Pak. j. sci. ind. res. Ser. B: biol. sci. 2015 **58** (2) 59-64

Antioxidant Activity of Date Palm Fruit (*Phoenix dactylifera* L.) Extract for Oxidative Stabilisation of Butter Oil at Ambient Temperature

Fazal Rahman[a], Muhammad Nadeem[a], Sabir Khan[a], Shakeel Ahmad[a]* and Yasir Zahoor[b]

[a]Department of Dairy Technology, University of Veterinary and Animal Sciences, Lahore, Pakistan

[b]Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan

(received August 18, 2014; revised April 30, 2015; accepted May 5, 2015)

Abstract. In this study, long term preservation of butter oil was achieved through ethanolic extract of date palm fruit (*Phoenix dactylifera* L.). Butter oil was supplemented with date palm fruit extract (DPFE) at three different concentrations i.e. 250, 500 and 750 ppm (T_1, T_2 and T_3) and compared with a control. Total phenolic content, DPPH free radical scavenging activity and inhibition of linoleic acid peroxidation of the DPFE was 5.19 GAE, 74.2 and 81%, respectively. IC_{50} value of date extract for the inhibition of DPPH and linoleic acid peroxidation was 2.45 and 0.82 mg/mL, respectively. The loss of oleic acid and linoleic acid in control after six months of storage was 16 and 52% as compared to T_3 which was 4% and 14%. T_3 yielded the lowest concentration of primary and secondary oxidation products with no effect on sensory attributes. DPFE can be used to enhance the shelf life of butter oil at ambient temperature.

Keywords: date palm, oxidative stability, butter oil

Introduction

Uncontrolled free radical mechanism in the human body leads to a large number of biochemical complications. Free radicals and reactive oxygen species have been implicated in the oxidative breakdown of vital biochemical molecules such as DNA, proteins, lipids (Madhujit and Shahidi, 2008). Phenolic compounds protect the body from the continuous threats of reactive oxygen species (Silva *et al*., 2009; Yazdanparast and Ardestani, 2007). The antioxidant, anti-inflammatory, antiallergic, anticancer and antiviral activities of phenolic compounds of plant origin and their role as a protector in hepatic and cardio-vascular diseases have been well established (Shahidi, 1997). Autoxidation of fats in food systems is a result of free radical mechanism leading to the destruction of essential fatty acids, vitamins and induction of objectionable flavours (Mc Sweeney and Fox, 2003). Perceived carcinogenicity of synthetic antioxidants, safety and efficacy of natural antioxidants in the inhibition of reactive oxygen species has necessitated broadening their array of application (Anwar *et al*., 2007). Studies have shown that most of the natural antioxidants of plant origin are better soluble in methanol (Anwar *et al*., 2010). The application of methanol based antioxidants for the preservation of food systems has a health concern due to the toxicity of methanol, although most of the solvent is evaporated yet the residues can have a potential health concern. Therefore, it is the need of hour to find out the sources of natural antioxidants which are soluble in water rather than polar organic solvents. Autoxidation of fats deteriorates sensory characteristics and limits the shelf life (Gonzalez *et al*., 2003; Shiota *et al*., 2002). The effect of (DPFE) date palm fruit extract for the stabilization of fats and oils has not been studied previously. This study aimed to investigate the antioxidant activity of date palm fruit extract on oxidative stability of butter oil on the basis of selected chemical and sensory techniques.

*Author for correspondence; E-mail: shakeel.1@live.com

Materials and Methods

Materials. Dates (*Zahidi*, Iranian variety) were procured from local market and cream was purchased from Haleeb Foods Multan Road, Lahore. All the chemicals used in this study were HPLC grade and obtained from Sigma Aldrich, USA.

Preparation of antioxidant extract. After removing the stones, dates were washed with distilled water, cut into small chunks, 20 g date was weighed in the flask, 80% ethanol was added into the flask and shaken with magnetic stirrer at 100 rpm for 8 h, the contents of the flask were filtered over filter paper (Whatman 41), the residue was extracted twice following the similar conditions and concentrated with rotary evaporator (Buchi, Switzerland).

Determination of total phenolic content. 125 µL sample, 500 µL deionised water and 125 µL Folin-Ciocalteu were mixed together, followed by the addition of 1.25 mL 7% solution of sodium carbonate and then 1 mL deionised water was added. Absorbance was measured on a double beam spectrophotometer (Shimadzu, Japan) at 760 nm. The concentration of total phenolic contents in the ethanolic date extract was determined by constructing a calibration curve using 10 standards of different concentration of Gallic acid (R^2= 0.9921) as prescribed by Negi *et al*. (2003).

DPPH free radical scavenging activity. DPPH free radical scavenging activity was determined by following the method of Mansouri *et al*. (2005).

Linoleic acid oxidation. Linoleic acid 0.13 mL was mixed with 10 mL (99.8% ethanol) and 10 mL of sodium phosphate buffer (0.2 M, pH 7). The contents were diluted to 25 mL with distilled water in the volumetric flask, sealed and incubated at 40 ºC in an oven for 15 days. The oxidation status was evaluated by the determination of peroxide value (thiocyanate method). 10 mL ethanol (75%) and 30% solution of ammonium thiocyanate prepared in distilled water and 0.2 mL extract and 0.2 mL ferrous chloride (20 mM prepared in 3.5% HCl) were added, contents were stirred for 3 min, absorbance was measured on 500 nm in visible region of spectra on a spectrophotometer using butylated hydroxytoluene (BHT; 100 ppm) as a control according to the method described by Anwar *et al*. (2010).

Experimental plan. Date palm fruit extract (DPFE) was incorporated into butter oil at three different concentrations i.e. 250, 500 and 750 ppm (T_1, T_2 and T_3), filled in PET bottles and stored at ambient temperature for six months and sampled at 60 days intervals for studying various characteristics.

