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Full Length Research Paper

Comparative phytochemical and microbiological studies for oil extracts of Australian and Chinese garlic

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In this study the effect of distillation and solvent extraction methods were studied on many factors; oil percentage, physical and chemical properties, and percentage of decay of both Australian (white and red) and Chinese garlic. On the other hand, the antimicrobial effect of Australian and Chinese garlic plants was determined at various concentrations against *Staphylococcus aureus* ATCC®25923, *Pseudomonas aeruginosa* ATCC®9027, *Escherichia coli* ATCC®25922 and *Bacillus subtilis* ATCC®6633. Furthermore, the antimicrobial effect of Australian and Chinese garlic plants was compared with that of the antibiotics at definite minimum inhibitory concentrations. The highest percentage of extracted oil was obtained from the white Australian garlic followed by the red one. The solvent extraction method showed higher efficiency in oil extraction than the distillation method. Moreover, the white Australian garlic was found to have the best physical and chemical properties followed by the red one. It is worth mentioning that, the Chinese garlic has higher resistance to decay more than the red Australian garlic. Therefore, it is recommended to use the white Australian garlic due to its high oil content which has potential antibacterial activity and has the best physical and chemical properties, but it should not be stored to prevent loss on decay.

Key words: Antimicrobial activity, distillation, oil extraction, synergistic effect.

INTRODUCTION

Garlic was considered as one of the most important economic plants due to its efficient antimicrobial activity and contains powerful sulfur and other numerous phenolic compounds which have great interest (Rivlin, 2001; Griffiths et al., 2002). Moreover, FDA and WHO reported that the garlic plant had been recently extensively introduced in the treatment of many microbial infectious diseases as potential bactericidal (Gram-negative and Gram-positive) and fungicidal agent. Furthermore, it was considered a nutritive plant because it has sufficient content from essential mineral elements such as sulfur, which may be required as a cofactor for various enzymes which play important roles in the metabolism (Whitemore and Naidu, 2000). Moreover, allicin is a notable flavonoid in garlic forming with crushing (Ross et al. 2000). Han et al. (1995) reported that one mg of garlic allicin (allyl 2-propene thiosulfinate) has been equated to fifteen IU of penicillin. Garlic allicin exerted antibacterial activity against *Salmonella typhimurium*, primarily by interfering with RNA synthesis

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(Feldberg et al. 1988). Garlic also has been reported to produce various beneficial effects, including anti stress protection, growth promotion, appetite stimulation, immune stimulation and antimicrobial properties in fin fish and shrimp larviculture (Vaseeharan et al., 2011). Guo et al. (2012) investigated the *in vitro* antibacterial activity of garlic against *S. iniae* and the effect of garlic supplemented diets on growth and disease. Ruiz et al. (2010) evaluated in vitro the effects of two of these garlic derived compounds (PTS and PTS-O) on predominant faecal microbial populations of swine, and to determine the concentrations active against some of the most relevant populations of swine intestinal microbiota. Additionally, activity against *Escherichia coli* and *Salmonella typhimurium*, two common pathogens of pigs, was also tested. Karuppiah and Rajaram (2012) evaluated the antibacterial properties of *Allium sativum* (garlic) cloves and *Zingiber officinale* (ginger) rhizomes against multi drug resistant clinical pathogens causing nosocomial infection. Resistance in organge-spotted grouper challenged with *S. iniae*. Many investigations have also demonstrated an inhibitory effect of aqueous extracts on numerous bacterial and fungal species (Sivam et al., 1997; Hsieh et al., 2001; Ward et al., 2002). Garlic was used in China since many years ago as an efficient antibacterial agent against *Helicobacter pylori*. This pathogen led to an epidemic event where 241 Chinese adults were highly incident with gastric lesions, gastric dysplasia and gastric cancer (You et al., 2004). Koch and Lawson (1996) cited in their comprehensive book on garlic, at least 15 statistical studies and review articles that correlate garlic consumption to low cancer rates in Europe, Egypt, India, China, and other third world countries, which are favorable to garlic. Garlic also contains some sulphur-containing compounds such as alliin, ajoene, diallylsulphide, dithin, S-allylcysteine, enzymes and other non sulphur-containing compounds including vitamin B, proteins, minerals, saponins and flavonoids (Johnson et al. 2008). The root bulb of garlic has been used traditionally for thousands of years to treat many diseases because it has high concentrations of sulfur containing compounds (Tattelman, 2005). The elucidation of the chemical structures of these compounds has led to the synthesis and production of more potent and safer drugs (Bhattacharjee et al., 2005). The aim of this work is studying the antimicrobial activity and phytochemical properties of Australian and Chinese garlic plants such as oil content, physochemical properties, percentage of decay on storage.

MATERIALS AND METHODS

Extraction of crude oil from garlic using steam distillation method

Garlic pills were collected from the market. Two hundreds grams of garlic cloves were grounded in a mixer and placed in the distillation flask, 400 ml of distilled water were added and distillation was carried out for 4 h. The distillate was collected and dried over anhydrous sodium sulphate then filtered and kept in dark brown glass containers in the refrigerator at 2 to 5°C (Benkeblia, 2004).

Extraction of garlic oil using organic solvent method

The organic solvent was first distilled; garlic pills were then grounded into coarse particles using the mixer and soaked for 24 h in organic solvent (ethanol) in reflux condenser apparatus. The extract was dried over anhydrous sodium sulfate, and then filtered through Bunchner funnel using filter paper No. 52. The solvent was evaporated at 60°C (Bektas et al., 2005).

Determination of specific gravity of oil

The specific gravity of the oil was determined according to the USP 2006 using the pyknometer.

Determination of refraction index of the oil

The refraction index of the oil was determined according to USP 2006 using the refractometer digital ABBE.

Determination of Acid and Peroxide Values

The acid value of oil was determined according to USP 2006. The peroxide value of oil was determined according to the EP 2005.

Microorganisms

The bacterial cultures used in the present study are *Pseudomonas aeruginosa* ATCC®9027, *Bacillus subtilis* ATCC®6633, *Staphylococcus aureus* ATCC®25923 and *Escherichia coli* ATCC®25922.

Preparation of inoculums

A loopful of inoculum was taken from pure culture of the respective bacteria grown on slants and inoculated into 10 ml of nutrient broth. The broth suspension was then incubated at 37°C. The growth so obtained was inoculum for the sensitivity test.

Sensitivity of bacteria to garlic extract

The antibacterial activity of various extracts was tested by agar diffusion method. The plates containing Mueller Hinton agar were spread with 0.2 ml of bacterial inoculum (about 10^5 – 10^6 CFU/ml). Wells (8 mm diameter) were cut from agar plates using sterilized stainless steel borer and were filled with 0.1 ml of garlic extract. The plates were incubated at 37°C. The diameter of any resultant zone of inhibition was measured. Each combination of microorganism and antibiotic was repeated three times. Microorganism showing clear zone was considered to be inhibited.

Comparative sensitivity of bacteria to garlic extract and antibiotic

Comparative activity of different antibiotics and garlic extract on bacteria was assayed by agar diffusion method. The Muller Hinton

Table 1. Effect of extraction methods on garlic oil percentages and physicochemical properties.

