



Potential of Marine-Derived Fungi to Remove Hexavalent Chromium Pollutant from Culture Broth

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Abstract Chromium (Cr) released from industrial units such as tanneries, textile and electroplating industries is detrimental to the surrounding ecosystems and human health. The focus of the present study was to check the Cr(VI) removal efficiency by marine-derived fungi from liquid broth. Amongst the three Cr(VI) tolerant isolates, #NIO SN-SK56-S19 (*Aspergillus sydowii*) showed Cr-removal efficiency of 0.01 mg Cr mg⁻¹ biomass resulting in 26% abatement of total Cr with just 2.8 mg of biomass produced during the growth in 300 ppm Cr(VI). Scanning Electron Microscopy revealed aggregation of mycelial biomass with exopolysaccharide, while Electron Dispersive Spectroscopy showed the presence of Cr₂O₃ inside the biomass indicating presence of active Cr(VI) removal mechanisms. This was further supported when the Cr(VI) removal was monitored using DPC (1,5-diphenylcarbazide) method. The results of this study point to the potential of marine-derived fungal isolates for Cr(VI) removal.

Keywords Biosorption · Halotolerant · Heavy metal · Hexavalent chromium · Marine-derived fungi

Introduction

Heavy metals mostly belong to the transition element category in the periodic table having densities of more than 5 gm cm⁻³ [1]. Anthropogenic activities such as effluent release from electroplating, tannery, mining and other industries lead to the release of toxic heavy metals into the environment, thereby polluting groundwater and soil [2, 3]. One such toxic heavy metal is Cr which occurs in + 3 and + 6 oxidation states, the latter being more toxic as it is water soluble and redox active. Various physical and chemical effluent treatment methods employed by industries have high costs and poor heavy metal removal efficiencies [4, 5]. Microbes such as fungi, bacteria possess cellular mechanisms to tolerate toxic metals such as extracellular/intracellular chelation using ligands, metallothioneins, and glutathione; enhanced efflux; subcellular compartmentation; enzymatic reduction; etc. [6–8]. This ability of fungi could be exploited for metal waste remedial purposes such as bio-mining and bioleaching. Fungi, either living or dead, have been reportedly used to bioremediate toxic metals, including biosorption of metals by isolates belonging to genera *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium* and *Saccharomyces* [5].

Fungi inhabiting marine habitats such as mangroves and salt pans have been less explored for their metal tolerance potential [9]. These eukaryotes can tolerate high amounts of salts and have excellent efflux mechanisms to exclude cations [10]. Therefore, they can be expected to tolerate high concentrations of heavy metals as well. Various halophilic and halotolerant bacteria have already been reported to be multi-metal resistant to high salt concentrations [11]. Though the biosorption efficiency of heavy metals by terrestrial fungi is well-documented [12–14], marine fungi are sparsely studied for the same. The aim of

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the present study was to isolate fungi from selected marine niches, followed by screening them for various metal tolerance. Chromium being one the most toxic metals, Cr removal efficiency and its reduction rate have been studied using marine-derived fungi.

Materials and Methods

Sampling Site and Isolation of Halotolerant Fungi

In the present study, fungal cultures were previously isolated from sediments collected from Divar mangroves (15°29'N, 74°12'E at low tides during the year 2012–2014, Supplementary Fig. 1, unpublished work), India and Arabian Sea [cruise track of different sampling stations at 15°30'N, 73°40'E to 12°47'N, 74°01'E (Supplementary Fig. 2)]. The sediments from the Arabian Sea were collected with the help of box grab from depths ranging up to 3000 m, during February and October 2013 onboard ORV Sindhu Sankalp (SSK046 and SSK056 resp.]. The sediment salinity of mangroves sediment varied from 32 to 35 PSU, and pH close to 6–6.5 with temperatures between 29 and 40 °C depending on the season. Approximately 0.5 g sediment samples were aseptically suspended in 10 mL of sterile seawater. Soil (sediment) suspension of 0.1 mL was spread-plated on 10, 20 and 30% of sea-salt (data in paper under review) containing different media (HiMedia) like potato dextrose Agar (PDA), Czapek Dox agar (CDA), malt extract agar (MEA), corn meal agar (CMA), Sabouraud's dextrose agar (SDA), Zobell marine agar (ZMA) and 50% sediment agar (SA), so as to isolate maximum numbers of halotolerant fungi. All the media supplemented with antibiotic (Cloxacillin and Ampicillin—750 µg/mL) and fungicide (Cycloheximide—100 µg/mL) to prevent the growth of rapid proliferators. In case of Arabian Sea sediments, the suspension aliquots were spread-plated on 1:5 strength media as above prepared in seawater. The main objective of the study was to isolate halotolerant fungi and check their tolerance to Cr, Cu, Ni, and Cd. All plates were incubated at room temperature (28 °C) for 5 days to a month. Morphologically distinct fungal colonies were sub-cultured to get pure cultures and maintained on CDA at 5 °C.

Screening for Heavy Metal Tolerance

Based on growth and colony morphology, 234 salt tolerant fungal isolates were selected for primary screening at 500 ppm of metal (Nickel, Copper, Chromium and Cadmium) concentration in CDA. Filter sterilized metal solution [stock solutions of Ni (1 M—58,693 ppm), Cu (1 M—63,546 ppm), Cr (100 mM—10,400 ppm) and Cd (2 M—

224,820 ppm)] was added to autoclaved agar media to give a desired concentration of the concerned metal. Fungal isolates were initially screened at 500 ppm of all four metals in batches of 15 isolates at a time. As the number of Cr-tolerant isolates was very less compared to other metals, the concentration for checking Cr-tolerance was reduced to 250 ppm. One week old isolates grown in CDA prepared in seawater were cut in the form of 7 mm discs and placed inverted on metal agar plates for 10 days. The subsequent screening was carried out at 750 ppm of Cu, Cd, Ni and 500 ppm of Cr for selected 75 isolates that grew in previous screening. Sixteen isolates showing growth in the first screening, were further screened at 1000 ppm of Cu, Cd and Ni and 750 ppm of Cr. Further, 15 isolates from these were screened and 1250 ppm of Cu, Cd and Ni and at 1000 ppm of Cr. Metal Tolerance Index (MTI) in percent, was calculated for the selected 15 isolates using the following formula [15]:

$$\text{MTI} = \frac{\text{Colony diameter of isolate in presence of metal}}{\text{Colony diameter of isolate in absence of metal (control)}} \times 100$$

