removal of pericarp, (b) Immobilization culture after 24 h, (c) Immobilization culture after 4 days, (d) Loofah pieces completely covered with immobilized fungal hyphae, (e) Loofah pieces before and after immobilization process.

Table 3: Percentage of chromate removal and amount of chromate uptake in mycelium by *C. cladosporioides* immobilized on loofah.

Intervals (day)	Chromate concentration (ppm)	Uptake of chromate in mycelium (mg/g)	Removal of cr^{6+} (%)
1	53.94 ± 0.45	NC	73.00 ± 1.12
3	0.00 ± 0.00	8.76 ± 0.23	100.00 ± 0.00

NC = not calculated.
Data represents mean value ± SD of three replicate samples.

and efficiency of bioremediation from 81% at 7 days (4.68 mg/g) using free cells to 100% at 3 days (8.76 mg/g) (Tables 2 and Table 3). This may be attributed to the immobilization of hyphae along the surface of the fibrous thread with no aggregate formation and the open network of loofah that increases the surface area and permitted contact of metal ions to the sorption sites (Akhtar *et al.*, 2004). Moreover, the surface functional groups on the fibers of loofah are primarily acidic (carboxylic, lactonic, enolic and phenolic), making them more available for ion exchange reactions (Saeed and Iqbal, 2013). The use of loofah material for the immobilization of algae, fungal hyphae and yeast cells have been effectively demonstrated by several researchers previously (Iqbal and Zafar, 1994; Ogbonna *et al.*, 2001; Akhtar *et al.*, 2008; Li *et al.*, 2008).

Toxicity assessment of treated and untreated effluents on the germination behavior of *B. napus*

The effects of various concentrations of treated and untreated tannery wastewater on seed germination and seedling growth of *B. napus* are recorded in (Table 4) and depicted in (Figure 10). Results showed that Ut100 and Ut75 tannery wastewater had the highest toxicity and completely prevented the germination of seeds. In contrast, the germination percentage (G %) of the seeds which received Ut25, T100, T75, T50 and T25 were no significant different compared to control (Table 5). The germination parameters such as germination percentage, germination index, peak value, germination value and seedling vigor index were gradually increased with the decrease in the concentration of treated tannery effluents. Moreover, the elongation of radicle and shoot was reduced with an increase in the concentrations of treated effluents which affect the values of seedling vigor index (Table 4).

The results of statistical analysis at significance level (p value=0.05) revealed a significant difference between the control and Ut50 and between Ut50 and T100, T75, T50 and T25 (p<0.05) which accepted to research hypothesis, whereas no significant differences were found between the control and Ut25, T100, T75, T50 and T25 and between Ut25 and T100, T75, T50 and T25 (p>0.05) which accepted to null hypothesis (Table 5). This indicates there is enhancement of germination parameters at Ut25, T100, T75, T50 and T25 when compared to untreated Ut50 effluent, thus confirming the efficacy of *C. cladosporioides* CEL14 in the treatment and bioremediation of toxic chromium from tannery wastewater (Table 5). However, there is decrease in germination parameters such as germination index, mean daily germination percentage, peak value, germination value, speed of germination and seedling vigor index when all the treatments compared to control. The deleterious effects of tannery wastewater on germination

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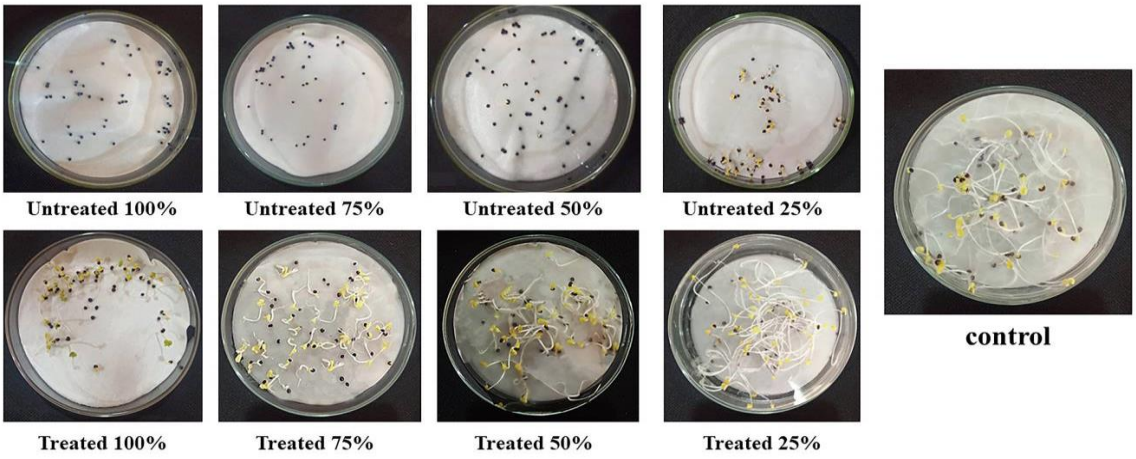

Figure 10: Seed germination of different sets of *B. napus* in response to irrigation by different concentrations of treated and untreated chromium contaminated effluents.