Extraction method	Distillation			Solvent ethanol		
Garlic	Australian		Chinese	Australian		Chinese
	White	Red		White	Red	
Oil %	0.058	0.046	0.044	6.730	5.971	5.658
Specific gravity	1.0588	1.0547	1.0549	1.0545	1.0555	1.0528
Refractive index	1.4658	1.4669	1.4650	1.4712	1.4710	1.4705
Acid value	0.00	0.00	0.00	0.50	0.52	0.48
Peroxide value	0.08	0.05	0.05	0.30	0.33	0.41

agar plates were inoculated with 0.2 ml of bacterial standard commercial antibiotic discs (OXOID). Inoculated plates with antibiotic discs were incubated for 24 h and the diameter of any resultant zone of inhibition was measured. Each combination of microorganism and antibiotic was repeated three times. Interpretation of resistance was based on the National Committee for Clinical Laboratory Standards (1999) criteria. The antibiotics discs used were penicillin pen, 10 µg; amikacin AK, 30 µg; tetracycline TE, 30 µg; amoxicillin clavulanate AC, 30 µg; ciprocin CIP, 30 µg; erythrocin E, 10 µg; chloramphenicol C, 30 µg; piperacillin/tazobactam TZP, 110 µg.

RESULTS AND DISCUSSION

Garlic oil percentage

Data recorded in Table 1 showed that the percentage of oil differed according to the type of garlic; Australian (white and red), and the Chinese garlic. The oil percentage differed also according to the extraction method used (distillation and solvent extraction method), and it was obtained by the solvent extraction method in all garlic types, whereas the distillation method yielded the lowest oil percentage for all garlic types. It was concluded that the white Australian garlic yielded the highest oil percentage by either solvent extraction method (6.73%) or by distillation method (0.058%), whereas the Chinese garlic yielded the lowest oil percentage either by using the distillation method (0.044%) or by solvent extraction method (5.658%).

Physical properties

Specific gravity

The specific gravity of oil showed by determined especially during the transport or the storage of those oils in order to design the specific pipes and tanks. The specific gravity values of garlic oil extracted from different types of garlic by steam distillation and organic solvent as shown in Table 1. The highest specific gravity was 1.0588 for the white Australian garlic oil extracted by using the distillation method whereas the Chinese garlic oil extracted by solvent extraction method gave the lowest value of the specific gravity (1.0528).

Refractive index

Refractive index is an important physical parameter used for the identification of oils, fats and liquid wax, as it can be used to estimate the degree of their purity of oil. Results in Table 1 showed the effect of extracting method on the refractive index of garlic oil. It was found that the refractive index values differed according to the extraction method of garlic oil. The values were higher in case of using the solvent extraction method than those obtained by the distillation method. The highest value was (1.4712) for the Australian white garlic oil extracted by organic solvent, whereas the lowest refractive index was 1.4650 for Chinese garlic oil extracted by steam distillation.

Chemical properties

Acid value

Acid value is a parameter for the content of free fatty acids in oil and is used to detect the hydrolysis of fatty acids under the influence of lipase enzyme. Table 1 showed the effect of oil extracting method on the acid value of garlic oil. It was found that garlic oil extracted by distillation value has no acid value whereas the oil extracted by solvent method gave only low acid values it ranged from 0.48 to 0.52 for oil extracted from Chinese garlic and Australian red garlic respectively.

Peroxide value

Peroxide value is the quantity of active oxygen absorbed by one kilogram of oil; peroxide value of garlic was slightly affected by the extraction methods. Data in Table 1 showed that peroxide values ranged from 0.3 for oil extracted from Australian white garlic by ethanol to 0.41 for oil extracted by ethanol from Chinese garlic; but when

Table 2. Effect of storage on the decay percentage of garlic cloves.

Days	Australian garlic		Chinese garlic
	White	Red	
60	5.31	1.10	0.51
120	9.82	3.56	1.07
180	10.77	4.05	2.10
240	11.34	5.50	3.72

using the distillation method for extraction, the peroxide value was 0.08 for the oil extracted from white Australian garlic and 0.05 for the oil extracted from red Australian garlic, respectively.

Percentage of garlic decay

It was found that the Chinese garlic has a higher ability to resist decay which affects the cloves on storage followed by the red Australian garlic which was found to be more resistant than the white Australian garlic. The results in Table 2 showed that the percentage of decay after 60 days was 0.51% for the Chinese garlic, whereas it was 1.1% for the red Australian garlic, then the white Australian garlic in which the percentage of decay was 5.31%. There was also a relation between the percentage of decay and the duration of storage, the percentage of decay increased after 240 days of storage to be 3.72% for the Chinese garlic, 5.5% for the red Australian garlic and 11.34% for the white Australian garlic.

Sensitivity of bacteria to different concentrations of garlic extracts

The result of our present study shows that the antimicrobial activity of both of garlic (Chinese and Australian) extracts exhibited different inhibition levels against four medically important pathogens; *P. aeruginosa* ATCC®9027*, B. subtilis* ATCC®6633*, S. aureus* ATCC®25923 and *E. coli* ATCC®25922, as shown in Table 3. In dose response study, the inhibition zone increased with increasing concentration of extracts. Low concentration (50 – 100 ml/L) inhibited weakly the growth of bacteria and this result agreed with previous study (Benkeblia, 2004). At high concentrations (200, 300, and 500 ml/L) of garlic extract exhibited significant inhibition activity against bacteria and the inhibition of Chinese extract was more than Australian extract.

The result of our present study shows that, the inhibition of Chinese extract of garlic was more than those of Australian garlic extracts. Comparatively, *S. aureus* ATCC®25923 was less sensitive to the inhibitory activity of garlic extracts than *E. coli* ATCC®25922 which were more inhibited at same concentrations of Chinese extracts as shown in Table 4. *E. coli* ATCC®25922 > *B. subtilis* ATCC®6633 > *P. aeruginosa* ATCC®9027 > *S. aureus* ATCC®25923. Kyung et al. (2002) reported that, allicin of garlic extract showed strong antibacterial activity against *S. aureus* ATCC®25923 at 150 ml/L concentration. The antibacterial activity of other and close, chemically cysteine sulfoxide (S-methyl-1-CS and methyl methane-CS) of cabbage also was markedly observed, particularly concentrations of 10, 20 and 50 mg/L (Kyung et al. 1997). In another study, 1% of oregano essential oil incorporated into a calcium caseinate WPI-carboxymethyl cellulose film was found to be effective against *E. coli* O157-H7 and *Pseudomonas* spp on the surface of beef muscle pieces (Oussallah et al. 2004). Previous studies on antimicrobial activity of garlic essential oils in culture media show some inhibitory effect on certain pathogen bacteria (O'Gara et al. 2000).

The mode of action of carvacrol was explained by Burt (2004) that it disintegrates the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP. The result of our present study indicates the antimicrobial activity of two kinds of garlic extract in culture media has shown some inhibitory effect in certain order *S. aureus* ATCC®25923 > *B. subtilis* ATCC®6633 > *E. coli* ATCC®25922 > *P. aeruginosa* ATCC®9027. Alrozeky and Nakahara (2002) reported weak antibacterial activity of extracts from some edible plants commonly consumed in Asia. Combined extracts of *Corni fructus*, cinnamon and Chinese chive (1:6:6, vol/vol/vol) exhibited low inhibitory effect against this bacteria than other combined ratios and against other bacterial species (Hsieh et al. 2001).

Comparative sensitivity of bacteria to garlic extracts and antibiotics

In the present study some bacteria showing resistance to certain antibiotic were sensitive to extract of garlic oil especially Chinese one. *S. aureus* ATCC®25923 was resistant to the antibiotics; penicillin, erthrocin, chloramphenicol, and tetracycline while sensitive to extract of garlic oil. On the other hand, *E. coli* ATCC®25922 was resistant to penicillin, erthrocin, and piperacillin/tazobactam. *B. subtilis* ATCC®6633 was resistant to only two antibiotics Amoxicillin/clavulanate and penicillin. The multi-antibiotics resistant *P. aeruginosa* ATCC®9027 was sensitive to garlic extract as shown in Table 5. It is known that the multi-antibiotic resistant bacteria can cause nosocomial infection which needs not only expensive antibiotic to treatment but also to development of agents with marked antibacterial activity and greater sensitivity and less toxicity. In the present study garlic was more effective in inhibiting multi-antibiotic resistant bacteria when compared with the

Table 3. Sensitivity of bacteria to different concentrations of garlic extract.