Identification of Selected Isolates by ITS Sequencing

DNA extraction for three isolates that showed high tolerance to Cr was done using ZR Fungal/Bacterial DNA MicroPrep Kit (Zymo Research Corp., USA). PCR amplification of the ITS region was performed using ITS primers (ITS1—TCCGTAGGTGAACCTGCGG and ITS4—TCCTCCGCTTATTGATATGC) [16]. Amplification program followed was—initial denaturation step at 95 °C for 5 min; denaturation step at 95 °C for 1 min; annealing temperature of 52 °C for 45 s; elongation step at 72 °C for 1 min and a final elongation step at 72 °C for 10 min. Step 2, 3 and 4 were repeated for 35 cycles. The amplicons were stored at 4 °C and then purified using AxyPrep PCR Cleanup Kit (Axygen Biosciences, USA). The purified PCR products were then uni-directionally sequenced using Applied Biosystems (ABI) 3730 DNA stretch sequencer, with the XL upgrade and the ABI prism big dye terminator version 3.1 Cycle sequencing ready reaction kit. The raw sequences were analyzed using the BLAST tool [17] to get the closest reference sequences available at NCBI. The partial ITS sequences were then submitted to GenBank database. Phylogenetic tree was constructed for the ITS sequences of all selected three isolates along with ITS sequences of type strains and other isolates showing closest hits, using the Neighbor-Joining method at bootstrap value of 1000 using the MEGA software version 5.05 [18].

Determination of Chromium Content Residual Broth Using Atomic Absorption Spectroscopy (AAS)

Three isolates that showed tolerance to chromium (750 ppm screening) were selected to estimate their Cr removal efficiency from liquid broth. Starter inoculum was prepared by incubating one 7 mm disc of isolate cut from the periphery of growing culture, in 100 mL Erlenmeyer flasks with 20 mL medium (CDB in distilled water). Before the onset of sporulation, the mycelia were broken evenly using glass beads and, 1 mL of this inoculum suspension was inoculated into 20 mL of CDB prepared in distilled water containing increasing concentrations of Cr(VI) (0, 50, 100, 200, and 300 ppm) in triplicates. The pH of the broth was adjusted to 5 using 6N HCl. After incubation for one week at 80 rpm at 28 °C (room temperature), the contents of the flasks (duplicates) were filtered through 0.45 µm Millipore filter paper to separate broth from biomass. The filtrate was acidified using 2% of trace metal grade HNO₃ and Cr (total) content in broth was estimated using AAS (Thermo Scientific Solar S series, Thermo Electron Corporation, Cambridge). Further, the biomass was dried, weighed and acid digested using 1:1 ratio of HNO₃: HClO₄ [modified from 19] to check if biomass was involved in the removal of Cr (total). The remnants of digestion were dissolved in 2% of trace metal grade HNO₃ prepared in MilliQ water, and Cr (total) content was estimated using AAS.

Estimation of Hexavalent Chromium Reduction Rate

The selected isolates were inoculated in 20 mL medium (CDB in distilled water) in the form of a disc (as mentioned before) as starter inoculum. The mycelial matt was crushed using sterile glass beads before the onset of sporulation. One mL of this crushed mycelial suspension was inoculated in 20 mL of CDB supplemented with 50 ppm of Cr (maximum biomass was obtained at this concentration from the previous experiment, therefore this concentration was chosen). The pH of the medium supplemented with Cr was adjusted to 5 using 6N HCl, and these flasks with mycelial inoculum were incubated for 1 week at 80 rpm at room temperature. Cr(VI) was estimated spectrophotometrically at 540 nm (UV2450, Shimadzu Corporation, Japan) using DPC method [20] every day. Further to detect whether varying chromium oxidation states gave different absorption spectra, the selected isolates were inoculated in 250 mL Erlenmeyer flasks with 100 mL medium (CDB in distilled water) amended with 50 ppm of Cr (pH adjusted to 5). The flasks with mycelial inoculum were incubated for 20 days at 80 rpm at room temperature. Every alternate day 1 mL of medium was filtered using syringe filter (0.22 µm) and spectrophotometrically

analyzed using UV–VIS spectrophotometer (UV-2450, Shimadzu Corporation, Japan).

Morphological Analysis of Mycelia Grown in the Presence of Chromium Using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDS) Analysis

The selected isolates were grown in CDB prepared in distilled water and supplemented with Cr(VI) to compare their mycelial morphology in presence and absence of Cr(VI), using scanning electron microscopy following a modified protocol from [21]. The biomass fixed in 2% glutaraldehyde was dehydrated with series of ethanol concentrations. The dehydrated mycelia were then gold-coated (SPI Module Sputter coater, USA) and observed using scanning electron microscope (JSM 5800 LV SEM and EDS, Japan). The same set of samples were used for EDS analysis.

Results and Discussion

Isolation of Marine-Derived Fungi from Mangrove and Sea Sediments

Divar mangroves being located along the Mandovi estuary (Supplementary Fig. 1), face a direct tidal effect along with the constant influence of disturbances caused by barge movement carrying metal ore, across the estuary. The offshore sediment samples collected from the Arabian Sea (Supplementary Fig. 2) sea-bed are comparatively less affected by anthropogenic activities. A total of 380 fungal isolates were obtained, out of which 145 isolates from mangrove sediments and 175 isolates from Arabian Sea sediments survived on sub-culturing, while 60 isolates did not grow further. The isolates were maintained on CDA plates till further studies. Coastal marine habitats such as mangroves and mudflats are rich in organic matter [22] and harbor diverse forms of fungi [23], which are the initiators for any decomposition processes [22]. Due to the presence of recalcitrant organic material, these sediments act as bio-filters for heavy metals [24] and other toxic substances. Mangroves and salt pans are expected to harbor heavy metal-tolerant bacteria and fungi as these microbes are well adapted to exclude sodium ions out of their system [25] and can also do the same with metals.