Table 4: Effect of different treated and untreated effluent concentrations on various germination parameters of *B. napus*.

Treatment	Parameters								
	G %	MGT	MGR	GI	MDG	Peak value	G value	Speed of germination	Seedling vigor index
Control	100.00 ± 0.00	1.06 ± 0.12	0.94 ± 0.12	48.50 ± 2.35	11.11 ± 1.25	94.00 ± 2.32	1044.44 ± 15.12	48.50 ± 2.10	958.00 ± 7.45
Ut100	NR	NR	NR	NR	NR	NR	NR	NR	NR
Ut75	NR	NR	NR	NR	NR	NR	NR	NR	NR
Ut50	12.00 ± 0.12	5.50 ± 0.04	0.18 ± 0.02	1.20 ± 0.01	1.33 ± 0.02	1.71 ± 0.01	2.29 ± 0.25	1.20 ± 0.13	4.80 ± 0.32
Ut25	92.00 ± 1.30	1.35 ± 0.06	0.74 ± 0.01	42.58 ± 1.34	10.22 ± 1.04	82.00 ± 2.21	838.22 ± 22.21	42.57 ± 1.15	174.80 ± 6.60
T100	74.00 ± 2.12	2.84 ± 0.21	0.35 ± 0.01	16.29 ± 1.02	8.22 ± 2.02	26.00 ± 1.52	213.78 ± 3.52	16.29 ± 0.22	97.12 ± 1.25
T75	92.00 ± 1.25	1.43 ± 0.01	0.69 ± 0.03	39.00 ± 2.13	10.22 ± 1.54	68.00 ± 3.05	695.11 ± 8.21	39.00 ± 1.12	215.28 ± 6.11
T50	98.00 ± 0.21	1.16 ± 0.01	0.86 ± 0.05	45.75 ± 1.12	10.89 ± 1.62	86.00 ± 1.32	936.44 ± 7.25	45.75 ± 1.82	321.44 ±10.50
T25	100.00 ± 0.00	1.04 ± 0.03	0.96 ± 0.02	49.00 ± 0.01	11.11 ± 1.35	96.00 ± 1.01	1066.67 ± 25.21	49.00 ± 1.25	690.00 ± 8.54

Ut = untreated effluent, T = treated effluent. G % = germination percentage, MGT = mean germination time, MGR = mean germination rate, GI = germination index, MDG = mean daily germination percent, G value = germination value, NR = not reported.
Data represents mean value ± SD of three replicate samples.

parameters might be due to the entrance of toxic heavy metals into the protoplasm, resulting in the loss of intermediate metabolites, which are essential for further growth and development of plants. In fact, chromium has been specifically reported to exert inhibitory effects on root growth compared to that on coleoptile growth before the germination of seeds (Ahsan *et al.*, 2007). The elevated salt content and metal ions of wastewater could also result in high osmotic pressure and anaerobic conditions, which inhibit several physiological processes involved in seedling mechanism, such as movement of solute, respiration and numerous enzymatic steps (Bharagava and Chandra, 2010; Malaviya and Sharma, 2011).

CONCLUSION

In this study, *Cladosporium cladosporioides* isolate CEL14 shows high capabilities in removal of toxic hexavalent chromium as it can completely remove toxic chromate within 3 days after permanently immobilized on natural loofah pieces and under favored operating conditions at 30 °C, pH 6, 0.1% malt extract and 0.2% glucose with 20% inoculum size. The evaluation of toxicity criteria of the treated effluent compared with the untreated effluent using *Brassica napus* seeds revealed that 74% of seeds were germinated upon exposure to the treated effluent, whereas the untreated effluent completely inhibited germination. Therefore, it is suggested that

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Table 5: Mann-Whitney test performed using germination percentage value (G %) between different untreated and treated effluent concentrations.

Treatment	*p*-value upon statistical analysis between control and different concentrations of treated and untreated effluent	*p*-value upon statistical analysis between Ut50 and different concentrations of treated effluent	*p*-value upon statistical analysis between Ut25 and different concentrations of treated effluent
Ut50	*0.02	NC	NC
Ut25	0.61	NC	NC
T100	0.44	*0.01	0.55
T75	0.61	*0.01	0.86
T50	0.67	*0.02	0.73
T25	0.86	*0.02	0.67

p-value is the probability of the test, the mark * indicates significant differences (α=0.05).
NC = not calculated.

loofah immobilized with *C. cladosporioides* CEL14 is a potential bioremediating agent for hexavalent chromium in tannery wastewater. This method is highly recommended for the use in wastewater treatment of small-scale tanneries after onsite trials.

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