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Investigation of Cr (VI) Uptake in Saline Condition Using Psychrophilic and Mesophilic *Penicillium* sp.

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A B S T R A C T

Salt tolerant and chromium resistant fungal strains were isolated from two different sites i.e. psychrotolerant fungus PLF1, was isolated from Pangong lake of Laddakh, India and mesophilic strain PMS2, was isolated from garden soil. Present study was carried out on interaction of *Penicillium sp.* with chromium and NaCl and reports ability of *Penicillium sp*. to bind with chromium and NaCl in aqueous solution. Biosorption of the chromium ion Cr(VI) onto the cell surface of *Penicillium* fungal species in aerobic condition was also investigated. Results displayed 42.85 and 40.38% inhibition of mycelial growth in presence of 10% NaCl and 100 ppm concentration of Cr (VI), whereas 63.15 and 60.65% inhibition of mycelial growth was observed in absence of NaCl and 100 ppm concentration of Cr (VI) in PLF1 and PMS2 fungal isolates respectively on 10^{th} day of incubation. It is demonstrated in the present study that NaCl reduced the extent of Cr biosorption and promote the fungal strains of *Penicillium* for better growth. FTIR spectrum was used to evaluate the membrane surface binding of chromium and NaCl by fungal strains of *Penicillium.*SEM was used to obtain the morphological as well as structural characterization of fungal pellets. The present finding suggests that NaCl might reduce the toxicity of hexavalent chromium in *Penicillium* sp. and may be used as a potential organism for remediation of chromium in high salinity environments.

Introduction

Chromium (Cr) toxicity is one of the major causes of environmental pollution which enter the agroecosystem from different sources like sewage sludge, tannery effluents and industrial operations such as smelting, mining etc. (Bai and Abraham, 2002). Of the various forms of chromium only hexavalent Cr (VI) and trivalent chromium Cr (III) have primary environmental significance because they are the most stable oxidation forms in the environment (Smith *et al.*, 2002). The increasing concentrations of chromium in the soil affect adversely both the microbes and plants (Ahmad *et al.*, 2005). High saline wastes are produced in different industrial

activities, such as chemical manufacture, oil and gas production and waste minimization practices. This waste is composed of water containing high concentration of salts, oil, organic acids, heavy metals, and radionuclides (Woolard and Irvine, 1995) therefore, the potential of halotolerants to remediate pollutants such as heavy metals in the presence of salt is useful for biological treatment without damage to the physically sensitive ecosystem. Physico-chemical methods such as reverse osmosis, solvent extraction, lime coagulation ion exchange and chemical precipitation (Rich and Cherry, 1987) for removal of heavy metals from wastewater are agonize from extremely costs connected with energy and chemical consumption. Recently, microbes have been reported as the exploration for new and advanced technology for the remediation of heavy metal pollution (Kapoor and Viraraghvan 1995; Doelman *et al.,* 1994). The present study attempts to isolate and identify heavy metal tolerant fungal strains from two different environments and to evaluate their chromium removal efficiency from liquid media in saline and non-saline conditions.

Materials and Methods

Isolation of Chromium Tolerant Fungi

Fungal strains were isolated from two different sites i.e. psychrotolerant fungus PLF1, was isolated from Pangong lake of Laddakh, India and mesophilic strain PMS2, was isolated from garden soil of BBAU Campus, Lucknow by serial dilution method using potato dextrose agar containing 100 ppm of Cr and 10% of NaCl. The 1000 ppm stock solution Cr was made in double distilled water using $K_2Cr_2O_7$. The stock solution of heavy metal was sterilized separately through bacteriological filters and added to sterilized potato dextrose agar (PDA) medium to make the concentration at 50 ppm. Twenty milliliter of PDA medium (Hi Media, India) containing 50 ppm of chromium and 10 % of NaCl poured in these petri plates and incubated at 28°C for 72h. The colonies of predominant genera of fungi were picked up and purified by pour plate method. Isolates were tested for morphological, physiological and biochemical characters according to mycological literature. Fungal strains were purified and maintained on potato dextrose agar slants and plates at 4°C for further use. Psychrotolerant PLF1 can grow at 20-40°C, but also able to tolerate lower temperatures (up to 4°C) with slower growth rates. However, low temperatures are not the only conditions that fungal strain PLF1 of glacial environments has to endure in order to survive.