Screening of the Fungal Isolates for Heavy Metal Tolerance

Majority of the isolates showed tolerance to 500 ppm concentration of Ni, Cu, and Cd, but as the concentration

increased further, the number of isolates tolerating the heavy metals decreased. Cadmium and Chromium showed the least MTI values, proving to be more toxic than the other two metals. It was observed that as the concentration of metal increased in the agar plate, the diameter of the fungal colony decreased. Incipient inhibition of mycelial growth due to Ni stress was reported to be 50–100 ppm for *A. niger* and *Trichoderma viride*, while total inhibition of growth was attained at 1000 ppm [26]. In such cases, only metal tolerant and metal resistant species survive, while the rest cease to grow. Similar results were seen with respect to the toxicity of metals, where the number of lead-tolerant isolates decreased with increase in lead concentration on PDA [4]. Most of the fungal isolates were tolerant to Ni on the agar plate (152 isolates) followed by Cu (61 isolates) and Cd (54 isolates), while least tolerant to Cr (16 isolates) at 500 ppm concentration. Chromium was found to be the most toxic amongst all the metals under consideration, where only four isolates were able to grow at 750 ppm concentration, and one isolate grew at 1000 ppm concentration. Some of the isolates showed a reduction in sporulation at higher metal concentrations, while some completely stopped growing (Supplementary Fig. 3). Reduction in sporulation has been reported when *A. terreus* and *Alternaria alternata* were grown in increasing concentrations of Cu on CDA [27]. Some of the fungal colonies showed blue coloration on Cu-containing agar plates. Change in colour of the biomass can be attributed to the accumulation of metal inside the mycelia as reported in

case of formation of copper oxalate crystals when subjected to copper stress [28].

On calculating MTI percentage for 15 selected isolates (Table 1), it was observed that MTI% decreased with increasing metal concentration, while some isolates (#NIOSN-M98, #NIOSN-SK56-S19) showed a sudden increase in metal tolerance at higher metal concentration and later showed a reduction in tolerance to metal. Some isolates like #NIOSN-M20, #NIOSN-M29, #NIOSN-M110, and #NIOSN-SK56-S19 also showed 100% MTI, as the growth in metal plate exceeded growth in control plate. Tolerance index more than 1.0 was reported in a study where the fungal culture was subjected to adaptive tolerance to metal [29]. Another study reported up to 7 times higher fungal activity in soil supplemented with Cu when compared to control without metal [30]. In both these studies, it appears that the presence of metal is stimulating the growth, which might be happening in some cases in the present study where there is a sudden increase in metal tolerance. MTI values were used to further short-list the isolates for molecular identification.

Identification of Selected Cr-Tolerant Fungal Isolates Using ITS Sequencing

On phylogenetic analysis of ITS rDNA of selected isolates, #NIOSN-SK56-S19, #NIOSN-SK56-S52 and #NIOSN-SK56-S76, they grouped with similar sequences of type strains and were identified as *Aspergillus sydowii*

Table 1 Metal tolerance index for 15 fungal isolates subjected to different concentrations of metals

Culture nos.	Metal tolerance index (MTI%)															
	Nickel (ppm)				Copper (ppm)				Cadmium (ppm)				Chromium (ppm)			
	500	750	1000	1250	500	750	1000	1250	500	750	1000	1250	250	500	750	1000
#NIOSN-M20	100	70	58	46	64	76	36	22	50	Nil	Nil	Nil	Nil	Nil	Nil	Nil
#NIOSN-M29	100	70	36	40	64	80	38	22	40	36	38	46	Nil	Nil	Nil	Nil
#NIOSN-M38	34	43	Nil	Nil	40	43	Nil	Nil	34	43	Nil	Nil	Nil	Nil	Nil	Nil
#NIOSN-M56	36	24	Nil	Nil	Nil	Nil	Nil	Nil	22	Nil	Nil	Nil	36	42	Nil	Nil
#NIOSN-M84	Nil	84	34	48	Nil	60	52	Nil	44	48	34	56	32	Nil	Nil	Nil
#NIOSN-M86	90	44	Nil	Nil	30	Nil	Nil	Nil	Nil	Nil	Nil	Nil	30	42	34	Nil
#NIOSN-M98	43	57	68	45	75	81	57	28	34	32	36	51	Nil	Nil	Nil	Nil
#NIOSN-M110	100	82	48	50	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	42	42	Nil	Nil
#NIOSN-SK56-S19	88	100	58	42	Nil	Nil	Nil	Nil	30	Nil	Nil	Nil	34	40	22	32
#NIOSN-SK56-S43	78	72	Nil	Nil	Nil	68	30	Nil	24	Nil	Nil	Nil	22	Nil	Nil	Nil
#NIOSN-SK56-S52	94	90	60	36	Nil	Nil	Nil	Nil	32	Nil	Nil	Nil	46	46	32	Nil
#NIOSN-SK56-S54	75	55	34	Nil	34	53	30	Nil	28	Nil	Nil	Nil	36	32	Nil	Nil
#NIOSN-SK56-S57	76	56	58	24	50	76	32	Nil	26	Nil	Nil	Nil	Nil	Nil	Nil	Nil
#NIOSN-SK56-S76	53	58	49	42	27	53	31	Nil	33	Nil	Nil	Nil	42	36	29	Nil
#NIOSN-SK56-C15	94	72	64	Nil	Nil	Nil	Nil	Nil	28	Nil	Nil	Nil	32	40	Nil	Nil

The isolates marked in bold were selected for further studies

(KT956258), *Aspergillus terreus* (KT956259) and *Purpureocillium lilacinum* respectively (KY788339). The evolutionary tree was constructed using Neighbour-Joining method (Fig. 1). All the isolates belonged to phylum *Ascomycota* and are reported to be rapid proliferators. The isolates #NIOSN-SK56-S19 (*A. sydowii*), #NIOSN-SK56-S52 (*A. terreus*) and #NIOSN-SK56-S76 (*P. lilacinum*) belonged to orders of *Eurotiales* and *Hypocreales* respectively. It has been reported that most of the isolates that could effectively tolerate metal, either as live or dead biomass, belong to these genera [13, 29–31].