Analysis. For the determination of fatty acid composition, 50 µL representative sample was taken in 11 mL screw capped test tube and 2 mL *n*-hexane was added to dissolve the sample. Methylation was performed by adding 2 mL, 0.5 N methanolic sodium methylate and tubes were vortexed for 3 min at 2200 rpm, after 5 min of settling time the supernatant was dried over anhydrous sodium sulphate, transferred to GC vials and injected into gas chromatograph model Shimadzu, Japan 17-A, fitted with a methyl lignoserate-coated (film thickness 0.25 µm), SP-2330 (SUPELCO Inc. USA) polar capillary column (30 m × 0.32 mm) using flame ionisation detector as per standard IUPAC method (1987) 2301. Fatty acids were identified and quantified by using FAME-37 internal standards (Sigma Aldrich, UK). Peroxide and anisidine values were measured by following the standard method of AOCS (1995). The sensory evaluation of butter oil supplemented with various concentrations of the date extract was performed by a panel of 10 trained judges who were selected and training sessions were conducted for them for standarisation of sensory language and familiarisation of flavour evaluation process. The flavour evaluation was performed on a 9-point scale in the sensory evaluation booths at 20±3 ºC as suggested by Larmond (1986). Each treatment was run in triplicate, the data were analysed by using analysis of variance technique (one way and two way). For the determination of significance difference among the treatments, Duncan Multiple Range Test was used. P-values ($P \leq 0.05$) were used to express the significant difference (Steel *et al*., 1997).

Results and Discussion

Total phenolic content. Total phenolic content of DPFE was 5.19% GAE. The higher concentration of phenols was due to the better solubility of antioxidants of date palm fruit in the ethanolic system, it makes date palm fruit extract superior to other natural antioxidants which are better soluble in methanol and other organic solvents. The application of natural antioxidants extracted by organic solvents for the preservation of food stuffs is questionable due to a great deal of potential health hazards associated with them. For the extraction of natural antioxidants, methanol has been considered to be a better solvent over others (Anwar *et al.,* 2010) but from commercial point of view methanolic based natural antioxidants have limited application due to high toxicity of methanol to humans. The aqueous date palm fruit extract showed higher concentration of phenolic substances and can provide better stabilisation of food systems without putting a question mark on the food safety. The total phenolic contents in this study were even higher than in methanolic extract of barley seeds (*Hordeum vulgare* L.) reported by Anwar *et al*. (2010). The higher total phenolic content of date palm fruit extract has also been reported in some Saudi Arabian date verities (Saleh *et al*., 2011).

DPPH free radical scavenging activity. The DPPH free radical scavenging activity of butter oil supplemented with DPFE increased in a concentration dependent manner and were in the order of $T_3 < T_2 < T_1 <$ control. The DPFE also exhibited good antioxidant activity in the

butter oil (Fig. 1). The DPPH free radical scavenging activity of the extract was 74.2% as compared to the BHT 94% (100 ppm) (1 mL concentration). IC_{50} value of the extract was 2.45 mL; the supplementation of butter oil with DPFE significantly decreased the IC_{50} value of butter oil which was shown in fresh and stored butter oil, the lower doses of the extract exhibited higher IC_{50} value. A longer storage period also had a negative influence on IC_{50} value. The disappearance of DPPH radicals is most likely due to presence of phenolic compounds in the extract. Strong DPPH free radical scavenging activity of date fruit extract has been described in literature (Singh *et al*., 2002).

Linoleic acid oxidation. In this study, the % inhibition of oxidation in linoleic acid system was also determined, the percentage inhibition of lipid peroxidation of DFPE was 81% (R^2=0.9934) as compared to BHT 100 ppm (91.5%) which was used as positive control. IC_{50} value of date fruit extract and butter oil supplemented with extract was also determined at different storage intervals (Fig. 2). The IC_{50} value of ethanolic date fruit extract was 0.82 mg/mL. IC_{50} value of butter oil cream (mL of butter oil to decrease 50% lipid peroxidation) decreased in a dose dependent manner and were in the order of $T_3 < T_2 < T_1 <$ control at zero day and all the determination frequencies. The lower the IC_{50} value, better is the antioxidant activity. The IC_{50} value of butter oil (mL of butter oil causing 50% decrease in lipid peroxidation) in T_2 and T_3 was significantly less than in the control and increased for all butter oil samples during storage period

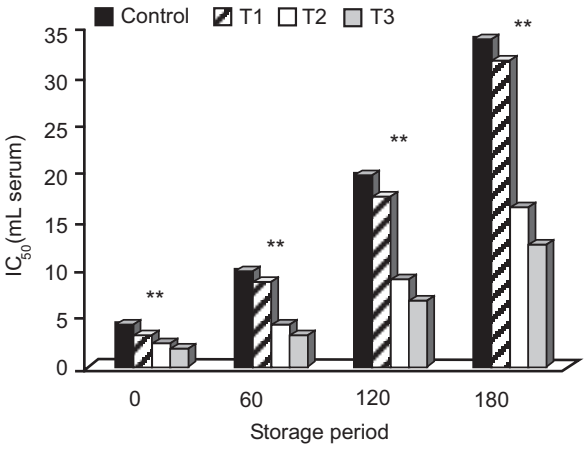

Fig. 1. IC_{50} Value for the inhibition of linoleic acid peroxidation **Highly significant ($p<0.01$) Refer Table 2 for the detail of treatment.

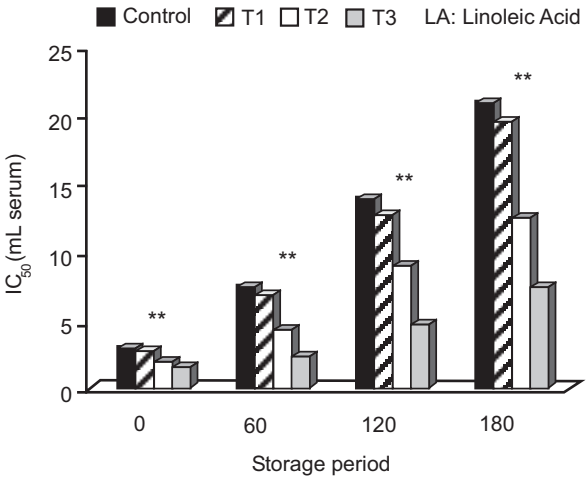

Fig. 2. IC_{50} Value for the elimination of DPPH free radicals. **Highly significant ($p<0.01$) Refer Table 2 for the detail of treatment.

of 180 days. The strong antioxidant activity of the extract could be attributed to the solubility of natural antioxidants in the ethanolic phase. The results regarding lower IC_{50} value of ethanolic date fruit extract in this study are also supported by the findings of Al-Farsi *et al*. (2005) howerver, little information is available on IC_{50} value of foodstuffs supplemented with natural antioxidants.