Screening of Fungal Isolates for Chromium Tolerance

Chromium tolerant (50 ppm) fungal isolates were further screened for tolerance to Cr at 100, 200 and 400 ppm of heavy metal on PDA in presence and absence of 10% NaCl. The discs of fungal isolates were kept on PDA medium containing 100, 200 and 400 ppm of chromium separately. Discs of fungal isolates on normal PDA medium served as control (normal growth) for comparison of growth of fungal isolates on PDA medium containing different concentration of heavy metal in presence and absence of 10% NaCl. Observations on growth of fungal isolate were made after 72 h of incubation. The growth of fungal isolates was recorded as normal, moderate or absent in comparison to control. The isolated screened fungal isolates were identified on the basis of cultural and morphological characters (Gilman, 1957; Barnett and Hunter, 1972).

Uptake of Chromium by Fungal Isolates from Liquid Media

The highly tolerant fungal isolates to heavy metal were evaluated for uptake of chromium in potato dextrose broth medium containing 20, 40, 60, 80 and 100 ppm concentration of Cr in presence and absence of 10% NaCl. Potato dextrose broth with and without 10% NaCl and containing 20,40, 60, 80 and 100 ppm of Cr was dispensed in 100 ml lots to 250 ml conical flasks and sterilized at 15 lbs/psi for 15 min. These flasks were inoculated with 1 ml of freshly prepared spore suspension (10^6 –10^7 spores/ml) of each fungal isolate and kept in static condition at 28°C for 96 h. Un-inoculated flasks containing PD broth of 20,40, 60, 80 and 100 ppm concentration of Cr in the presence and absence of NaCl, served as control. Fungal growth was harvested after 96 h through filtration using Whatman filter No. 42. The harvested fungal biomass was rinsed with double distilled water 3–4 times and dried in hot air oven at 80°C for 18 h. The dried fungal biomass was weighed and heavy metal concentration in it was estimated by digestion with nitric acid and perchloric acid (3:1 ratio). The digested fungal biomass was filtered through Whatman filter No. 42 and made the volume of filtrate to 50 ml in volumetric flask.

The heavy metals concentration in filtrate was estimated (Greenberg *et al.*, 1985) by Atomic Absorption Spectrophotometer (AA 240 FS: Fast Sequential AAS Varian, Netherland) All the experiments were conducted in triplicate and data were analyzed statistically. Growth and uptake of Cr by both mesophilic (PMS2) and psychrophilic (PLF1) fungi in presence and absence of 10% NaCl in PD broth was studied. The uptake of chromium by fungal biomass was calculated using the following equation:

$$qe\ (mg/g) = \frac{C \times V \times 1000}{W}$$

qe concentration of chromium cumulated by fungal biomass, (mg/g); C concentration of chromium (ppm); V (ml) the volume of the liquid medium and W (g) is the dry weight of the fungal biomass.

Microscopic Characterization

Scanning Electron Microscopic Observations

The fungal mycelia harvested during the mid-exponential phase and taken in to eppendorf tube and were fixed with 2.5 % of glutaraldehyde (Loba Chemi, INDIA) solution in Milli-Q (M.Q) water for 1- 2 hours at room temperature. Fixed culture were washed twice with M.Q water, and post-fixed with 0.5 ml of 2% Osmium tetra oxide for 1 hour. The culture was subsequently dehydrated using a series of 10, 30, 50, 70, 90 and 100% ethanol in M.Q water for 5 minutes. The final dehydration in 100% of ethanol was carried out for 10 minutes. The dehydrated culture drop was fixed on the cover slip and then dried overnight in an oven and desiccator till mounting.

The specimen was mounted over stainless steel stab with double-stick carbon adhesive tapes and coated with platinum using a sputter coater prior to viewing using a scanning electron microscope of JEOL (JSM 6490 LV) JAPAN. (Fig 6 and Fig:7).

Fourier Transform Infrared Spectra (FTIR) Analysis

The cell surface binding characteristics of PMS2 and PLF1biomass before and after adsorption of Cr (VI) with and without NaCl were analyzed by using Fourier Transform

Infra-Red Spectrometer (FTIR) of Thermo-Scientific (Nicole 6700). The mid-exponential phase growing fungal biomass was harvested by using Whatman No 1 filter paper. Biomass of both the strains treated with 60 ppm of Cr (VI) with and without NaCl was analyzed. The fungal biomass without treatment of chromium and NaCl was treated as control. Biomass was washed three times with M.Q water and then dried in hot air oven at 60°C for 24 hour. The dried fungal biomass samples and Potassium bromide (KBr) (1/100 ratio) were grinded pressed to form pellet by using a manual hydraulic press (150 lb).The IR spectra (4000-400 cm^{-1}) were obtained with resolution of 5-7 $cm^{-1}$with the 32 scan number for each spectrum, using (FTIR) (Thermo- Scientific Nicole 6700, USA).