Bacteria	Control	Antibacterial activity of Chinese garlic (mm)					Antibacterial activity of Australian garlic (mm)				
		50	100	200	300	500	50	100	200	300	500
P. aeruginosa ATCC®9027	18	15	16	18	19	21	13	13	16	17	20
B. subtilis ATCC®6633	18	18	21	22	23	25	15	17	18	19	22
S. aureus ATCC®25923	18	18	15	17	18	19	14	13	16	17	18
E. coli ATCC®25922	18	18	22	24	26	29	15	17	21	25	28

Table 4. Sensitivity of bacteria into two types of garlic extract.

Bacteria	Antibacterial activity of garlic extracts (mm)					
	C.O.S.	A.R.O.S.	A.W.O.S.	C.W.E.	A.R.W.E.	A.W.W.E.
P. aeruginosa ATCC®9027	28	30	22	28	27	23
B. subtilis ATCC®6633	35	30	22	26	25	24
S. aureus ATCC®25923	36	24	22	25	30	23
E. coli ATCC®25922	27	32	24	28	26	23

C.O.S. = Chinese organic solvent, A.R.O.S. = Australian red organic solvent, A.W.O.S. = Australian white organic solvent, C.W.E. = Chinese water extract, A.R.W.E. = Australian red water extract, A.W.W.E. = Australian white water extract.

Table 5. Sensitivity of bacteria to garlic extracts and antibiotics.

Bacteria	Antibacterial activity of garlic extracts and antibiotics (mm)									
	Pen 10 µg	AK 30 µg	TE 30 µg	AC 30 µg	CIP 30 µg	E 10 µg	C µg 30	TZP 110 µg	C.G. 500 ml/L	A.R.G. 500 ml/L
P. aeruginosa ATCC®9027	None	25	None	None	15	None	None	25	21	20
B. subtilis ATCC®6633	None	30	23	None	25	25	35	28	25	22
S. aureus ATCC®25923	None	28	None	13	35	None	None	30	19	18
E. coli ATCC®25922	None	18	28	23	27	None	25	None	29	28

None = No inhibition zone, Pen = Penicillin, AK = Amikacin, TE = Tetracycline, AC = Amoxicillin/Clavulanate, CIP = Ciprocin, E = Erthrocin, C = Chloramphenicol, TZP = Piperacillin/Tazobactam, C.G. = Chinese Garlic, A.R.G. = Australian Red Garlic.

tested antibiotic. O'Gara et al. (2000) indicated a relationship between lower MICs and number of sulfur atoms/molecules for diallyl sulphides against *H. pylori* and suggested that the number of sulphur atoms/molecules and/or disulphide bonds in the diallyl sulphides was an important factor in determining their antimicrobial activity. Garlic oil and its component possessed in vitro antibacterial activity against multi-antibiotic resistant *P. aeruginosa* and *K. pneumoniae*. Both additive and synergic effects were observed in combinations of ceftzidine, gentamicin, imipenem and meropenem with garlic sulphide agent, therefore, garlic oil and both sulphides DAT and DATS may have the potential to prevent or treat nosocomial infections caused by *P. aeruginosa* and *K. pneumoniae* (Tsao and Yin, 2001).

Conclusion

White Australian garlic is a best type to use due to its high oil content which has potential antibacterial activity

and it has the best physical and chemical properties, but it should not be stored to prevent loss on decay. However, the Chinese garlic has higher resistance to decay more than the red Australian garlic.

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Characterization of heavy metal (cadmium and nickle) tolerant Gram negative enteric bacteria from polluted Yamuna River, Delhi

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The presence of heavy metals in aquatic environments poses a serious environmental risk. The usage of heavy metal-tolerant bacteria may serve as a cost-effective tool for bioremediation of polluted water bodies. Thus, the present study aims at the isolation and characterization of heavy metal tolerant gram-negative bacteria collected from the polluted water of the river Yamuna, Delhi, India. The water samples were collected from the downstream river and enriched separately in the nutrient broth. Appropriate dilutions were then plated in eosin methylene blue (EMB) agar supplemented with cadmium (10 to 4000 µg/ml) and nickel (10 to 3000 µg/ml). The colonies having different morphologies that were formed in the EMB agar were selected, and their metal tolerance concentration (MTC) was determined. Based on their MTC values, two isolates were identified by using morphological, biochemical, and molecular (16S rRNA gene sequencing) methods. Besides, their growth kinetics, co-metal tolerance, and antibiotic resistance were also determined. The identified isolates 2 and 8 were tolerant to cadmium (3000 µg/ml) and nickle (2000 µg/ml), respectively. The isolates were found to be closely related to *Pantoea agglomerans* JCM 1236 (sample 2) and *Enterobacter asburiae* JCM 6051 (sample 8). They were not only co-tolerant to cadmium (3000 µg/ml) and nickle (2000 µg/ml) but also showed resistance to various antibiotics. Scanning electron microscopy (SEM) showed changes in surface morphology of nickle and cadmium-treated samples 2 and 8, and the energy dispersive X-ray spectroscopy (EDX) studies revealed the presence of cadmium and nickel in both the isolates after metal treatment. The results conclude that the identified heavy metal tolerant bacteria could be useful for the bioremediation of contaminated wastewater and industrial effluents.

Key words: Heavy metal removal, gram-negative bacteria, 16S rRNA gene sequencing, maximum tolerance concentration, bioremediation.

INTRODUCTION

The Yamuna river is one of the most polluted rivers in India due to the presence of high concentrations of toxic heavy metals (Ahmad, 2009; Misra, 2010; Sehgal et al., 2012; Kaur and Mehra, 2012). Anthropogenic and industrial activities add non-biodegradable environment pollutants like heavy metals, such as lead (Pb), chromium

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(Cr), cadmium (Cadmium), mercury (Hg), iron (Fe), cobalt (Co), and nickel (Ni), and their derivatives into the water bodies (Islam et al., 2007). In developing countries, rapid and unorganized industrialization and urbanization have also contributed to the elevated levels of heavy metals in the water bodies (Sharma et al., 2008). In India, most of the industries are situated along the river banks for the easy disposal of the effluents that contain heavy metals (Kaushik et al., 2009; Malik et al., 2014). The industrial waste dump grounds present in the vicinity of rivers also add to the heavy load of toxic metal ions, organic ions, organic wastes, and antibiotics in the rivers (Mohiuddin et al., 2011; Haq et al*.,* 1999).

Cadmium and nickel are among the most widespread contaminants in the aquatic environment (Kaushik et al., 2001) and they can cause serious health problems to all organism. Human consumption of such contaminated water is extremely dangerous as these heavy metals have carcinogenic and mutagenic effects. Cadmium can affect the kidney, causing renal dysfunction, especially in the proximal tubular cells as it is the main site of cadmium accumulation. It can also cause bone demineralization, either directly by damaging the bones or indirectly as a result of renal dysfunction (Blessy and Krishnamurthy, 2015; Bernard, 2008). Moreover, Ni is also known for its hematotoxic, immunotoxic, neurotoxic, genotoxic, reproductive toxic, pulmonary toxic, nephrotoxic, hepatotoxic, and carcinogenic effects (Duda-Chodak and Blaszczyk, 2008).