Determination of Total Chromium Content in Biomass and Residual Broth

It was observed that the isolate *P. lilacinum* (#NIOSN-SK56-S76) showed substantial growth at all Cr concentrations, whereas isolates *A. sydowii* (#NIOSN-SK56-S19) and *A. terreus* (#NIOSN-SK56-S52) showed a decrease in biomass production with an increase in metal concentration (Table 2). Less biomass at higher metal concentrations can be attributed to the ceasing of growth and death due to stress. The concentration of Cr in biomass was also found to decrease with increase in metal concentration in these two isolates, which suggested that the Cr concentration in biomass is directly proportional to the active growth of culture and their biosorption capabilities.

Interestingly in the present work, it was found that even minute quantity of biomass of 2.8 mg, as in case of isolate *A. sydowii*, resulted in up to 26% removal of Cr from the broth at 300 ppm. But the Cr removal efficiency of biomass, in this case, was 0.01 mg Cr mg⁻¹ biomass, when compared to 25.1 mg biomass obtained from 50 ppm flask that resulted in 22% decrease with an efficiency of 0.007 mg Cr mg⁻¹ biomass. Further, isolate *P. lilacinum* did not show high percent removal although it produced more biomass as compared to other isolates. Despite showing highest total Cr-content in biomass at 300 ppm, the decrease in total Cr content was 14% with an efficiency of 0.008 mg Cr mg⁻¹ biomass. The dead or severely affected biomass, which is also known to be a good biosorbent as reported previously [32, 33] could be responsible for high percent removal of Cr (Table 3). Untreated biomass of the isolates #NIOSN-SK56-S19 and #NIOSN-SK56-S52 had more Cr removal efficiency than previously reported in pre-treated *R. arrhizus* [34]. Jakubiak et al. [35] reported that live biomass shows more biosorption than dead biomass. Apart from the type of culture affecting metal uptake, pH of the broth also plays an important role in bioavailability of metals. At very low pH, H⁺ ions compete with metal ions for binding sites, while metal solubility decreases as the pH increases. Therefore an acidic pH is most suitable for these types of studies [36], because of which, a final pH of 5 was chosen

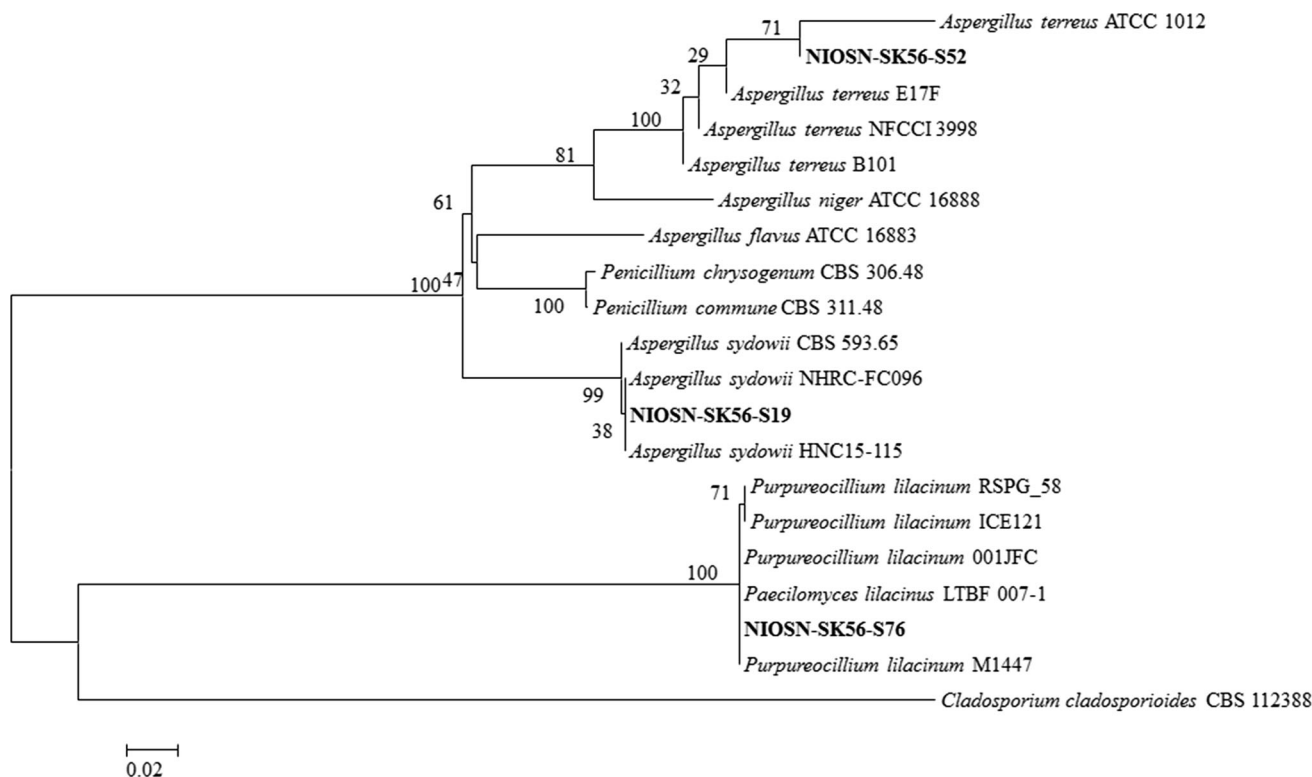


Fig. 1 Phylogenetic analysis of fungal isolates (ITS sequences) inferred from NJ analysis using MEGA software

Table 2 Determination of total Chromium content in residual broth and biomass of Cr-tolerant isolates