Changes in the fatty acid composition. The changes in the fatty acid composition of butter oil supplemented with DPFE and control are presented in Table 1. At 250 ppm supplementation level DPFE was virtually inactive to inhibit the lipid peroxidation. The lipid peroxidation inhibition of DPFE was in the order of $T_3 > T_2 > T_1$. Some difference was observed in the fatty acid composition of fresh and six months stored butter oil. The extent of difference was dependent upon three factors; storage period, supplementation and the supplementation level of ethanolic date fruit extract. DPFE in T_2 and T_3 significantly inhibited the free radical mechanism in the stored butter oil, better antioxidant activity was seen in T_3. Unsaturated fatty acids decreased during storage due to their breakdown into primary and secondary oxidation products and saturated fatty acids increased on percentage basis. The loss of oleic acid and linoleic acid in control was 16 and 52% as compared to T_3, 4 and 14% after six months of storage. DPFE significantly retarded the autoxidation process in T_2 and T_3. The strong antioxidant activity of DPFE could be attributed to the higher concentration of polyphenolic compounds. The concentration of unsaturated fatty acids decreased during storage of 42-days when flax seed oil

was added in the formulation of ice cream (Lim *et al*., 2010). The fatty acid composition of fresh and stored butter was slightly different (Mallia *et al*., 2008). The strong antioxidant activity of date palm has been shown in literature (Mansouri *et al*., 2005) but little information is available regarding the application of DPFE for the stabilisation of fat rich dairy products.

Peroxide value. The results regarding increase of peroxide value in the DPFE supplemented and control are presented in Table 2. Supplemented and control butter oils showed varying degree of rise in peroxide value. The magnitude of rise in peroxide value during storage period was in the order of control > T_1 > T_2 > T_3. The peroxide value and concentration of DPFE were highly correlated (R^2=0.9749). The addition of 750 ppm date extract was more effective in the stabilisation of butter oil over other treatments. Peroxides are the products of free radical mechanism, and phenolic compounds can terminate the free radical mechanism by donating protons, the lower peroxide value of T_3 could be attributed to the better proton donating capability of DPFE. Shiota *et al*. (2004) used peroxide value as important parameter to characterise the photochemical oxidation of butter oil. Supplementation of chicken meat mince with date palm extract significantly retarded the lipid peroxidation for 20-days at 4 ºC (Biglari *et al*., 2009). Peroxide value of ice cream prepared from modified and unmodified milk fat increased during storage (Shiota *et al*., 2004; Gonzalez *et al*., 2003). The stabilisation of butter fat through the application of natural antioxidant has been reported by Nadeem *et al*. (2013).

Anisidine value. The data of anisidine value of supplemented and control butter oil is given in Table 3. Anisidine value numerically increased throughout the storage period of 180 days, the classical rise in anisidine value varied considerably among the treatments and control, T_3 revealed the lowest concentration of secondary oxidation products at all the determination intervals followed by T_2. Determination of anisidine value reflects the concentration of aldehydes produced as a course of free radical mechanism (McGinely, 1991). The antioxidant activity of natural antioxidants for the stabilisation of edible oils has been extensively studied by the researchers. In other studies, supplementation of barley extract and wheat bran extract significantly inhibited the generation of secondary oxidation products in sunflower and canola oils (Chatha *et al*., 2011; Anwar *et al*., 2010).

Table 1. Effect of DPFE on fatty acid composition of fresh and six months stored butter oil

Fatty acid	Fresh%	Control-6M%	T_1-6M%	T_2-6M%	T_3-6M%
C4:0	4.68±0.13^c	5.36±0.09^a	5.27±0.06	4.93±0.04^b	4.72±0.013^c
C6:0	2.89±0.14^b	3.27±0.03^a	3.18±0.06^a	2.97±0.07^b	2.92±0.02^b
C8:0	1.75±0.05^c	2.41±0.04^a	2.33±0.06^a	2.04±0.02^b	1.82±0.04^c
C10:0	4.17±0.11^c	4.83±0.08^a	4.71±0.07^a	4.53±0.03^b	4.24±0.12^c
C12:0	5.33±0.19^c	5.79±0.015^a	5.73±0.09^a	5.58±0.07^b	5.41±0.16^c
C14:0	12.19±0.12^d	12.72±0.48^a	12.59±0.22^a	12.43±0.31^b	12.32±0.19^c
C16:0	20.47±0.35^a	20.98±0.84^a	20.84±0.49^a	20.61±0.17^a	20.53±0.28^a
C18:0	5.43±0.10^c	5.87±0.61^a	5.80±0.16^a	5.69±0.21^b	5.40±0.36^c
C18:1	31.68±1.24^a	26.64±0.44^e	27.37±0.38^d	28.66±0.13^c	30.48±0.46^b
C18:2	3.22±0.15^a	1.54±0.05^e	1.99±0.04^d	2.43±0.12^c	2.79±0.02^b

Within a row, means represented by the same letter are not statistically different; T_1 = ethanolic date palm fruit extract 250-ppm; T_2 = ethanolic date palm fruit extract 500-ppm; T_3 = ethanolic date palm fruit extract 750-ppm; 6M = six months stored butter oil

Table 2. Effect of ethanolic date extract on peroxide value of butter oil ($M_{eq}O_2$/kg)

Treatments	0-D	60-D	120-D	180-D	Increase
Control	0.24±0.02^d	0.68±0.05^c	1.85±0.06^b	3.77±0.19^a	3.53
T_1	0.24±0.02^d	0.51±0.09^c	1.43±0.11^b	2.92±0.25^a	2.68
T_2	0.24±0.02^d	0.44±0.10^c	1.15±0.16^b	2.48±0.15^a	2.24
T_3	0.24±0.02^d	0.35±0.04^c	0.82±0.14^b	1.37±0.12^a	1.13

Within the rows and columns, means carrying different letter are statistically different; Increase = increase in PV from the start; D = storage days

Table 3. Effect of ethanolic date extract on anisidine value of butter oil

Treatments	0-D	60-D	120-D	180-D	Increase
Control	4.52±0.13^{d}	7.35±0.15^{c}	13.62±0.38^{b}	21.64±0.45^{a}	17.12
T_1	4.52±0.13^{d}	5.42±0.22^{d}	10.58±0.29^{b}	15.76±0.51^{a}	11.24
T_2	4.52±0.13^{d}	6.75±0.17^{d}	8.49±0.18^{b}	12.92±0.34^{a}	8.40
T_3	4.52±0.13^{d}	5.29±0.13^{d}	7.36±0.15^{b}	9.53±0.26^{a}	5.01

Within the rows and columns, means carrying different letter are statistically different; Increase = increase in PV from the start; D = storage days