Results and Discussion

Isolation of Chromium Tolerant Fungi

Five fungal isolates tolerant to Chromium (50 ppm) were isolated from garden soil of BBAU Campus, Lucknow and only one chromium tolerant fungal strain was isolated from Pangong lake of Laddakh, India using standard methods (Gilman, 1957). Isolate PLF1 and PMS2 were selected from the collection of all strains as they displayed maximum salinity tolerance capacity and high chromium tolerance. Isolate PLF1 and PMS2 were identified according to the mycological literature and assumed to be a member of the genus *Penicillium*. Based on slide preparation, biochemical and physiological characteristics isolate PLF1 and PMS2 was reaffirmed and identified as *Penicillium*.

Screening of Fungal Isolates for Salt and Chromium Tolerance

All fungal isolates were further screened for their tolerance to at 50, 100, 200 and 400 ppm of Cr. Data indicated decrease in number of isolates tolerant to Cr at higher concentration of metal. Among all fungal isolates tolerant to Cr at 50 ppm, only two isolates could tolerate Cr at 400 ppm. Similar trend was observed for screening of fungal isolates for their tolerance to salinity. Inhibition of some of the fungal isolates at higher concentration of Chromium is observed. Similar observations about toxic effect of higher concentration of heavy metals on growth of fungi have been reported. (Malik A 2004 and Rama Rao *et al.,* 1997)

Uptake of Chromium by Fungal Isolates from Liquid Media

The maximum dry weight (0.61 g) was observed in mesophilic strain of *Penicillium*-PMS2, followed by psychrophilic strain of *Penicillium*-PLF1 (0.57 g) in potato dextrose broth containing 60 ppm of Cr. The maximum uptake (0.062 mg/g) of Cr was observed in PMS2. Minimum uptake Cr (0.032 mg/g) found in PLF1 in PD broth containing 60 ppm of Cr respectively (Table 3). The highest uptake of Cr (0.062 mg/g) by PMS2 indicated its efficiency to remove Cr from aqueous solution in non-saline medium containing higher concentration of Cr (60 ppm). These results with respect to uptake of Cr by fungi are in agreement with those reported earlier (Gopal *et al.,* 2002; Preetha andViruthagiri 2007; Congeeraram *et al.,* 2007).

Effect of Chromium and Salt Concentration on Mycelial Growth

Isolates PLF1 and PMS2 could tolerate salinity level upto 0-15%. Salt shock upto 5% NaCl did not affect the growth, but higher osmotic stress of above 10% brought significant reduction in fungal biomass by

27.63% and 33.07% in PLF1 and PMS2 respectively, in comparison to non-saline conditions (Fig 1). Results also displayed 63.15 and 60.65% inhibition of mycelial growth in absence of NaCl and 100 ppm concentration of Cr (VI), in PLF1 and PMS2 respectively on 10^{th} day of incubation (Fig 2), whereas 42.85 and 40.38% inhibition of mycelial growth was observed in presence of 10% NaCl and 100 ppm concentration of Cr (VI), in PLF1 and PMS2 respectively on 10^{th} day of incubation (Fig3).

Growth and Uptake of Chromium in Saline and Non-saline Medium

The maximum dry weight (0.40 g) was observed in mesophilic fungi PMS2 followed by psychrotolerant strain of *Penicillium* PLF1 (0.38 g) in presence of 10% NaCl. There was 0.032 and 0.041 mgg^{-1} reduction of chromium was observed by psychrotolerant fungi PLF1 and 0.046 and 0.062 mg g^{-1}reduction of chromium was observed by mesophilic fungi PMS2 in presence of saline and non-saline medium respectively, invitro conditions. (Table: 1) These results with respect to uptake of Cr by fungi are in agreement with those reported earlier (Congeeraram *et al.,* 2007).