Stainless steel manufacturing units, electroplating factories, etc. discharge Ni into the river. Whereas, industrial processes such as electroplating of iron metals, preparation of Cadmium-Ni batteries, control rods and shields within nuclear reactors, and preparation of television phosphors discharge cadmium into the river (Singh, 2014). Therefore, it is important to remove the heavy metals from industrial effluents before they are discharged into the rivers. Chemical methods, including reverse osmosis, electrodialysis, ultrafiltration, ion-exchange, phytoremediation, etc., used for heavy metal removal from the wastewater/industrial effluents are expensive and inefficient (Ahalya et al., 2003). Many authors have screened and studied different micro-organisms, such as bacteria, fungi, and algae, over the past decades to identify their highly efficient metal removal biological systems (Vijayaraghavan and Yun, 2008). These microorganisms can grow in the presence of high concentrations of heavy metals and have a great potential for bioremediation of heavy metals discharged in industrial effluents (Ansari and Malik, 2007).

Previous studies have well documented the occurrence and abundance of metal-tolerant microbes in metal-polluted water bodies (Abyar et al., 2012; Jankowska et al. 2006; Wong et al. 2003). Bacterial species such as *Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis* and *Staphylococcus sp.* isolated from wastewater samples were found to be resistant to heavy metals and antibiotics (Filali et al., 2000). Similarly, cadmium-resistant *Klebsiella* and *Enterobacter* species and nickel resistant *Actinomycetes* and other bacteria have also been isolated from industrial effluents and soil samples (Alboghobeish et al., 2014; Karakagh et al., 2012). These microorganisms develop a variety of resistance mechanisms to survive in different heavy metal concentrations, but the resistance is often specific to one or few metals (Mejare and Bulow, 2001; Nies, 2003; Piddock, 2006). These heavy metal tolerant bacterial species may serve as an important and cost-effective bioremediation tool for the removal of heavy metals from wastewater and industrial effluents, preventing the contamination of water bodies (Jan et al., 2014). Thus, the present study was envisaged to isolate and characterize heavy metal tolerant gram-negative bacteria from the water samples collected from the Yamuna river, Delhi, India.

MATERIALS AND METHODS

Sample collection

Water samples were collected from the Yamuna river from three different sites: a) Sarita vihar barrage, b) Income tax office (ITO) barrage, and c) Okhala barrage. The samples were collected in sterile plastic bottles following standard sampling techniques and sent to the laboratory for further processing. Sampling bottles were treated with 1% nitric acid followed by rinsing with deionized water three times. After washing, bottles were sterilized by autoclaving and dried. At first, the collected samples were added to the nutrient broth (250 ml) and incubated overnight at 37°C. Serial dilution and spread plating method was used to isolate the colonies. Appropriate dilutions were plated in eosin methylene blue (EMB) agar (Hi Media, India) supplemented with cadmium (10 to 4000 µg/ml) and nickle (10 to 4000 µg/ml). The colonies with different morphologies formed on EMB agar plates were further studied. For long-term preservation and maintenance, the microbial cultures were stored as 15% glycerol stocks in airtight vials at −80°C.

Determination of maximum tolerance concentration (MTC) of heavy metals

After preliminary selection of cadmium and nickle tolerant isolates the MTC of Cadmium and Ni was determined. For the quantitative determination of heavy metal tolerance, the isolated strains were inoculated in EMB broth supplemented with increasing concentrations of cadmium and nickle (10 to 5000 µg/ml). The broth tubes were incubated at 37°C for 48 h in an incubator shaker (150 rpm). The highest concentration of metal ions at which bacterial growth was observed (as indicated by optical density, OD, at 600 nm) and was defined as the maximum tolerance concentration (MTC) of the respective heavy metal for the isolated strain (Schmidt and Schlegel, 1994). EMB broth without the metal ions was also inoculated with the isolated strains to serve as controls. All experiments were performed in triplicates.

Identification of the bacterial isolates

Two isolates, samples 2 and 8 were selected on the basis of their MTC values for further identification and characterization. The

isolates were characterized morphologically (Gram staining). Biochemical characteristic like indole test, MR-VP, urease, catalase, citrate were also taken into consideration for characterization of these isolates (Cappucino and Sherman, 2002).

16S rRNA gene amplification

After biochemical characterization, isolates were identified on the molecular basis using 16S rDNA sequencing for the accurate identification of bacterial isolates. First, the genomic DNA was extracted from the bacterial culture by Triton prep Method. The partial 16S rDNA was amplified by using the universal primers, 27 F (5'- AGA GTT TGA TCC TGG CTC AG – 3') and 1492R (5'- CGGTTACCTTGTTACGACTT- 3'). Polymerase chain reaction (PCR) was performed using thermal cycler (Applied Biosystem, USA) with 50 µl reaction mixture containing 1 µl (10 ng) of DNA extract as a template, each primer with a concentration of 10 picomole/µl, 25 mM $MgCl_2$, and 2 mM dNTPs along with 1.5U of *Taq* polymerase and buffer as per the manufacturer's recommendations (GCC Biotech, Kolkata, India). The initial denaturation was performed at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 1 min, extension at 72°C for 1 min and 30 s, and final extension at 72°C for 10 min. The PCR products were analyzed by 1.5% w/v agarose gel electrophoresis (1× TAE) buffer with ethidium bromide (0.5 µg/ml).

Molecular characterization

The PCR product was sequenced by capillary sequencing method using ABi 3500 Genetic Analyzer machine as per the manufacturer's instructions. The 16S rRNA gene sequences were compared with the known bacterial 16S rRNA gene sequences using NCBI ADVANCED BLAST so as to identify the most similar sequence alignment (Altschul et al., 1997). Based on the scoring index, the most similar sequences were aligned with the sequences of other representative bacterial 16S rDNA regions by using multiple alignment software program, ClustalW (Thompson et al., 1994). A phylogenetic tree and a similarity index were generated and compared with the known sequences. The phylogenetic tree was constructed using the UPGMA method, and the tree reliability was tested by Bootstrap method using MEGA version 4 (Tamura et al., 2007).

Determination of co-metal tolerance

The bacterial isolate tolerant to one heavy metal was tested for its tolerance to other heavy metals included in the study. The cadmium-tolerant bacterial isolate (sample 2) was inoculated in EMB broth supplemented with nickle (10 to 4000 µg/ml). The tubes were incubated at 37°C for 48 h. Similarly, the nickle-tolerant strain (sample 8) was grown on Cadmium supplemented broth (10 to 4000 µg/ml). The cometal tolerance was confirmed by agar plate grid method. Series of EMB agar plates supplemented with various concentration of cadmium (10 to 4000 µg/ml) and nickle (10 to 4000 µg/ml) were made. A grid of 5 × 5 was made on each plate and each box was numbered. Selected isolates were streaked in same numbered box on each plate. Plates were incubated at 37°C for 48 h. The isolates that grew on both cadmium and nickle supplemented plated were selected and the maximum metal concentration of each metal on which they were also noted.

Effect of heavy metals on bacterial growth

The bacterial isolates were grown in 20 ml nutrient broth (HiMedia) and incubated overnight at 37°C in an incubator shaker (150 rpm) for preparing the inoculum. One ml of this culture was further inoculated into 50 ml nutrient broth containing 0 (control) to 3000 µg/ml of cadmium and 0 (control) to 2000 µg/ml of nickle. The flasks were then incubated at 37°C in an incubator shaker. The bacterial growth was monitored at specific time intervals by recording absorbance (OD) at 600 nm. All the experiments were performed in triplicates, and the results are represented as average values. Analysis of the variance was performed using the statistical software GraphPad Prism (version 4.00 for Windows, GraphPad Software, Inc., San Diego, CA) to compare the growth curves at different metal concentrations.