Isolate number	Concentration of chromium (ppm)	Absolute Cr content (mg)	Dry weight of biomass (mg)	Cr content in digested biomass (mg)	Cr content per mg of dried biomass (mg)	Residual Cr content (mg) in broth	% removal of Cr in broth
#NIOSN-SK56-S19	Control-0	0	141.8	0	0	0	Nil
	50	1	25.1	0.186 ± 0	0.007	0.779 ± 0.011	22.1
	100	2	19.4	0.157 ± 0	0.008	1.947 ± 0	2.65
	200	4	1.6	0.007 ± 0	0.004	3.417 ± 0.023	14.575
	300	6	2.8	0.029 ± 0	0.01	4.450 ± 0.023	25.833
#NIOSN-SK56-S52	Control-0	0	73.2	0	0	0	Nil
	50	1	50.4	0.39 ± 0	0.008	0.592 ± 0.016	40.8
	100	2	34.6	0.364 ± 0	0.01	1.975 ± 0.046	1.25
	200	4	6.5	0.053 ± 0	0.008	3.565 ± 0.023	10.875
	300	6	4	0.05 ± 0	0.013	4.598 ± 0.140	23.367
#NIOSN-SK56-S76	Control-0	0	169.1	0	0	0	Nil
	50	1	76.6	0.15 ± 0	0.002	0.751 ± 0.032	24.9
	100	2	80.7	0.28 ± 0	0.004	2.134 ± 0.040	Error
	200	4	50.6	0.264 ± 0.1	0.005	3.286 ± 0.040	17.85
	300	6	63.6	0.523 ± 0	0.008	5.154 ± 0.161	14.1

Table 3 Comparison of Chromium removal using fungal isolates in the present study with that reported in literature

S. no	Fungal isolate	Isolated from	Sample/effluent	Cr concentration	Cr removal/recovery	References
1	<i>Aspergillus niger</i> (pre-treated)	Industrial waste contaminated soil	K ₂ Cr ₂ O ₇ solution	4 mM	18.05 mg Cr g ⁻¹ biomass	[2]
2	<i>Penicillium sp.</i> (pre-treated)	Industrial waste contaminated soil	K ₂ Cr ₂ O ₇ solution	4 mM	19.30 mg Cr g ⁻¹ biomass	[2]
3	<i>Rhizopus arrhizus</i> (pre-treated)	Not mentioned	K ₂ Cr ₂ O ₇ solution	100 ppm	9.02 mg Cr g ⁻¹ biomass	[35]
4	<i>Rhizopus arrhizus</i> (pre-treated)	Not mentioned	K ₂ Cr ₂ O ₇ solution	300 ppm	23.07 mg Cr g ⁻¹ biomass	[49]
6	<i>Rhizopus nigricans</i> (pre-treated)	Not mentioned	K ₂ Cr ₂ O ₇ solution	50 mM	47 mg Cr g ⁻¹ biomass	[50]
7	<i>Trichoderma longibrachiatum</i>	Industrial and sewage waste contaminated water	Potato dextrose broth	50 ppm	0.55 mg Cr g ⁻¹ biomass	[4]
8	<i>Trichoderma viridae</i>	Industrial and sewage waste contaminated water	Potato dextrose broth	50 ppm	2.55 mg Cr g ⁻¹ biomass	[51]
9	<i>Aspergillus sydowii</i> (#NIOSN-SK56-S19)	Arabian Sea sediment	Czapek Dox broth	300 ppm	10 mg Cr g ⁻¹ biomass	Present study
10	<i>Aspergillus terreus</i> (#NIOSN-SK56-S52)	Arabian Sea sediment	Czapek Dox broth	300 ppm	13 mg Cr g ⁻¹ biomass	Present study
11	<i>Purpureocillium lilacinum</i> (#NIOSN-SK56-S76)	Arabian Sea sediment	Czapek Dox broth	300 ppm	8 mg Cr g ⁻¹ biomass	Present study

for this experiment. At acidic pH, Cr(VI) gives anionic species like Cr₂O₇²⁻, HCrO₄⁻, and Cr₂O₄²⁻ which get attracted to the protonated surface of fungal mycelia [36, 37]. The acidic pH keeps the Cr ions in soluble form and also facilitates adsorption on the mycelial cell walls. Apart from chitin and melanin components of cell walls,

production of extracellular material such as organic acids is also responsible for metal binding [6, 7]. Considering the experimental and instrumental error, it was observed that, on adding the Cr content in broth and biomass, some amount of Cr was left unaccounted, which needs to be investigated further.

Reduction in Cr(VI) Content During the Growth of Fungal Isolates as Determined by DPC Method

On determining the chromium reduction using DPC, which is a method for specific detection of Cr(VI) state, it was observed that isolate *A. terreus* (#NIOSN-SK56-S52) was the most efficient culture (Fig. 2). It reduced the total

Cr(VI) state to Cr(III) in 3 days while isolate *A. sydowii* reduced the same in 7 days. Surprisingly, isolate *P. lilacinum* did not participate significantly in the reduction of Cr(VI) to Cr(III) and was able to survive in toxic Cr(VI) state indicating the presence of an alternate mechanism for survival.