Table 4. Effect of ethanolic date extract on flavour score of butter oil

Treatments	0-D	60-D	120-D	180-D	Decrease
Control	8.2±0.2^{a}	7.7±0.24^{b}	7±016^{d}	5.9±0.10^{d}	2.3
T_1	8.1±0.2	7.7±031^{b}	7.2±0.25^{c}	6.3±0.15^{c}	1.8
T_2	8±0.1^{a}	7.9±0.19^{a}	7.7±0.27^{b}	6.8±0.12^{b}	1.2
T_3	8±0.15^{a}	8±0.11^{a}	7.9±0.35^{a}	7.4±0.24^{a}	0.6

Within the rows and columns, means carrying different letter are statistically different; D = storage days

Sensory evaluation. The results of sensory evaluation of butter oils supplemented with DPFE and correlation between peroxide value flavour score are given in Table 4. When fresh, flavour score of the treatments and control were not different from each other (P>0.05). Flavour score decreased during storage period of 180-days, the decline in the flavour score of butter oil was observed in the order of control > T_1 > T_2 > T_3. The decline in the flavour score was not due to the addition of DPFE but due to the development of oxidised flavour. Peroxide value and flavour score were highly correlated (R^2=0.9803), the smallest drop in flavour score of the control, T_1, T_2 and T_3 was 2.3, 1.8, 1.2, and 0.6, respectively from the initial value. The lowest drop in the flavour score of T_3 was due to the generation of considerably lower amounts of primary and secondary oxidation products by the strong antioxidant potential of ethanolic DPFE. Shiota *et al*. (2004) reported a strong correlation between peroxide value and flavour score of butter oil. Nadeem *et al*. (2013) also reported a decline in the flavour score of butter stored for three months.

Conclusion

Characterisation of ethanolic date palm extract revealed strong antioxidant activity; the addition of aqueous date palm fruit extract at 750 ppm concentration significantly inhibited the changes in the fatty acid composition, generation of primary and secondary oxidation products with minimum decline in the flavour score during storage period of 6 months. Date palm extract at 750 ppm can be added for better storage stability of butter oil with acceptable flavour characteristics.

References

Al-Farsi, M., Alasalvar, C., Morris, A., Baron, M., Shahidi, F. 2005. Comparison of antioxidant activity, anthocyanins, carotenoids, and phenolics of three native fresh and sun-dried date (*Phoenix dactylifera* L.) varieties grown in Oman. *Journal of Agriculture and Food Chemistry,* **53:** 7592-7599.

Anwar, F., Qayyum, H.M.A., Hussain, A.I., Iqbal, S. 2010. Antioxidant activity of 100% and 80% methanol extracts from barley seeds (*Hordeum vulgare* L.): stabilization of sunflower oil. *Grasas y Aceites* **61:** 237-243.

Anwar, F., Siddiq, A., Iqbal, S., Asi, M.R. 2007. Stabilization of sunflower oil with *Moringa oleifera* leaves under ambient storage. *Journal of Food Lipids,* **14:** 35-49.

AOAC. 1997. *Official Methods of Analysis,* 16th edition, Association of Official Analytical Chemists, AOAC Internat. Gaithersburg, MD, USA.

AOCS, 1995. *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th edition, AOCS, Champaign, IL, USA.

Biglari, F., Alkarkhi, A.F.M., Essa, A.M. 2009. Cluster analysis of antioxidant components in dates (*Phoenix dactylifera*). Effect of longterm cold storage. *Food Chemistry*, **112:** 998-1001.

Chatha, S.A.S., Hussain, A.I., Bajwa, J.R., Sherazi, S.T.H., Shaukat, A.2011. Wheat bran extracts: a potent source of natural antioxidants for the stabilization of canola oil. *Grasas y Aceites*, **62:** 190-197.

Gonzalez, S., Duncan, S.S.E., Keefe, S.F., Sumner, S.S., Herbein, J.H. 2003. Oxidation and textural characteristics of butter and ice cream with modified fatty acid profiles. *Journal of Dairy Science,* **86:** 70-77.

IUPAC, 1987. *Standard Methods for the Analysis of Oils, Fats and Derivatives*, International Union of Pure and Applied Chemistry, 7th revised and enlarged edition, C. Paquot and A. Hautfenne (eds.), Blackwell Scientific, London, UK.

Larmond, E. 1986. Sensory evaluation can be objective. In: *Objective Methods in Food Quality Assessment*, J. Kapsalis (ed.), pp. 3-14, CRC, Press, Boca Raton, Florida, USA.

Lim, C.W., Norziah, M.H., Lu, H.S. 2010. Effect of flaxseed oil towards physicochemical and sensory characteristic of reduced fat ice creams and its stability in ice creams upon storage. *International Food Research Journal*, **17:** 393-403.

Madhujith, T., Shahidi, F. 2009. Antioxidant potential of barley as affected by alkaline hydrolysis and release of insoluble-bound phenolics. *Food Chemistry,* **117:** 615-620.

Mallia, S., Piccinali, P., Rehberger, B., Badertscher, R., Escher, F., Schlichtherle-Cerny, H. 2008. Determination of storage stability of butter enriched with unsaturated fatty acids/conjugated linoleic acids (UFA/CLA) using instrumental and sensory methods. *International Dairy Journal,* **18:** 983-993.

Mansouri, A., Embarek, G., Kokkalou, E., Kefalas, P. 2005. Phenolic profile and antioxidant activity of the algerian ripe date palm fruit (*Phoenix dactylifera*), *Food Chemistry,* **89:** 411-420.

Mc Sweeney, P.L.H., Fox, P.F. 2003. *Advanced Dairy Chemistry*, *Proteins*. vol. **1:**, pp. 129-135, 3rd edition, Kluwer Academic Plenum Pub., NY, USA.

Mcginely, L. 1991. Analysis and Quality Control for Processing and Processed Fats. In: *Analysis of Oilseeds, Fats and Fatty Foods,* J. B. Rossell and J.L.R. Pritchard (eds.), pp. 460-470, Elsevier Applied Science, The Netherlands.

Nadeem, M., Abdullah, M., Khalique, A., Hussain, I., Mahmud, A., Mahmood, T. 2013. The effect of *Moringa oleifera* leaf extract as antioxidant on stabilization of butter oil with modified fatty acid profile. *Journal of Agriculture Science and Technology,* **15:** 919-928.

Negi, P.S., Jayaprakasha, G.K., Jena, B.S. 2003. Antioxidant and antimutagenic activities of pomegranate peel extracts. *Food Chemistry,* **80:** 393-397.

Potter, N.W., Hotchkis, J. 1998. *Food Science*. pp. 302-306, 5th edition, Spinger Publisher, USA.