Determination of antibiotic sensitivity and resistance pattern of selected isolates

Antibiotic sensitivity and resistance pattern of the bacterial isolates were determined by the single-disk diffusion method (Bauer et al., 1966) using the following antibiotics: Rifampicin (10 µg/ml), Ofloxacin (10 µg/ml), Ciprofloxacin (10 µg/ml), Ceftriaxone (30 µg/ml), Levofloxacin (10 µg/ml), Trimethoprim (30 µg/ml), Augmentin (30 µg/ml), Cefpodoxime (10 mcg), Cefixime (30 mcg), Vancomycin (30 mcg), Ampicillin (10 mcg), Streptomycin (25 mcg), Amoxicillin (20 mcg), Imipenem (10 mcg), Ticarcillin-clavulanic acid (10 mcg), Aztreonam (30 mcg), and Gentamicin (10 mcg). The inhibition zone was recorded after 20 h of incubation at 37°C. The organism was classified as sensitive or resistant according to the diameter of inhibition zone given in standard antibiotic disc chart (Baeur et al., 1966).

Effect of metal (Ni and Cadmium) on Morphology of bacteria:

In order to determine the effect of metals on the morphology, the metal-treated and untreated bacterial cells were analyzed by scanning electron microscope (SEM). The samples 2 and 8 were grown in nutrient broth containing cadmium (3000 µg/ml) and nickle (2000 µg/ml) in separate flasks for 24 h at 37°C. The bacterial cultures grown without the metal in a separate flask were taken as normal control. Post growth, cells from treated and control were harvested at a density of 10^8 to 10^9 cells ml. An aliquot of each culture was centrifuged at 5000 rpm for 5 min. The supernatant was discarded, and the pellet was washed with phosphate buffer saline. The samples were fixed with 2.5% glutaraldehyde for 1 h at 37°C and pelleted again and then washed thrice with de-ionized water. The samples were then dehydrated by suspending in a series of acetone and alcohol (30 to 100%) solution for 5 min each and were kept in 100% alcohol each for 1 h. Then, each sample was centrifuged and dried and spread on a small glass slide followed by its coating with a very thin layer of gold alloy. The morphology of prepared specimen was analyzed by using SEM, and the metal concentration was determined by energy dispersive spectrometer (EDS) (Zeiss, operating voltage 15 kV).

RESULTS

Screening, isolation, and MTC of bacteria

The main goal of this study was to isolate and characterize cadmium and nickel tolerant gram negative bacteria from different sites of polluted Yamuna river. For this purpose, 50 isolates were selected on the basis of color, size and morphology from EMB plates. Further screening was done on the basis of maximum metal

Table 1. Morphological and biochemical characterization of the two selected bacterial isolates.

S.No.	Identification	Sample 2	Sample 8
1	Colony Characteristics	Pink colored, smooth, glistening colonies	Pink colored, smooth, glistening colonies
2	Gram staining	Gram negative Rods	Gram negative Rods
3	Motility	+	-
4	Indole production	-	-
5	Methyl Red	-	+
6	Vogus Proskauer Test	+	-
7	Citrate Utilization	+	+
8	Urease Production	-	-
9	Catalase test	+	+

tolerance for cadmium and nickel, a total of 18 bacterial isolates tolerant to high concentration of cadmium tolerant and 12 to high concentration of nickel were selected. The two isolates (samples 2 and 8) were then selected for subsequent research based on the MTC and co metal tolerance of the metals selected. The results of the morphological and biochemical test revealed that two bacterial isolates were gram negative rods. Sample 2 showed negative response to indole production, methyl red and urease production but positive response to Vogus Proskauer test, citrate utilization and catalase test. Sample rod showing was also gram negative rods showing negative response to indole production, Vogus Proskauer test and urease test and positive response to methyl red, citrate utilization and catalase test (Table 1).

Molecular characterization

The partially amplified and sequenced 16S mRNA gene was analyzed for sequence homology by using BLAST. The partial nucleotide sequence of sample 2 revealed 99% similarity with *Pantoea agglomerans* JCM1 and of sample 8 with *Enterobacter asburiae* JCM 6051. Phylogenetic analysis characterized them within the gram-negative bacteria. The phylogenetic trees derived from 16S rRNA sequence data of the two samples with other related species are represented in Figures 1 and 2, respectively. The partial sequences of samples 2 and 8 were submitted to GenBank NCBI database under the accession numbers KP410394 and KP233878, respectively (Table 2).

Co-metal tolerance

The cadmium-tolerant bacterial isolate sample 2 demonstrated bacterial growths in nutrient broth supplemented with Ni, with an MTC of 2000 µg/ml. Similarly, the nickle-tolerant sample 8 showed MTC for cadmium at 3000 µg/ml. Co metal tolerance of the two metals was verified by grid method on EMB plate supplemented with various concentration of cadmium and nickel (10 to 5000 µg/ml).

Growth studies of isolated bacteria

The growth curve of samples 2 and 8 in the presence of increasing concentrations of cadmium and nickle (0 to 3000 µg/ml) are depicted in Figures 3 and 4, respectively. The growth curves of both samples exhibited similar phases in the presence of heavy metals. However, the growth rate was retarded in the presence of heavy metal ions relative to control (ANOVA, $p < 0.05$).

Antibiotic resistance

The resistance of cadmium and nickle tolerant bacteria towards popular antibiotics was estimated to investigate the relationship between metal resistant and antibiotic resistance. Antibiotic susceptibility of these for carbapenem group of antibiotics was also screened to ascertain the emergence of resistant varieties to these antibiotics. Both the isolates showed resistance to antibiotics, *viz.* vancomycin, augmentin, cefpodoxime, and rifampicin. Moreover, sample 8 also demonstrated resistance to aztreonam. The results of antibiotic susceptibility testing of the two isolates are presented in Table 3.

Effect of metals (Cadmium and Nickle) on morphology of bacteria

The effect of metals on cell morphology was demonstrated through the SEM analysis. Distinct changes were observed in the bacterial cell shape and surface features. There were some sticky appearances evident from the overlapping of bacterial cells with each other. Some cells even showed rough surface structure and blister-like protrusions. SEM photomicrographs of samples 2 and 8 cultured with and without heavy metals (at respective