Fig. 2 Determination of Cr(VI) reduction using Diphenylcarbazide (DPC) method

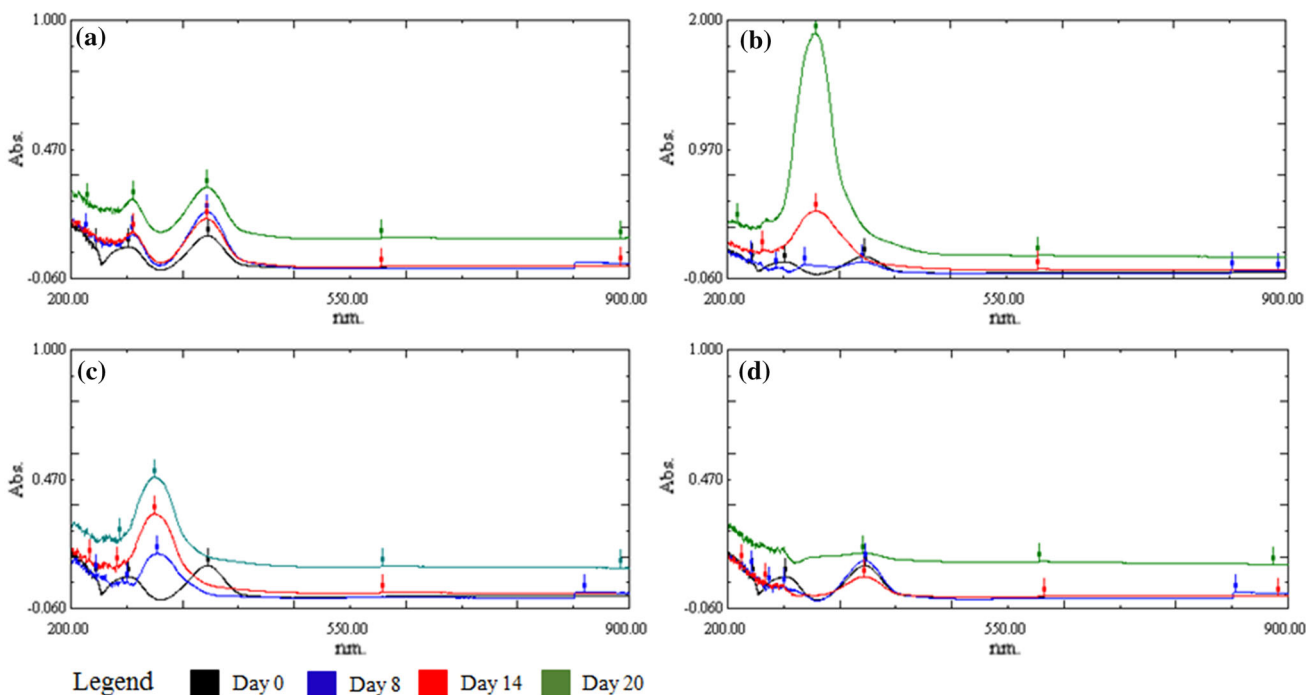
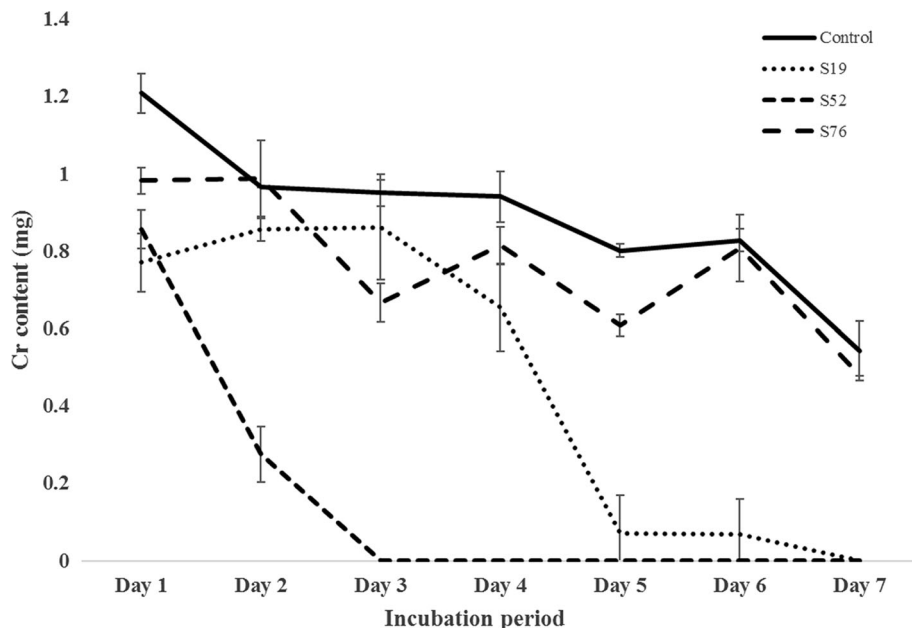


Fig. 3 Absorption spectra of cell-free culture broth obtained growth of fungal isolates in chromium supplemented culture broth using UV–VIS spectrophotometry. **a** Control. **b** Isolate *A. sydowii*. **c** Isolate *A.*

terreus. **d** Isolate *P. lilacinum*. The arrows indicate the Cr peaks in all the four spectra

To further investigate, whether chromium under different oxidation states showed different absorption peaks, filtered broth samples were subjected to UV–VIS spectroscopy. Both the isolates belonging to *Aspergillus* genera, #NIOSN-SK56-S19 (Fig. 3b) and #NIOSN-SK56-S52 (Fig. 3c), showed a shift in peak from 390 to 310 nm where #NIOSN-SK56-S52 showed a gradual increase in intensity while #NIOSN-SK56-S19 exhibited a rapid increase in intensity by the 20th day of incubation. It was observed that the control flask gave a peak at 370 nm at all days (Fig. 3a) indicating there is no change in chromium oxidation state due to any chemical redox reactions. #NIOSN-SK56-S76 (Fig. 3d) did not show any shift in peaks and was comparable to the control. Peak shift from 390 to 310 nm for #NIOSN-SK56-S52 and #NIOSN-SK56-S19 indicated change in Cr form due to biological reduction. Previous studies have also reported absorption peak at 300 nm in UV–VIS spectrum attributing to Cr nanoparticles [38–40]. A separate study reported nano-sized Cr_2O_3 particles showed absorption peak at 460 nm [41] while

another study [42] depicted that the inter-band transitions within the core electrons of chromium to chromium oxide give a peak at 445 nm indicating the presence of Cr_2O_3 nanoparticles. Cr(VI) being a highly oxidizing agent, easily gets transformed into Cr(III) in the presence of organic matter [43] which might have led to shifts in the peak position as well as its intensity. It was therefore concluded that absorption peaks for chromium changed with respect to oxidation state and not due to nanoparticle formation.