Saleh, E.A., Tawfik, M.S., Abu-Tarboush, H.M. 2011. Phenolic contents and antioxidant activity of various date palm (*Phoenix dactylifera* L.) fruits from Saudi Arabia. *Food and Nutrition Science,* **2:** 1134-1141.

Shahidi, F. 1997. *Natural Antioxidants Chemistry, Health Effects, and Applications*, vol. **41,** 321 pp., AOCS Press, Urbana, IL, USA. 321.

Shiota, M., Takahashi, N., Konishi*b*, H., Yoshioka, T. 2004. Impact of oxidized off-flavor of ice cream prepared from milk fat. *Journal of American Oil Chemists Society,* **8:** 455-460.

Shiota, M., Ikeda, N., Konishi, H., Yoshioka, T. 2002. Photooxidative stability of ice cream prepared from milk fat. *Journal of Food Science*, **67:** 1200-1207.

Siddiq, A., Anwar, F., Manzoor, M., Fatima, M. 2005. Antioxidant activity of different solvent extracts of *Moringa oleifera* leaves under accelerated storage conditions of sunflower oil. *Asian Journal of Plant Science,* **4:** 630-635.

Silva, E.M., Souza, J.N.S., Rogez, H., Rees, J.F., Larondelle, Y. 2007. Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian Region. *Food Chemistry*, **101:** 1012-1018.

Singh, R.P., Murthy, C.K.N., Jayaprakasha, G.K. 2002. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using *in Vitro* models. *Journal of Agriculture and Food Chemistry,* **50:** 81-86.

Steel, R.G.D., Torrie, J.H., Dickey, D.A. 1997. *Principles and Procedures of Statistics. A biometrical approach,* 3rd edition, Mc-Graw Hill Book Co. New York, USA.

Yazdanparast, R., Ardestani, A. 2007. *In vitro* Antioxidant and free radical scavenging activity of *Cyperus rotundus*. *Journal of Medicine and Food,* **10:** 667-674.

Pak. j. sci. ind. res. Ser. B: biol. sci. 2015 **58** (2) 65-71

Cadmium Tolerance and Bioremediation Potential of Bacteria Isolated from Soils Irrigated with Untreated Industrial Effluent

Rizwan Ahmad*, Muhammad Mahmood-ul-Hassan, Muhammad Yasin and Vishandas Suthor
Land Resources Research Institute, National Agricultural Research Centre, Islamabad 45500, Pakistan

(Received July 22, 2014; revised May 7, 2015; accepted May 13, 2015)

Abstract. The present study was aimed to investigate the Cd tolerance of bacteria isolated from municipal effluent irrigated soils. Thirty bacterial strains were isolated and screened for their Cd^+ tolerance by growing on nutrient agar plates amended with varying amount of Cd^+. Out of them four bacteria (GS_2, GS_5, GS_{10} and GS_{20}) were found highly Cd tolerant (600 ppm Cd). The minimum inhibitory concentration of Cd^+ was found 200 ppm. The isolates showed optimum growth at 30 °C and pH 7.5-8.5. Growth curve study against different concentrations of Cd (0-600 ppm) revealed that GS_2 was more tolerant among selected strains showing only 33% reduction in growth compared to 64% by GS_5 and 77% by both GS_{10} and GS_{20} at 600 ppm Cd. Inoculation of maize seeds with Cd tolerant bacteria for root elongation demonstrated up to 1.7 fold increase in root elongation (in the absence of Cd) and up to 1.5 fold (in the presence of 50 ppm Cd) compared to the un-inoculated plants. The results of the study revealed that the bacterial isolates exhibiting great Cd tolerance and growth promoting activity can be potential candidates for bioremediation of metal contaminated soils and wastewaters.

Keywords: soil contamination, Cd tolerance, tolerance index, bioremediation

Introduction

The contamination of the environment with toxic heavy metals is a serious problem because it is associated with heavy metal accumulation in the food chain which later has an impact towards human health (Hamzah *et al.,* 2009). Municipal/industrial effluents contain considerable amounts of different metals as: chromium (Cr), cadmium (Cd), lead (Pb), nickel (Ni) and copper (Cu) in various combinations depending upon their source and nature (Khan *et al.,* 2013; Mahmood-ul-Hassan *et al.,* 2012). Release of untreated municipal/industrial effluents to agricultural lands and water bodies is a common practice in big cities of developing countries like Pakistan (Khan *et al.,* 2013; Mahmood-ul-Hassan *et al.,* 2012). Its long-term application can adversely affect soil and ecosystem health, ultimately human health (Singh and Bhati, 2005). Contamination of soil with heavy metals negatively affects biodiversity and the activity of soil microbial communities (McGrath *et al.,* 1995). Continuous application of untreated wastewater elevates the metal concentrations in surface soil to toxic levels. As soil is a rich habitat of all major groups of microorganisms (bacteria, actinomycetes, fungi and algae), long-term exposure of microorganism to high metal concentration develop the immunity in the microorganisms (Akhtar *et al.,* 2013; Ezzouhri *et al.,* 2009).

Among metal pollutants of the surface soil, cadmium is one of the most toxic elements. Cadmium is used in industries like Ni-Cd battery manufacturing, electroplating, pigments manufacturing and stabilizers manufacturing. In plants, Cd affects nutrient uptake and homeostasis, inhibits root and shoot growth and frequently accumulated by agriculturally important crops (Sanita di Toppi and Gabrielli, 1999). Cadmium is the most dangerous heavy metal both to human and animal health as it is carcinogenic, embryo toxic, teratogenic and mutagenic (Hussain *et al.,* 2006). Excess Cd can damage kidney and lungs (Dhaliwal and Kukal, 2005). It may cause hyperglycemia, reduced immune potency and anemia, due to its interference with iron metabolism (Bueno *et al.,* 2008).

Several techniques (chemical and physical) are used for remediation of polluted soil and water. Chemical (precipitation and neutralisation) and physical (ion exchange, membrane separation and electro dialysis) techniques are applied to remove heavy metals from contaminated soils and waste water (Atkinson *et al.,* 1998). Such techniques have disadvantages like unpredictable metal ion removal, high reagent requirements, destruction of beneficial micro fauna and generation of toxic sludge (Ciba *et al.,*1999).