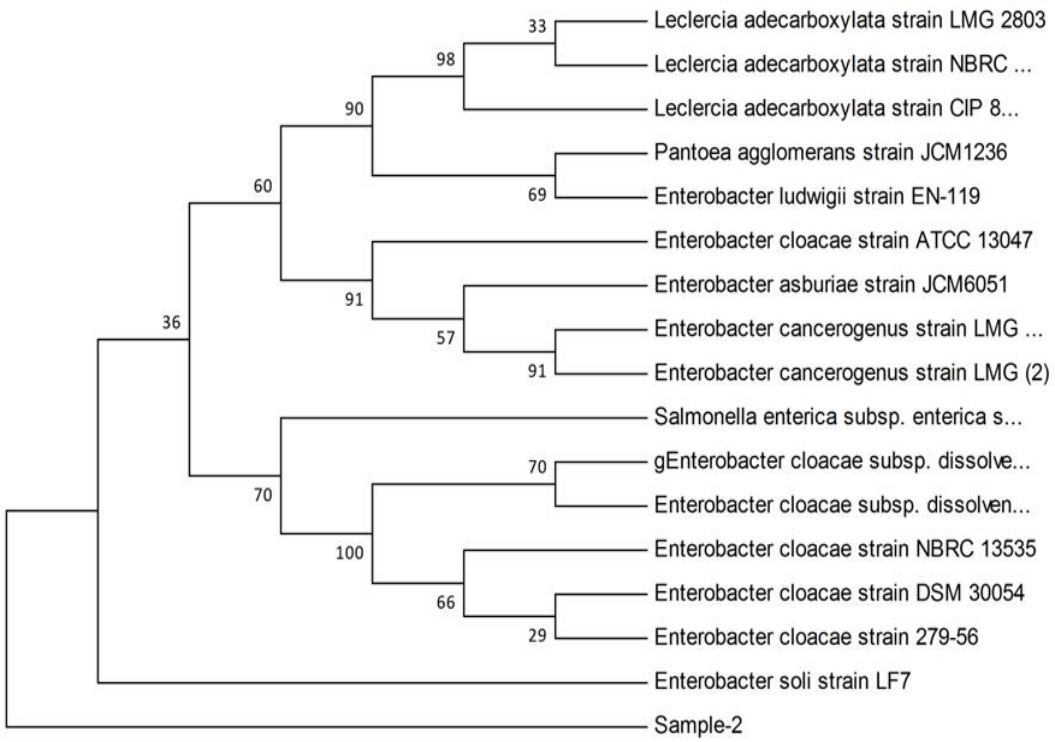

Figure 1. Phylogenetic relationship of sample 2 and closely related sequences based on partial 16S rRNA gene sequence. The phylogeny for the 16S bacterial identification data was constructed using the UPGMA method, and the tree reliability was tested by Bootstrap method for the Test of Phylogeny option in MEGA 4 software.

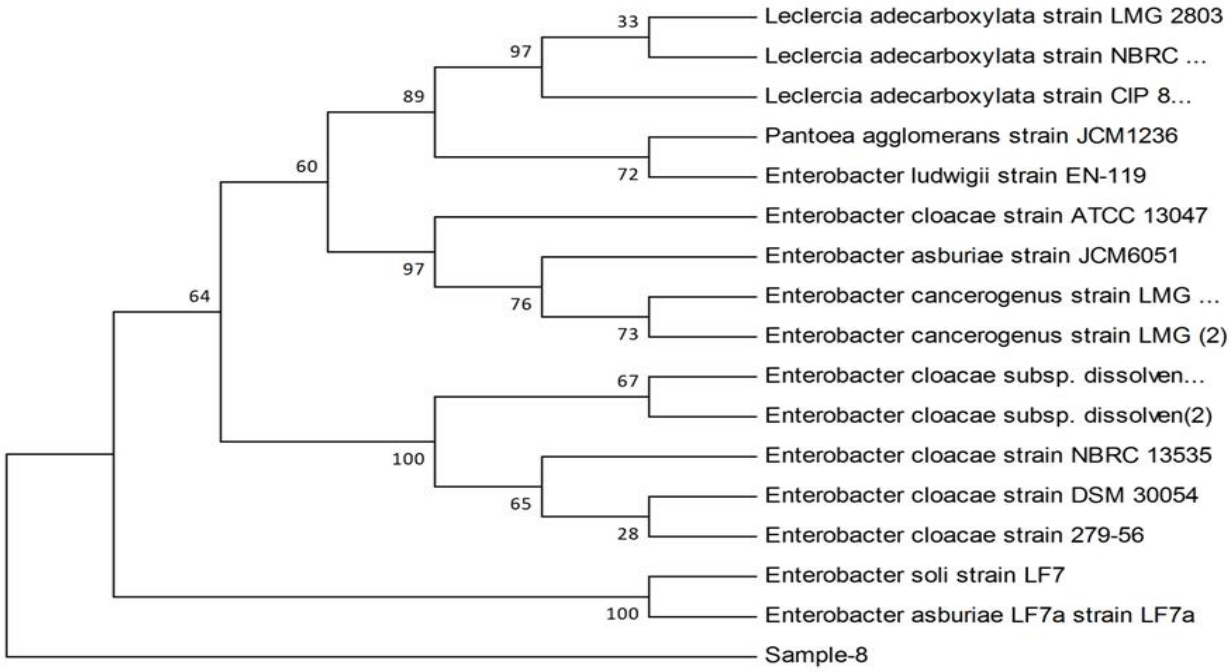

Figure 2. Phylogenetic relationship of sample 8 and closely related sequences based on the partial 16S rRNA gene sequence. The phylogeny for the 16S bacterial identification data was constructed using the UPGMA method, and the tree reliability was tested by Bootstrap method for the Test of Phylogeny option in MEGA 4 software.

Table 2. Molecular characterization of the bacterial isolates.

S.No.	Isolate	Organism Identified	Universal Primers Used	Identiity %	Accession No
1	Sample 2	*Pontoea agglomerans*	27 F (5 '- AGA GTT TGA TCCTGG CTC AG – 3') and 1492R (5'-GTTACCTTGTTACGACTT-3').	99% to *Pontoea agglomerans* JCM1236	KP410394
2	Sample 8	*Enterobacter asburiae*	27F (5 '- AGA GTT TGA TCCTGG CTC AG – 3') and 1492R (5'-GGTTACCTTGTTACGACTT- 3').	99% to *Enterobacter asburiae* JCM6051	KP233878

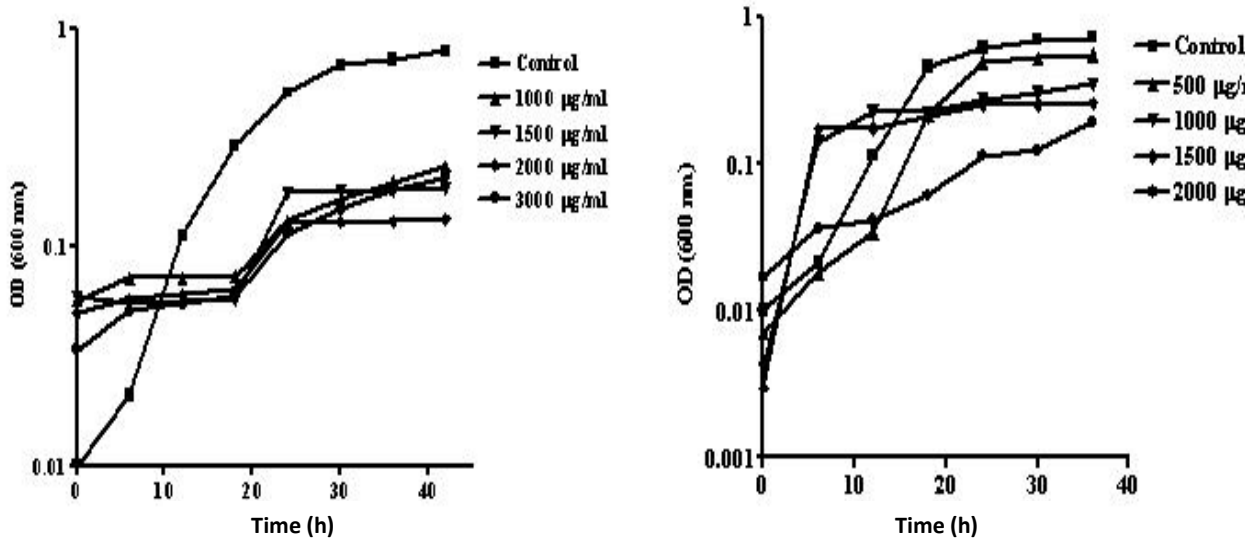

Figure 3. Growth pattern of the isolate sample 2 in the presence of a) Cadmium (1000 to 3000 µg/ml) and b) Ni (500 to 2000 µg/ml); control: Isolate grown in media without any metal supplementation. Anova was performed using GraphPad Prism (version 4).