SEM and EDS Analysis

The effect of metal stress on fungal surface morphology was studied by using SEM. Isolate *A. sydowii* was seen to produce an extracellular thread-like substance (Fig. 4b) at 50 ppm Cr stress, while such thread-like substance was not observed in control (absence of metal) (Fig. 4a). Extracellular aggregation in between the mycelial network was seen in *A. terreus* (Fig. 4d) when subjected to Cr stress and this focuses on an important point that aggregates could be

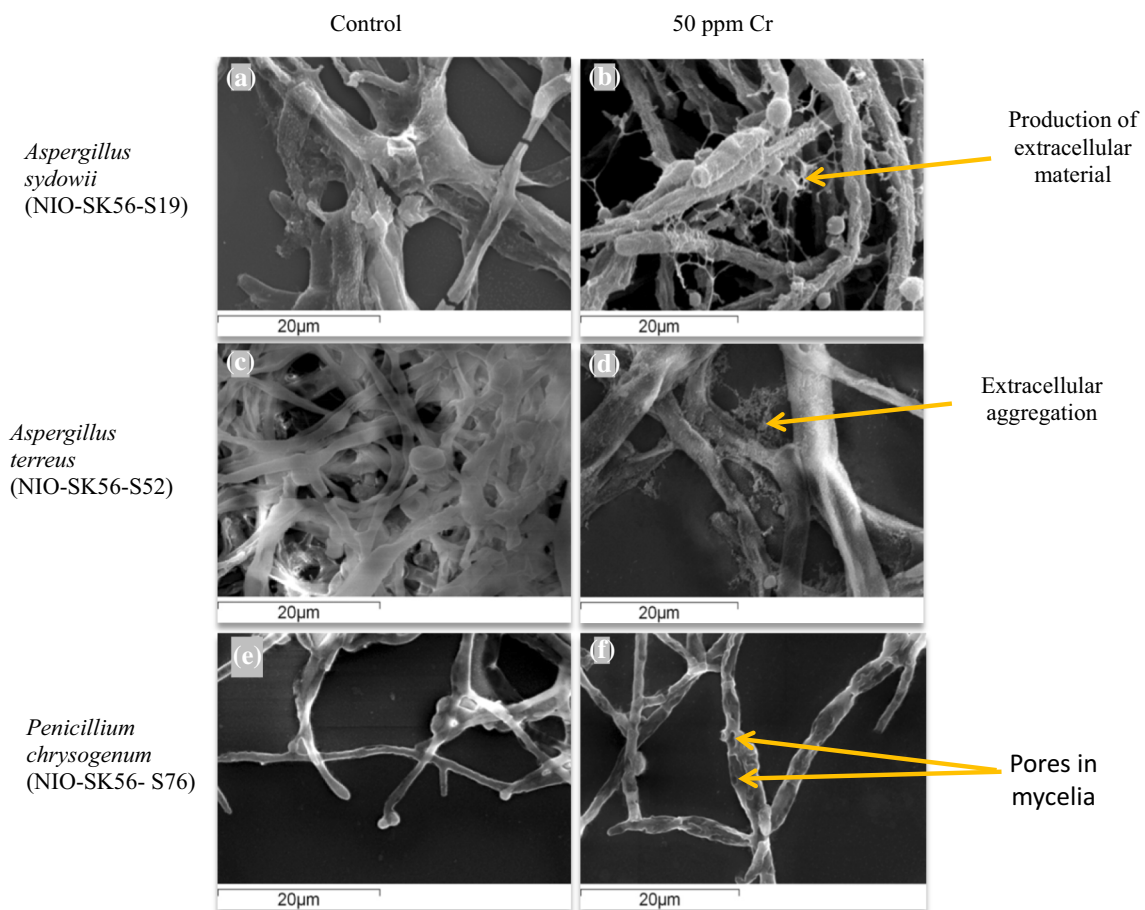


Fig. 4 SEM images fungal isolates grown at 50 ppm Cr and without Cr. **a** Isolate *A. sydowii* grown in 0 ppm Cr. **b** Isolate *A. sydowii* grown in 50 ppm Cr showing production of extracellular material. **c** Isolate *A. terreus* grown in 0 ppm Cr. **d** Isolate *A. terreus* grown in

50 ppm Cr showing extracellular aggregation within mycelial growth. **e** Isolate *P. lilacinum* grown in 0 ppm Cr. **f** Isolate *P. lilacinum* grown in 50 ppm Cr showing deflation of mycelia

the possible sites of biosorption in these cultures. SEM image of *P. lilacinum* (Fig. 4f—arrow) showed irregularly shaped mycelia, while the mycelia in control were found to be healthy and intact (Fig. 4e—arrow). Interesting observation for this particular isolate was that it efficiently produced more biomass (Table 2) under metal stress but did not produce any extracellular material that could possibly adsorb the metal onto its surface as evident from SEM image (Fig. 4f) to combat the stress. This particular isolate also did not aid in the reduction of Cr(VI) to Cr(III) as mentioned earlier. Primary sites for biosorption to occur are the cell walls that contain huge proportions of chitin, melanin and phenolic compounds which provide oxygen-rich metal-binding sites [31, 44, 45]. This extracellular material could be most probably responsible for biosorption, as exopolysaccharide (EPS) is known to biosorb heavy metals [46]. Nanoparticle aggregation was reported on the mycelial cell wall of *Pleurotus eryngii* exposed to

Al₂O₃ compared to the non-exposed mycelia [35]. Subbiah and Yun [14] reported the presence of pores and deformed mycelia when used as biosorbent in an aqueous solution of Ni.