*Author for correspondence; E-mail: rizwan_narc@yahoo.co.in

Biological approaches have been considered as an alternative remediation for heavy metal contamination (Khan *et al.,* 2009). Recent advances have been made in understanding metal-microbe interaction and their application for bioremediation of metal contaminated soils (Chibuike and Obiora, 2014). Bioremediation is the use of microbes like fungi and bacteria for removal of heavy metals and have been successfully used (Congeevaram *et al.,* 2007). The metal tolerant microorganisms are helpful to alter the chemical status of the metal ions and in turn metal ion mobility. They act through processes such as reduction, bioaccumulation, mobilisation and biotransformation (Khan *et al.,* 2009). Bioremediation is an efficient strategy due to its low cost, high efficiency and eco-friendly nature. It can be applied without removing and transporting contaminated soils. As soil matrix is not disturbed, soil micro flora and fauna are preserved.

Bacterial surfaces have several types of functional groups that can react with dissolved metals. Bacteria are important microorganisms to be used for biosorption and bioaccumulation of metals and hence are an important factor in controlling the mobility and distribution of metals in contaminated soil and water (Burnett *et al.,* 2007). Biosorption is removal of heavy metals using passive binding process of living and dead biomass while bioaccumulation is referred as metal uptake into the cell across the membrane using active cell metabolism (Kotrba *et al.,* 2011). Considering the importance of bacteria in bioremediation, this study was designed to isolate indigenous bacteria from polluted sites and assess their cadmium tolerance potential to use in bioremediation.

Materials and Methods

Samples collection. Six composite surface soil samples (0-15 cm) were collected from heavy metal polluted peri-urban areas of Gujranwala and Sialkot, Pakistan for this research work. Sampling sites have been continuously (more than 10 years) irrigated with untreated industrial/ municipal effluent with high metal contents. The surface soil samples were collected in sterilised plastic bottles and were transported to Soil Environment Laboratory, NARC, Islamabad, Pakistan in sealed containers. These containers were stored at 4 °C to ensure minimal biological activity till further process.

Isolation for cadmium tolerant bacteria. Dilution plate technique was used for isolation of microbes (Pepper and Gerba, 2004). Bacteria from soil were cultured with 10^{-4}- 10^{-7} dilutions on nutrient agar medium at 28±2 ºC for three days. Thirty prominent isolates (twenty from Gujranwala and ten from Sialkot sites) with some distinguished morphological characters (colony colour, size, shape etc.,) were further cultured and purified through repeated streaking on the same medium. The cultured strains were preserved on slants for Cd tolerance test at 4 ºC and refreshed within three months regularly.

Minimum inhibitory concentrations (MIC) of Cd. To determine MIC for Cd, the growth of isolated bacterial strains was tested on nutrient agar medium amended with ascending concentration of Cd starting from 50 ppm (Kalantri, 2008). Stock solution (1000 ppm) of Cd salt ($CdCl_2$) was prepared with sterile water and added to the nutrient agar in varying concentrations (50-600 ppm). The process was continued with 50 ppm interval till the growth was ceased. Highly tolerant strains (600 ppm Cd) were tested repeatedly for further confirmation.

Morphological and biochemical characterisation. For colony and cell morphology, bacterial strains were grown on nutrient agar medium at 28±2 °C for 36-48 h. Each colony was characterised on the basis of colour, margin, elevation and cell shape with ocular and light microscopy. For gram staining, the slides of tolerant bacterial strains were prepared according to Benson (1994). A small loop of bacterial culture was taken and a thin smear on glass slides was prepared. The smear was air dried and heat fixed, stained with crystal violet stain for one minute and washed with water. Then the smear was flooded with iodine solution for 30 sec. After 30 sec it was washed with water and smear was decolourised with 75% ethanol for 30 sec. After washing, safranin was used for counter staining. The slide was rewashed with water, air dried and observed under light microscope.

Indole acetic acid (plant hormone) production of Cd tolerant bacterial strains was detected by using the method stated by Brick *et al.* (1991). Bacterial cultures were grown in 250 mL conical flasks containing 50 mL nutrient broth (Lab-lemco powder 1.0; Yeast extract 2.0; Peptone 5.0; Sodium chloride 5.0, each was on g/L basis) for 3 days at 28±2 ºC. Flasks were inoculated with different bacterial strains individually. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 mL) was mixed with two drops of orthophosphoric acid and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1mL 0.5M $FeCl_3$ solution). Development of pink colour indicates IAA production.

For measurement of phosphate solubilising activity, a single colony of each strain culture was streaked on

Pikovskaya`s medium containing tricalcium phosphate (EL-Komy, 2005) and incubated at 28 ± 2 ºC for 3 days. Qualitative determination was done by optically observing clear P-zone (halo-zone) formation around the colonies.

Screening for acid producing ability was determined by using the bromothymol blue indicator along with nutrient broth. Each strain was grown in 250 mL conical flasks containing 50 mL nutrient broth for 3 days at 28±2 °C. Traces of bromothymol blue indicator were added to each flask at the time of inoculation. Dark green colour appeared at neutral pH. Appearance of bluish green colour indicated the acid producing ability of the bacterial strain (Dupree and Wilcox, 1977).

Determination of optimum growth conditions. For optimum growth of bacterial isolates, two parameters i.e. pH and temperature were considered. To determine optimum pH, 30 mL test tubes having 10 mL nutrient broth were prepared in 5 sets for pH 6.0, 7.0, 8.0, 9.0 and 10 (each containing three test tubes) and autoclaved. These tubes were inoculated with freshly prepared culture of each isolate one by one. The tubes were incubated at 28±2 °C and 70 rpm. After an incubation period of 24 h, their absorbance was taken at 600 nm wavelength on Spectronic Genesys 5 (Milton Roy Company, USA) and then a graph was plotted between pH (along x-axis) and absorbance (along y-axis).

For determination of optimum temperature, test tubes having 10 mL nutrient broth were prepared in 3 sets for 20, 30, and 40 °C. The pH of all the sets, each containing three test tubes was adjusted at 7 by using diluted HCl or NaOH solutions. Test tubes were autoclaved and then inoculated with freshly prepared cultures of different isolates individually. The tubes were incubated at 20, 30, and 40 °C, respectively, with 70 rpm. After an incubation period of 24 h, their absorbance was taken at 600 nm wavelength and then graph was plotted between temperature (along x-axis) and absorbance (along y-axis).