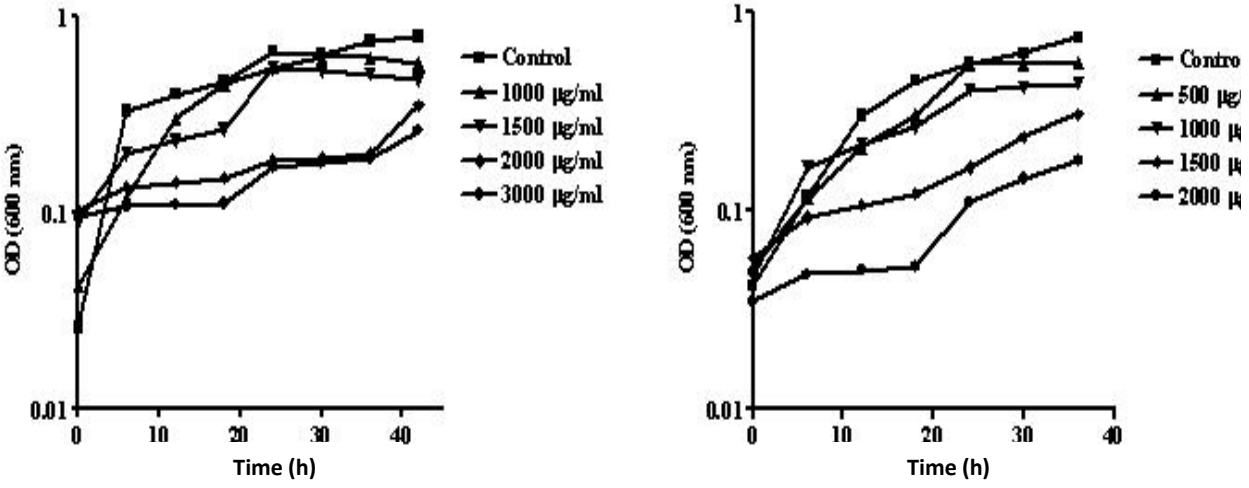

Figure 4. Growth pattern of the isolate sample 8 in the presence of a) Cadmium (1000 to 3000 µg/ml) and b) Ni (500 to 2000 µg/ml); control: Isolate grown in media without any metal supplementation. Anova was performed using GraphPad Prism (version 4).

Table 3. Antibiotic susceptibility of the two bacterial isolates.

S.No.	Antibiotics (mcg)	Zone of Inhibition (mm)	
		Sample 2	Sample 8
1	Ciprofloxacin (5)	34	34
2	Ceftriaxone (30)	26	32
3	Levofloxacin (5)	31	31
4	Ofloxacin (5)	34	31
5	Trimethoprin (1.25)	33	29
6	Rifampicin (15)	Nil	7
7	Augmentin (30)	9	Nil
8	Cefpodoxime (10)	8	8
9	Cefixime (30)	24.5	26
10	Vancomycin (30)	Nil	Nil
11	Ampicillin (10)	Nil	Nil
12	Streptomycin (25)	31	36
13	Amoxicillin (20)	13	16
14	Imipenem-EDTA (10)	24	21
15	Ticarcillin-clavulanic acid (10)	12	19
16	Aztreonam (30)	24	10
17	Gentamicin (10)	17	18

MTC values) are presented in Figure 5(a to f). It was revealed that both the bacterial isolates were loosely bound rod shaped with an average length of 1.878 ± 0.02 and 2.779 ± 0.18 µm, respectively under standard conditions. After growing in nickle (2000 µg/ml)-supplemented media, the sample 2 cells showed rods of 1.7 ± 0.288 µm length with a rough surface, and sample 8 showed rods of 2 ± 0.33 µm dimension. The nickle-treated cells also appeared in aggregates due to some extra polysaccharide secretions (EPS). Cadmium-exposed (3000 µg/ml) cells of sample 2 showed morphological changes, that is, small rods of dimension 1.517 ± 0.227 µm. Cadmium-treated (3000 µg/ml) cells of sample 8 also became smaller, that is, the rods were of 1.82 ± 0.35 µg/ml dimension, showing secretion of EPS around them. EDS analysis detected 4 ± 0.05% cadmium and 9.5 ± 1.01% Ni in metal treated sample 2. While cadmium and nickle-treated sample 8 showed a presence of 5.08 ± 0.88% cadmium and 7.18 ± 0.16% Ni, respectively (Table 4).

DISCUSSION

Heavy metal contamination of aquatic environments is a major global concern as huge amounts of heavy metals are discharged into water bodies as effluents of mining, metallurgy and electroplating industries (Mahato et al., 2014; Singh, 2014; Tiwari et al., 2015). Sehgal et al. (2012) studied heavy metal contamination in water and soil from 13 different sites of the Delhi segment of Yamuna basin. Average heavy metal concentration at different locations in the river water varied in the order of Fe > Cr > Mn > Zn > Pb > Cu > Ni > HgAs > Cd. So, heavy metal removal from industrial effluents is a crucial requirement in wastewater treatment plants. Several chemical methods used for this purpose are expensive and not environmental friendly. In contrast, bioremediation of heavy metals by microorganisms is cost-effective and compatible with the environment (Jan et al., 2014). Bacterial populations isolated from heavy metal contaminated sites may have the ability to tolerate a higher concentration of metals. Hence, in the present study, cadmium and nickle-tolerant bacteria were isolated from the polluted Yamuna river which has been contaminated with heavy metal. The enrichment of stagnant water samples collected from heavily polluted sites led to the isolation of bacteria that have significant potential to tolerate high cadmium and nickel concentrations. Isolate 2 was able to grow at 2000 µg/ml of cadmium ion concentration and isolate 8 at 3000 µg/ml in the liquid medium. The values are significantly variable than the tolerance values reported in some of the previous studies. Isolates *Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Bacillus cereus* were found to tolerate and exclude cadmium ion (Cd^{2+}) from the cell surface (Kafilzadeh et al., 2013). Earlier studies have reported cadmium-resistant bacteria community isolated from sewage sludge contaminated by cadmium ions (50 µg/ml) and the predominance of gram-negative bacteria with 5.08 ± 0.88% cadmium (Chovano et al., 2004). Similarly, isolate 8 demonstrated tolerance for nickel (2000 µg/ml). Ni-tolerant *Acinetobacter* isolates with MTC of 6.5 mM had been screened from Torsa