Fourier Transform Infrared Spectroscopy (FTIR)

The possible metal binding sites were identified by FTIR spectral analysis (wave numbers 400-4000 cm^{-1}) by comparing control and treated biomass of fungal strains (Fig 4 and Fig 5). Clear spectral changes were observed after biosorption of chromium on fungal biomass as shown in Table 2 and 3. The results showed that the cell surface of fungal strains contain several functional groups such as carbohydrate, amide, amine and hydroxyl groups which contribute to chromium binding by the cell surface of both *Penicillium* strains. The functional groups such as carboxyl, hydroxyl, amide, phosphate and sulphonate groups have been recognized as potential adsorption sites to be accountable for binding metallic ions to fungi (Das and Guha 2007).

Their potential for metal uptake depends on factors such as the abundance of sites, their accessibility, chemical state and affinity between adsorption site and metal. (Subbaiah *et al.,* 2008).The FTIR spectra of *Penicillium* after treatment of chromium and chromium with NaCl displayed changes in spectral peaks (Table 2 and Table 3). Shifting of the FTIR peak in the range of 3500-3300 cm^{-1}were indicating the presence of hydroxyl group (O-H stretch) and secondary amide (N-H stretch) which after adsorption of chromium shifted to 3384.9 and 3356.9 in PLF1 and PMS2 respectively. After adsorption of chromium the wave numbers 2358.5 cm^{-1}shifted to 2361.1 showed asymmetric CO_2 stretching (Schultz *et al.,*1996) in mesophilic *Penicillium* PMS2.

Peaks at 1740.2 cm^{-1} can be attributed to (C=O stretching) band of carboxylic or ester groups shifted to 1743.1.The wave numbers 1647.4, 1538.5and 1647.3, 1550.4 shifted due to the stretching in C=O of amide-I and N-H of amide-II band of proteins (Chandra *et al.,* 2014) after chromium adsorption in PMS2 and PLF1 strains respectively. A new peak emerged in the range of 1455.1 cm^{-1}in PMS2 strain is closely related to N-H bending and C-N stretching frequencies. Fourier transform infrared (FTIR) analysis was carried out to determine the involvement of the type of functional groups in metal adsorption. Fein *et al.* reported that some components of the cell wall serve as electron donors for the reduction reaction while metal binding.

Table.1 Growth and Uptake of Cr by Fungi from Liquid Medium Containing 60 ppm Chromium

PLF1		PMS2		
Cr 60 ppm	10% NaCl	No NaCl	10% NaCl	No NaCl
Dry weight (g)	0.38	0.30	0.40	0.34
Cr Uptake (mg/g)	.032	.041	.046	.062

Table.2 Assignment of Functional Groups Associated with Major Vibration Bands in Mid-IR Spectra of Psychrophilic *Penicillium* (PLF1)

Representing range of wavenumber in cm^{-1}	**PLF1 Control**	**PLF1 with Cr 60 ppm**	**PLF1 with 10% NaCl and Cr 60 ppm**	**Band assignment**
3500-3300	3391.1	3384.9	3389.1	O-H (asym.) and N–H stretching of secondary amides (Coates, 2000; Yang *et al.*, 2005; Paluszkiewicz and Kwiatek, 2001)
2950-2700		2927.1	2930.7	C–H stretching (Bellamy, 1954)
1660-1600	1647.3	1651.4	1647.6	Amide-I band arises from C=O stretching vibrations (Paluszkiewicz and Kwiatek, 2001)
1550-1510	1550.4	1550.0	1538.7	Amide-II band (arises from C-N & C-H bending vibrations (Sethuraman and Balasubramanian, 2010; Eckel *et al.*, 2001)
1377	1377.8	1376.6	1378.7	C-H bending modes of CH_2 (Erukhimovitch *et al.*, 2005)
1270-1230	1235.3	1236.9	1230.5	PO^{2-} stretching vibrations and phospholipids (Erukhimovitch *et al.*, 2005)
1090-990		1076.7		Carbohydrate and nucleic acid vibrations (Salman *et al.*, 2012)
	1037.0	1038.2	1076.6	P=O stretching (Das and Guha 2007)
630-530	562.4	570.1	571.8	Nitro compounds and disulfide groups (Ramrakhiani *et al.*, 2011)

Table.3 Assignment of Functional Groups Associated with Major Vibration Bands in Mid-IR Spectra of Mesophilic *Penicillium* (PMS2)