Growth curve and metal tolerance index. Nutrient broth with increasing concentrations of Cd i.e. 200, 400, 600 ppm was prepared and autoclaved. A control (0 ppm Cd) was also made. Growth of the selected heavy metal tolerant strains was studied at standard temperature (30 °C) and pH (7). Test tubes were inoculated and incubated for 24 h in water bath shaker at 70 rpm. After an incubation period of 24 h, their absorbance was taken at 600 nm wavelength and then a graph was plotted between Cd concentration (along x-axis) and absorbance (along y-axis). Metal Tolerance Index (T_i) was calculated as the ratio of the optical density of the treated colony to that of the untreated colony.

$$T_i = \frac{OD_t}{OD_u}$$

Where:

OD_t = optical density of treated colony and
OD_u = optical density of the untreated colony.

Root elongation assay on filter paper culture. The plant root elongation promoting activity of the isolated bacteria was determined using the modified root elongation assay of Belimov *et al.* (2005). The seeds of maize variety ISD-gold were surface sterilised with a mixture of ethanol and 30% H_2O_2 (1:1) for 20 min, washed with sterile water and placed on wetted filter paper. Bacteria were grown in nutrient broth for 48 h at 28 ± 2 °C. Bacterial suspensions 5 mL or sterile water (un-inoculated control) were added to petri dishes containing filter papers, both in the presence and absence of 50 mg/L Cd. Root length of seedlings was measured after incubation of closed petri dishes for 7 days at 28 ± 2 °C in the dark. The assay was repeated twice with three dishes with 10 seeds per dish for each treatment.

Results and Discussion

Total heavy metals in Gujranwala and Sialkot soils. Surface soil samples used in this study were collected from peri-urban area of Gujranwala and Sialkot being irrigated with untreated wastewater (Table 1). The wastewater was a mixture of cottage industries and domestic effluent. Soil organic matter content was 0.7-2.0 %. The soils were alkaline in reaction (pH from 7.0 to 8.5), non-saline (electrical conductivity from 0.3 to 1.2 dS/m) and calcareous in nature (lime contents from 1.5 to 15.2%). The concentrations of different metals in soil of the study areas were; Cd ranged from 2 to 8.4 mg/kg; Cu 60 to 380 mg/kg; Pb 205 to 250 mg/kg; Cr 80-330 mg/kg and Ni from 90 to 130 mg/kg. The total soil Cd, Cu, Cr, Pb and Ni content in almost all the soil samples were higher than the permissible limits, i.e., 3, 100, 100, 100 and 50 mg/kg, respectively, as proposed by FAO/WHO (2001). Heavy accumulation of these metals in the soils are results of their use in different industries like ceramics, sanitary fittings, electrical and gas appliances, detergent manufacturing, dry batteries, plastic-ware, kitchen-ware and tanneries. The elevated concentrations of heavy metals in the soils are most likely due to long-term continuous application of

Table 1. Geographical position of peri-urban sites of sample collection

Site	City	North	East
1	Gujranwala	32° 06'	74° 10'
2	Gujranwala	32° 07'	74° 10'
3	Gujranwala	32° 07'	74° 11'
4	Gujranwala	32° 09'	74° 11'
5	Sialkot	32° 28'	74° 30'
6	Sialkot	32° 29'	74° 32'

untreated municipal/ industrial effluent containing these heavy metals.

Screening and characterisation of Cd tolerant bacterial strains. In this study, a total of 30 soil bacteria were isolated from the heavy metal contaminated soils. Minimum inhibitory concentration of Cd was found 200 ppm. Similar results were also reported by Ansari and Malik (2007), who reported MIC of 200 ppm for Cd. Out of 30, 21 bacterial strains tolerated cadmium (Cd) up to 200 ppm, thirteen strains were moderately tolerant (400 ppm Cd) and only 4 strains (GS_2, GS_5, GS_{10} and GS_{20}) were found highly tolerant (600 ppm Cd).

The selected 4 bacteria were characterised morphologically and bacteria were also observed under microscope for cell shape (Table 2). Most of the strains had phosphorus solublisation and acid producing activity with bacillus cell shape and gram negative staining. None of the tolerant strain had IAA producing ability in the absence of L-tryptophan. IAA producing ability in the presence of L-tryptophan was not studied.

Results have shown that microorganisms in a contaminated environment could have adapted to that environment over a period of time. Piotrowska-Seget *et al*. (2005) also found in his study that, prolonged exposure of soil bacteria to Cd can develop resistance to its toxicity by activating the tolerance mechanism towards Cd. Most of the bacteria they studied were gram negative.

Optimum growth conditions, growth curve and tolerance index. The effect of pH on the growth of selected 4 bacterial isolates is shown in Fig. 1 and the effect of temperature is shown in Fig. 2. Optimum pH varied from 7.5 to 8.5 which was in accordance with the pH of the soils from which the strains were isolated (Mahmood-ul-Hassan *et al*., 2012). Regarding temperature, optimum growth of bacterial isolates was found at 30 °C ± 2. It shows that the selected bacteria are well adapted to soil as well as the climatic conditions of the regions from where they were isolated and can be reused in field conditions of the same ecology.

Growth curves of 4 highly Cd tolerant bacterial strains were made against different concentrations of Cd ranging from 0 to 600 ppm (Fig. 3). It is obvious from the result that at low concentration of Cd (200 ppm) the bacterial growth was high as compared to control; however, at 400 and 600 ppm Cd, the growth of all bacterial strains was also suppressed.

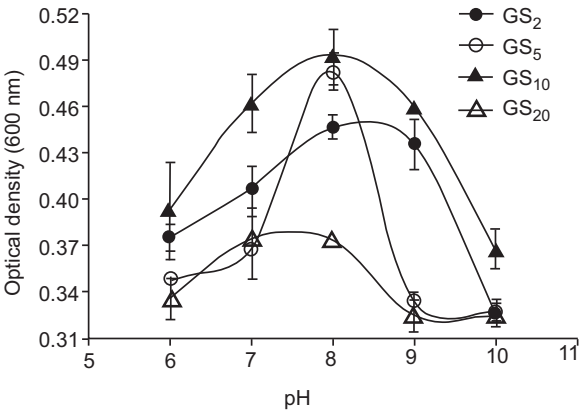

Fig. 1. Growth of Cd-tolerant bacteria as affected by different pH at 30 °C (N=3).