Gold-sputtered mycelia used for SEM analysis were also studied for EDS analysis to understand the form of Cr present within the biomass. The salt used in the broth was K₂Cr₂O₇ which gives Chromium anion with an oxidation state of + 6. On EDS analysis, the compound detected in the samples was Cr₂O₃, with an oxidation state of + 3 (Fig. 5). Therefore the presence of Cr₂O₃ in the mycelia of the isolates highlights the ability of the organisms to convert Cr⁺⁶ to Cr⁺³ state through a reduction process. Conversion of Cr⁺⁶ to Cr⁺³ state as seen from EDS analysis indicates that these organisms might have multiple metal tolerance mechanisms. This was also supported by the DPC method as discussed above, which is specific to the detection of Cr(VI). The decrease in concentration of

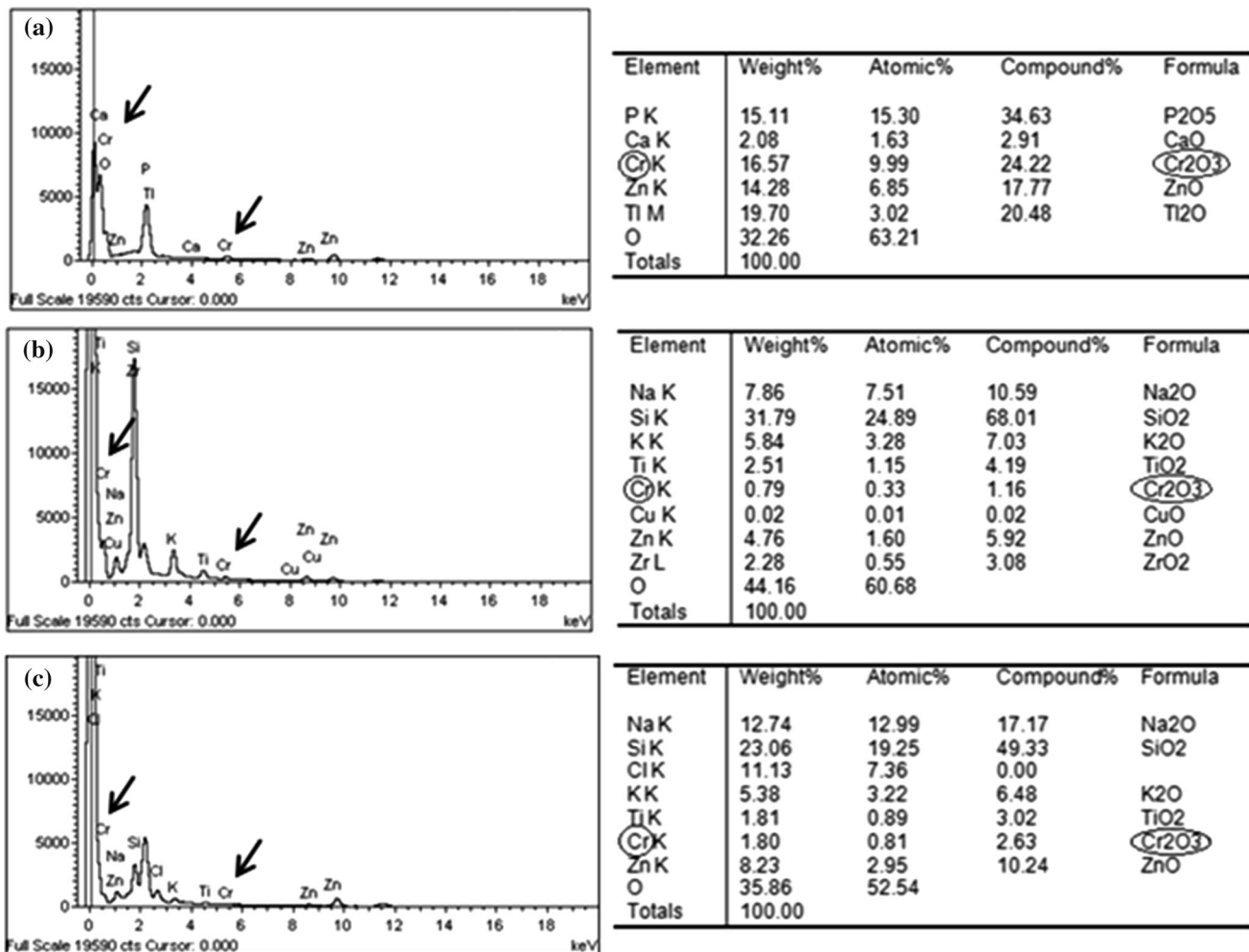


Fig. 5 EDS spectra of fungal isolates grown at 300 ppm Cr showing the presence of Cr₂O₃. **a** Isolate *A. sydowii*. **b** Isolate *A. terreus*. **c** Isolate *P. lilacinum*. The arrows indicate the Cr peaks in all the three spectra

Cr(VI) over an incubation period of a week till no more Cr(VI) was measured, indicated a process of conversion of detectable state of Cr(VI) to undetectable form (Cr(III)). This is the first report of fungi causing direct Cr reduction as determined by DPC method as opposed to the extensive literature available for bacteria. Zahoor and Rehman [20] reported up to 85% reduction of Cr(VI) by a *Bacillus* sp. within 96 h of incubation. In another study, *O. intermedium* and *Brevibacterium* sp. were able to reduce Cr(VI) entirely by 72 and 96 h respectively [47]. Reduction of Cr⁺⁶ to Cr⁺⁴ can occur with two electrons participating in the initial reduction stage and then further getting reduced to Cr⁺³ in the second step [8]. Reduction of Cr⁺⁶ to Cr⁺³ with Cr⁺⁵ as an intermediate in a two-step reaction has also been reported in *Pseudomonas ambigua* [48]. The results indicated towards not only single but multiple metal tolerance mechanisms existing in these isolates, which needs to be explored further to understand the conversion of toxic Cr(VI) to lesser-toxic form Cr(III).

Conclusion

The diversity of microorganisms from marine environments is of growing interest and have already found applications in various fields of science. The salt-tolerant species isolated from marine environments, able to grow at high concentrations of salt, possessing a high resistance to heavy metals have excellent potential as agents for reduction of pollution in saline conditions and also in non-saline environments. The analysis carried out in this study serves as an indication of the potential of various marine-derived fungal isolates for metal bioremediation. The fungi isolated from marine environments such as mangroves and open ocean sediments tolerated high concentrations of heavy metals and even converted toxic Cr⁺⁶ to lesser toxic Cr⁺³ form as seen in this study. Further studies are required before proposing the ability of the cultures to be used for designing bioremediation strategy to remove harmful metals from the effluent discharges of tanneries or dye industries which are rich in chromium.

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Compliance with Ethical Standards

Conflict of interest The authors declare no financial or commercial conflict of interest.

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