Observed wavenumber (cm^{-1}) at different concentration of Cr (VI)				
Representing range of frequency in cm^{-1}	**PMS2 Control**	**PMS2 with 60 ppm Cr (VI)**	**PMS2 with 10% NaCl and 60 ppm Cr (VI)**	**Band assignment**
3400-3300	3391.3	3356.9	3396.7	O–H and N–H stretching of sec-amides (Coates, 2000; Yang *et al.*, 2005; Paluszkiewicz and Kwiatek, 2001)
3050-2850	2926.9	2926.6	2927.4	CH_2 asymmetric stretching (Naumann 2011)
2862-2843	2856.7	2857.1	2857.5	(C-H) stretching (Bellamy, 1954).
2400-2300	2358.5	2361.1		Asymmetric CO2 stretching (Jackson *et al.*, 1998)
1760-1720	1740.2		1743.1	C=O stretching of ester (Dukor 2001;Chandra *et al.*, 2014)
1700-1600	1647.4	1649.9	1648.2	Amide-I band proteins arises from C=O stretching (Paluszkiewicz and Kwiatek, 2001);
1560-1530	1538.5	1542.6	1552.3	Amide II (N—H), C—N stretching (Sethuraman and Balasubramanian, 2010)
1450-1400		1455.1		C-N stretching and N-H bending of Amide- III band of protein (Eckel *et al.*, 2001))
1400-1360	1377.8	1377.2	1376.1	CH bending mode of CH_2 (Erukhimovitch *et al.*, 2005)
1270-1230	1234.8	1239.5	1237.1	PO^{2-} stretching vibrations and phospholipids (Yang *et al.*, 2005)
		1152.5	1151.9	CO—O—C stretch in polysaccharides (Maquelin *et al.*, 2002)
1100-1050	1076.7	1077.0	1077.6	Carbohydrate and nucleic acid vibrations (Salman *et al.*, 2012)
	1041.9	1040.3	1039.3	P=O stretching (Das and Guha 2007)
630-530	572.9	577.5	569.4	Nitro compounds and disulfide groups (Ramrakhiani *et al.*, 2011)

Figure.1 Growth of Fungal Strains with Different Conc. of NaCl (0-15%)

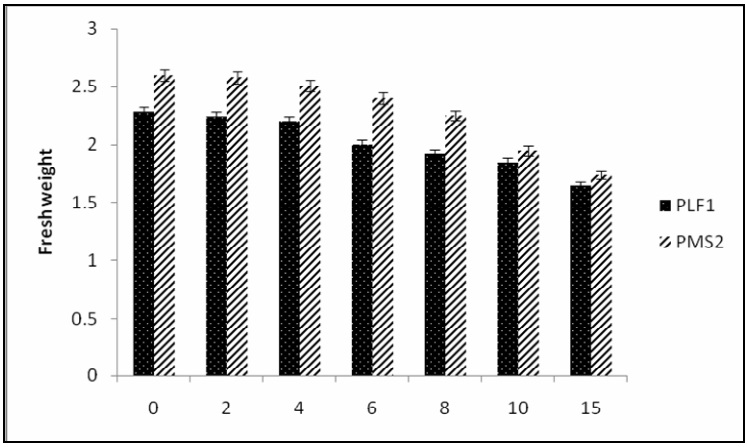


Figure.2 Growth of fungal Strains with Different Concentrations of Cr (VI) in Absence of NaCl

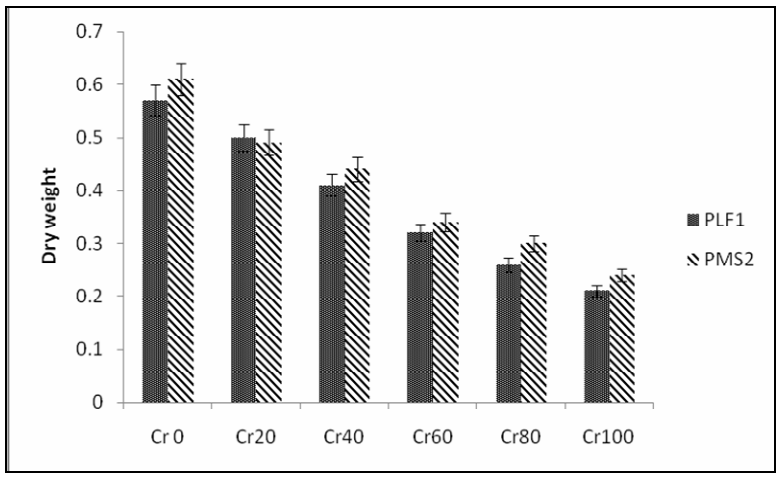


Figure.3 Growth of Fungal Strains with Different Chromium Concentrations and 10% NaCl