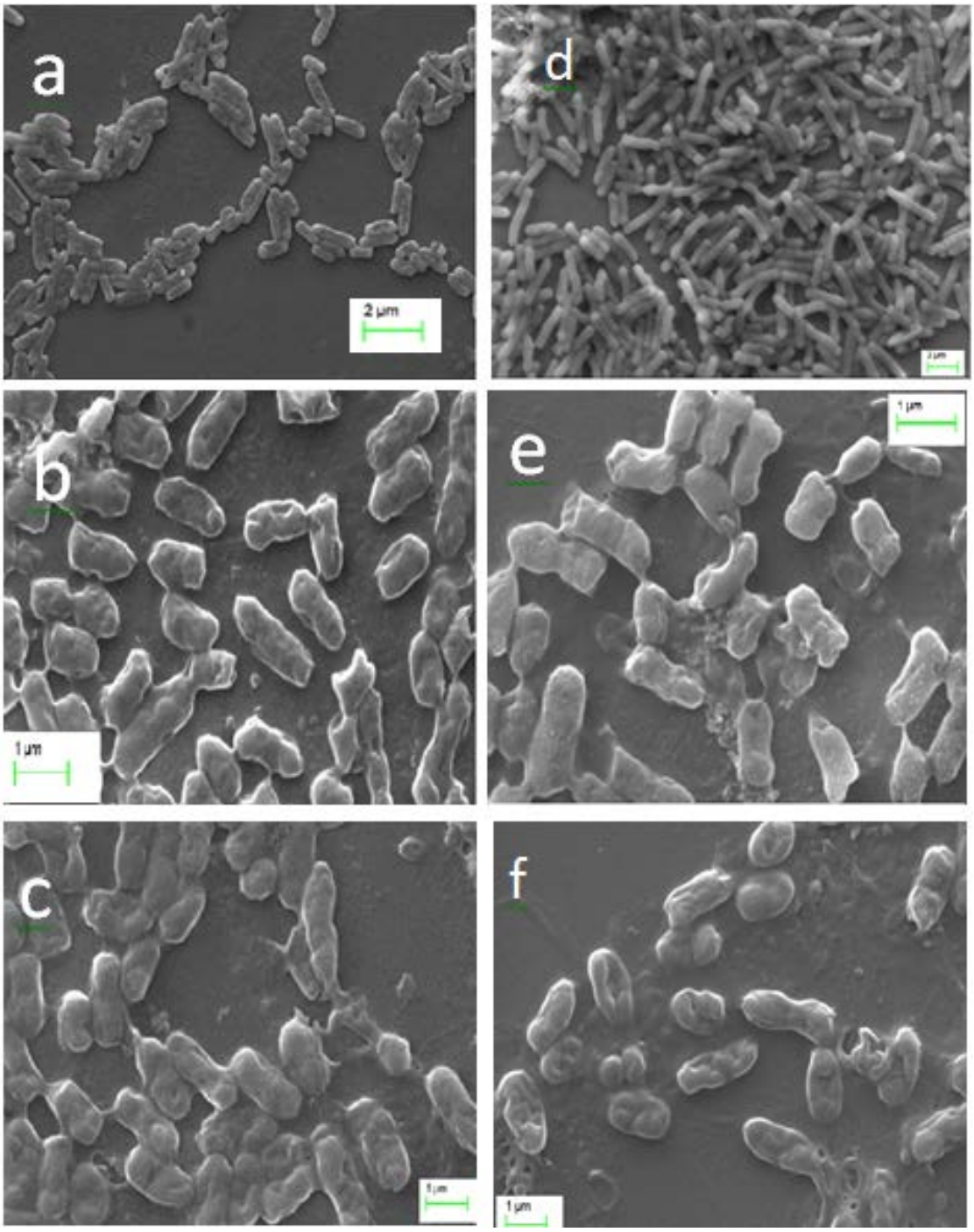

Figure 5. SEM analysis of untreated and nickle and cadmium-treated samples 2 and 8; a) Untreated sample 2; b) Sample 2 treated with nickle (2000 µg/ml); c) Sample 2 treated with cadmium (3000 µg/ml); d) Untreated Sample 8; e) Sample 8 treated with nickle (2000 µg/ml); f) Sample 8 treated with cadmium (3000 µg/ml).

Table 4. Metal concentration as detected by EDX analysis.

Sample	Ni concentration (%)	Cadmium Concentration (%)
Sample 2	9.5±1.01	4±0.05
Sample 8	7.18±0.16	5.08±0.88

River, West Bengal, India (Bhadra et al., 2006). Other studies have reported the gram-negative Ni-tolerant isolates, exhibiting MTC range of 1 to 24 mM for nickle, isolated from the industrial effluents wastewater treatment plant of Paonta Sahib, Himachal Pradesh, India (Virender et al., 2010). Multimetal tolerant *Alcaligenes xylosoxidans*, with the tolerating range of 2.0 to 4.0 mM for $NiCl_2$ and 1 mM cadmium was characterized by Sevgi et al. (2010). *Pseudomonas fragi*, *Staphylococcus* spp. and *Bacillus* spp. with MTC ranging from 150 to 500 µg/ml was isolated from industrial effluents (Patel et al., 2006; Rajbanshi, 2008). Thus, many such bacteria with a varying range of MTCs have been reported in various studies from different parts of the world. Our isolates (sample 2 and 8) showed tolerance to much higher concentration of cadmium and nickle (3000 and 2000 µg/ml).

The preliminary identification results suggested that isolates 2 and 8 are gram-negative rods representing the family *Enterobacteriaceae.* A combination of biochemical tests and 16S rRNA gene sequencing revealed that the two isolates share 99% similarity with *Pantoea agglomerans* JCM 1236 and *Enterobacter asburiae* JCM 6051, respectively. Both isolates exhibited co-tolerance to cadmium and nickel. The microbiota isolated from co-contaminated environments could exhibit tolerance to more than one metal ion and, thus, co-tolerance may be a common natural response (Gadd and Sayer, 2000; Malik et al., 2002). Since the heavy metals are similar in their toxic mechanisms; multiple tolerances were the common phenomena among the heavy metal tolerant bacteria (Mikolay and Nies, 2009; Ansari and Malik, 2007). The growth rate of the bacterial isolates in the presence of the heavy metals (cadmium and nickle) was consistently slower than that of the control ($p < 0.05$). The growth pattern suggests tolerance development or adaptation of bacteria to the presence of heavy metals in the aquatic environment.

All the isolates were tested for their resistance to common antibiotics, that is, rifampicin, augmentin, vancomycin, and cefpodoxime. A correlation exists between the two beneficial traits, that is, metal tolerance and antibiotic resistance, in bacteria. It may be because the resistance genes to both antibiotics and heavy metals may be located closely together on the same plasmid in bacteria. Thus, they are more likely to be transferred together in the event of conjugation (Filali et al., 2000; Malik and Aleem, 2011; Nies, 1992). This phenomenon would be favorable for bacteria to survive in heavy metal contaminated water bodies. The microbial tolerance to heavy metals is attributed to various detoxifying mechanisms such as complexation by exopolysaccharides, binding of metal in bacterial cell envelopes, metal reduction, metal efflux or using them as terminal electron acceptors in anaerobic respiration (Haferburg and Kothe, 2010). The SEM analysis (Figure 5) showed distinct changes in the cell size and surface features. The cellular changes and presence of exopolysaccharides around the cell can be interpreted as a possible strategy of the cell to accumulate more metals.

Several studies have reported the potential use of metal-resistant microorganisms in the treatment of heavy metal contaminated wastewater bodies (Karakagh et al., 2012; Shakibaie et al., 2010). Researchers have characterized *Pseudomonas aeruginosa* KUCADMIUM1 showing biological removal of cadmium at the level of 75 to 89% of total content (Sinha and Mukherjee, 2009) and *Klebsiella pneumoniae* CBL-1 at a concentration of 1500 mg/ml (Rehman and Shamim, 2012). Ni-tolerant *Micrococcus* sp. was also studied for its applicability in bioremediation of industrial waste water (Congeevarama et al., 2007). A bacterial consortium of metal-resistant bacteria including Enterobacteriaceae members, isolated from the river Yamuna, had been studied as a potential source for generation of electricity (Malik et al., 2014). Similarly, the bacteria isolated in the present study can be further explored for their ability to remove heavy metals.

Conclusion

The increased levels of heavy metals in the river water lead to accumulation of heavy metals in the adjoining soil and plants grown on these contaminated soils, leading to great harm to humans and animals. Such contaminated environment poses pressure on microbes to develop various mechanism to survive in high metal concentration. So these microorganism can be promising tool for removal of these metals from the contaminated river bodies. The present study reports the identification of two bacterial strains with 99% similarity to *Pantoea agglomerans* JCM1 and *Enterobacter asburiae* JCM 6051 with a tolerance to high concentrations of cadmium (3000 µg/ml) as well as nickel (2000 µg/ml). These isolates can be termed as heavy metal tolerant bacteria that may have the potential to remove heavy metals from contaminated effluents before discharging them in river waters.

Conflict of interest

The authors have not declared any conflict of interest.

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