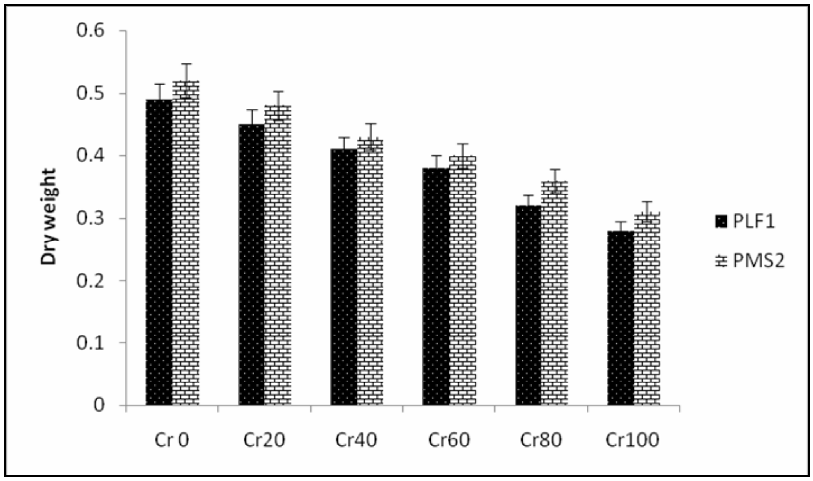


Fig.4 A- (Laddakh *Penicillium*) PLF1 Control, B- PLF1 with 10% NaCl and Cr 60ppm, C- PLF1 with Cr 60ppm

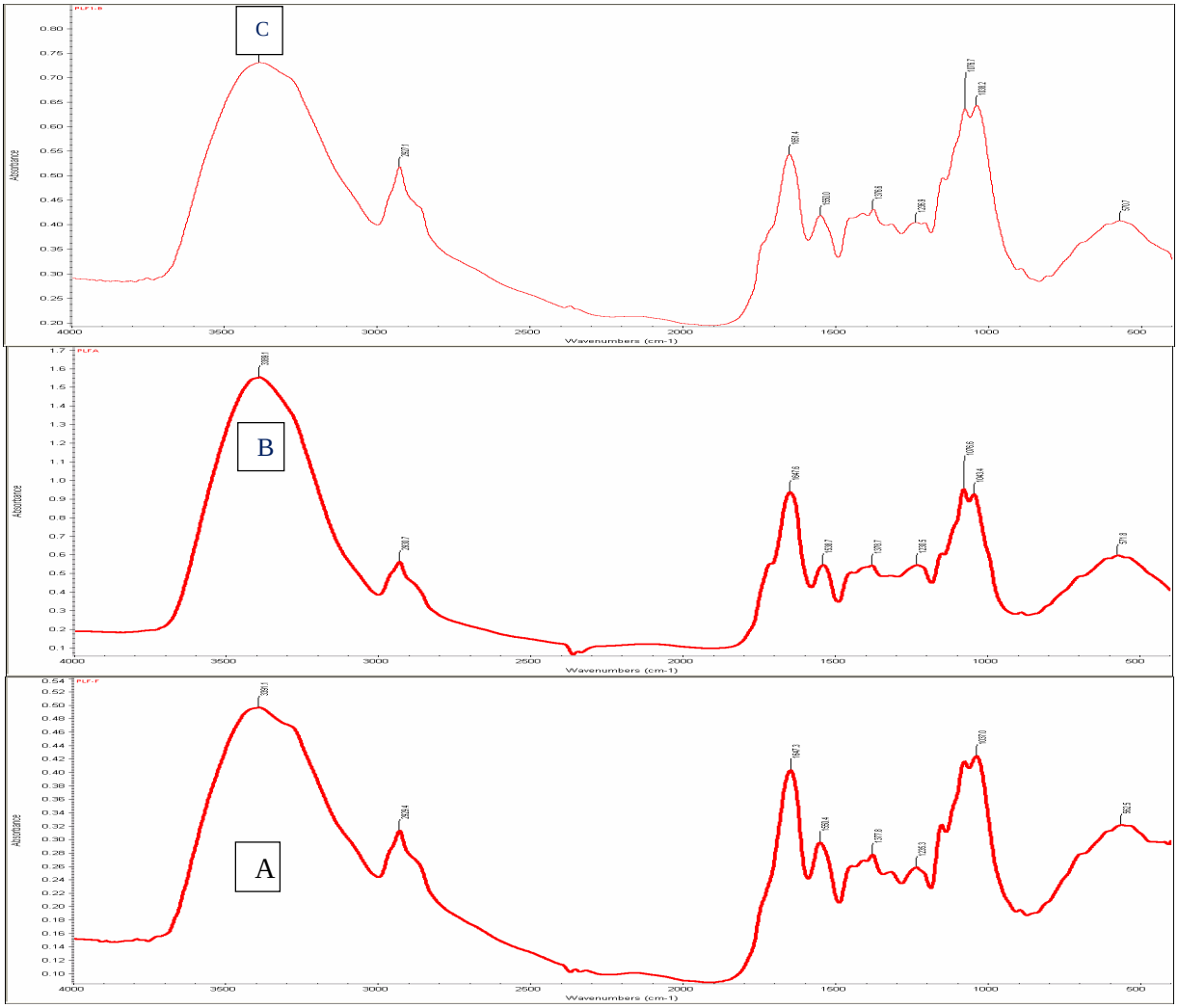


Fig.5 A- (Field soil *Penicillium*) PMS2 Control B- PMS2 with NaCl 10ppm and Cr 60ppm, C- PMS2 with Cr 60 ppm

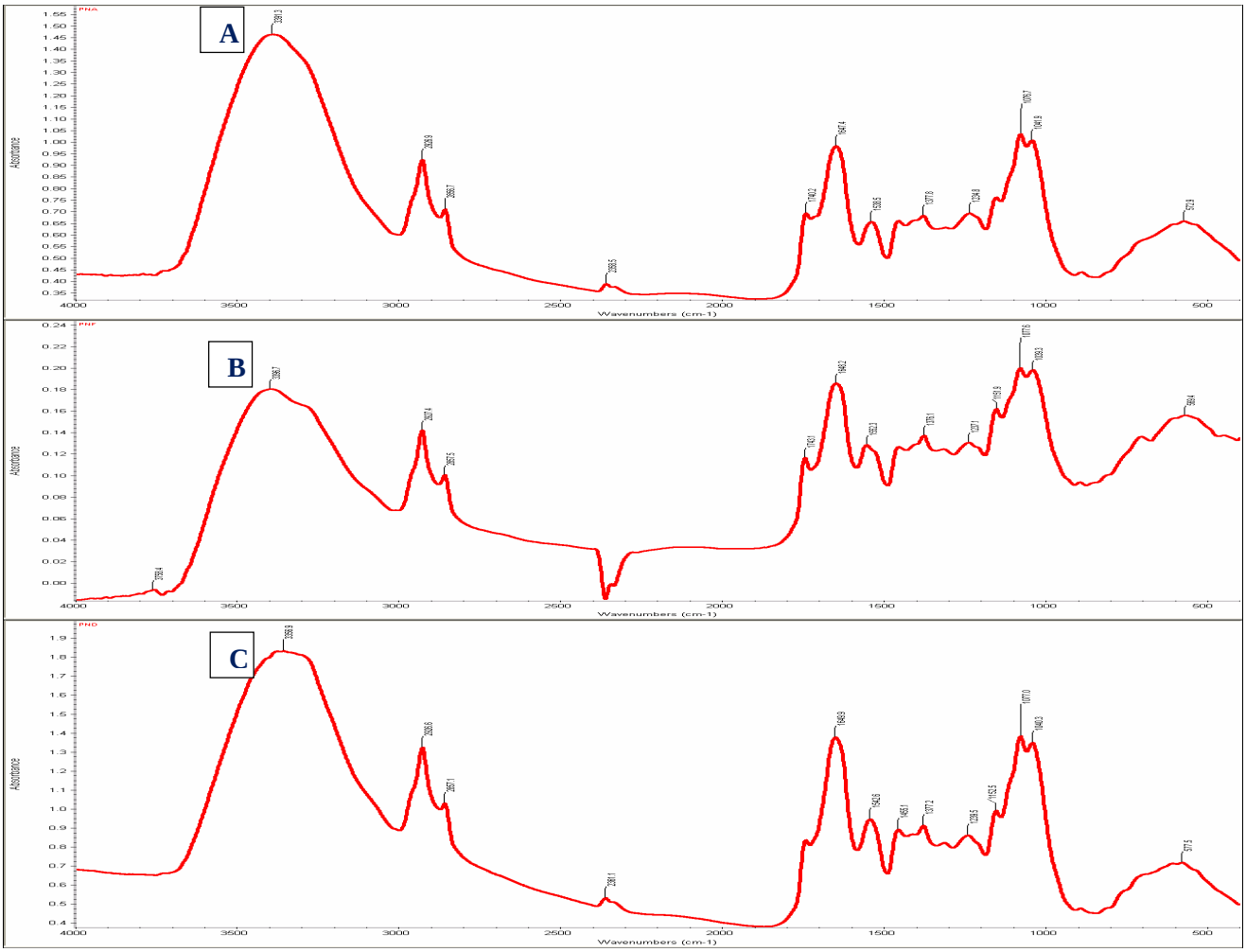


Fig.6 Scanning Electron Micrograph of Mesophilic *Penicillium* PMS2

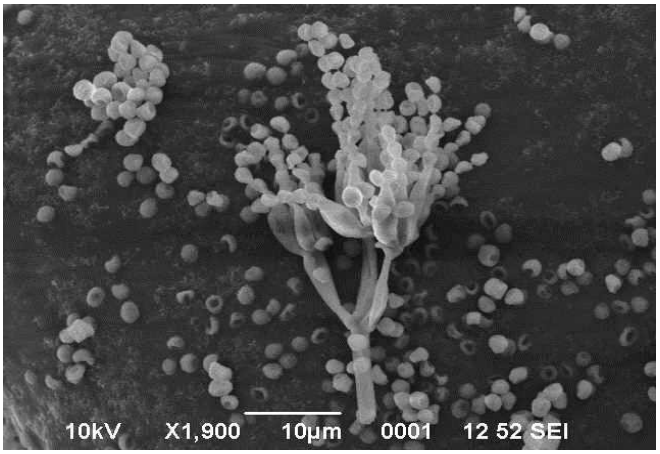
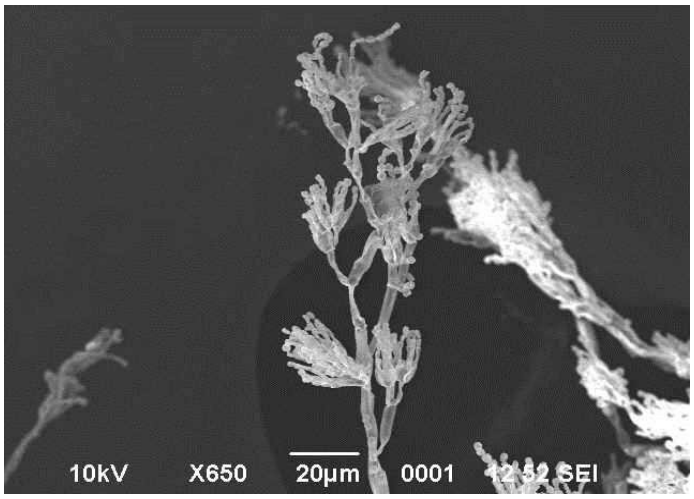


Fig.7 Scanning Electron Micrograph of Psychrophilic *Penicillium* PLF1



Biosorption of metals by microbial biomass is mainly based upon physicochemical interactions between metals and functional groups of the cell wall (Wu *et al.,* 2010).

To the best of our knowledge this is the first report on psychrotolerant Pangong Lake fungus PLF1 for the removal of Cr (VI) with comparison to other mesophilic soil fungi PMS2 in saline and non-saline conditions. Chromium contamination of the environment has become an important issue due to the potential health threat it poses. Conventional technologies to clean up heavy metal ions from contaminated waters have been utilized, but they remain cost-ineffective. Therefore, the use of such fungal biomass for the detoxification of Cr (VI) from metal contaminated sites may be a novel and cost-effective alternative. Data revealed that *Penicillium sp.* removed substantial amount of Chromium (.032 mg/g by PLF1) and (.046 mg/g by PMS2) in presence of 10% NaCl. This indicated the potential biosorption capacity of *Penicillium* to remove chromium metal in saline condition. The FTIR observation demonstrates the involvement of amino, carboxylic, phosphate, sulfonyl and carbonyl functional groups in Cr (VI) biosorption. It is concluded from this study that NaCl improved the chromium tolerance and bioaccumulation properties in *Penicillium sp.* and could be used as a potential organism for chromium remediation in high salinity environments.

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