Table 2. Morphological, biochemical and microscopic characteristics of Cd-resistant bacterial strains

Characteristics	Bacterial strain			
	GS_2	GS_5	GS_{10}	GS_{20}
Colony shape	Filamentous	Filamentous	Filamentous	Irregular
Colony colour	Yellowish white	White	Yellowish white	Creamy
Elevation	Umbonate	Concave at centre	Concave at centre	Umbonate
Margin	Undulate	Lobate	Erose	Undulate
Cell shape	Bacillus	Bacillus	Coccus	Bacillus
Gram staining	-	-	-	-
P-solublising	+	-	+	+
Acid production	+	-	+	+
IAA-production	-	-	-	-

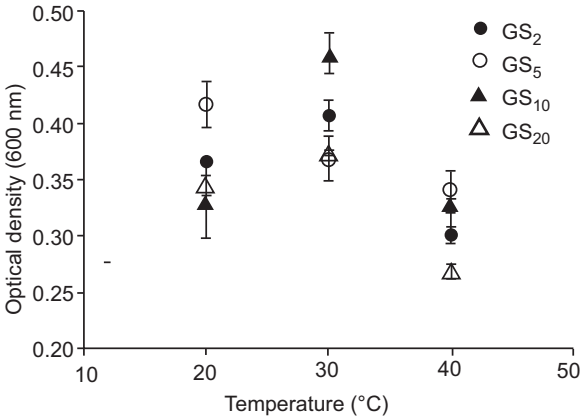

Fig. 2. Growth of Cd-tolerant bacteria as affected by different temperatures at pH 7 (N=3).

At 200 ppm Cd, optical density of GS_2, GS_{10} and GS_{20} showed an increase of 30, 12 and 13%, respectively, over control (0 ppm Cd). However, there was 7% reduction in case of GS_5. The results show that except GS_5 all other bacterial strains have so much adapted to Cd that they have bio-accumulated it to some extent. At 400 ppm Cd, growth of all the bacterial strains was reduced. GS_2 was found more tolerant with 10% reduction in growth and GS_5 least tolerant with 64% reduction. Reduction of growth in case of GS_{10} and GS_{20} was 35 and 55%, respectively, (Fig. 3).

Contrary to other bacterial strains, GS_2 again was found more tolerant at 600 ppm Cd with 33% reduction in growth. The growth of GS_5 at 400 and 600 ppm Cd was almost similar (64% reduction); however there was a sharper decrease in the tolerance indices of GS_{10} and GS_{20} at 600 ppm than at 400 ppm. There was up to 77% decrease in the growth of GS_{10} and GS_{20} at 600 ppm Cd (Fig. 3).

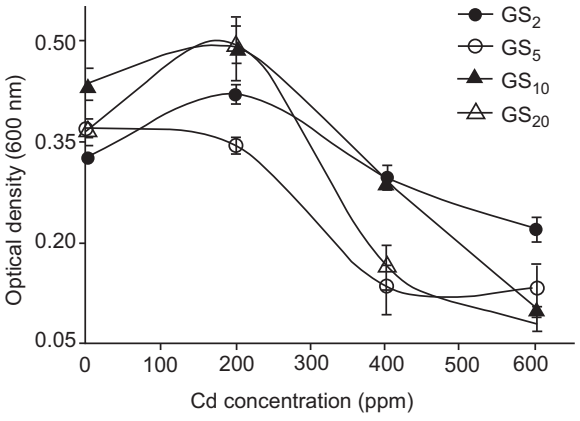

Fig. 3. Growth curve of Cd-tolerant bacteria as affected by different concentration of Cd at pH and temperature 30 °C (N=3).

Cadmium tolerance indices of all tested bacterial strains at different Cd concentrations are presented in Fig. 4. GS_2 being more tolerant among all strains showed highest tolerant index at highest concentration of 600 Cd. Tolerant indices of other bacterial strains revealed the order of tolerance as; $GS_5 > GS_{10} > GS_{20}$.

Findings of other researchers also revealed that, Cd has inhibitory effect on bacterial growth (Kalantri, 2008; Laddaga and Silver, 1985). Higher Cd concentration can reduce the activities of essential enzymes such as protease, urease, arylsulphatase and alkaline phosphatase (Lorenz *et al.,* 2006).

Root length promotion. The effects of 4 Cd-resistant bacterial strains on root elongation of maize variety ISD-gold in the absence of Cd is shown in Table 3. Addition of 50 mg/L Cd to the filter paper culture inhibited root elongation of un-inoculated seedlings by 33%. Inoculations with Cd-resistant bacteria in the absence and presence of Cd significantly increased the root length of maize seedlings over un-inoculated seedlings. The maximum root length promoting effect on Cd-treated plants was observed after inoculation with strains GS_2 (150% over control). It was followed by GS_{10} and GS_{20}; both produced 130% increase over control. The minimum increase in root length where seeds were treated with Cd was observed after inoculation with strains GS_5 (78%). Statistically similar trend was observed where seeds were inoculated in the absence of Cd.

Sheng and Xia (2006) and Belimove *et al*. (2005) also observed root growth promotion of Indian mustard inoculated with Cd resistant bacteria over un-inoculated seedlings in the presence of Cd. Rhizobacteria belonging

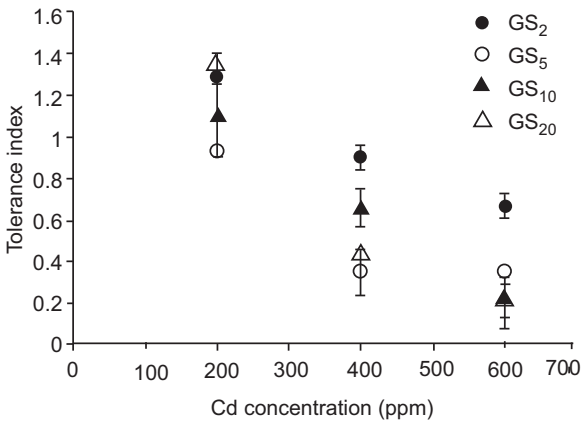

Fig. 4. Tolerance index of Cd-tolerant bacteria as affected by different concentration of Cd (N=3).

Table 3. Root length of maize seedlings inoculated with Cd-resistant bacterial strains grown in absence or presence of Cd in nutrient solution (N=3)

Bacterial strains	Untreated seedlings		Treated with 50 mg/L Cd	
	Root length (mm)	Bacterial effect (%)	Root length (mm)	Bacterial effect (%)
Uninoculated control	33.0[e]	-	22.4[d]	-
GS_2	92.1[a]	+176.6	56.0[a]	+150.0
GS_5	54.5[d]	+ 63.7	40.0[c]	+78.6
GS_{10}	63.0[b]	+ 89.2	51.5[b]	+129.9
GS_{20}	59.8[c]	+ 79.6	51.5[b]	+129.9
LSD	3.3	-	2.7	-

to different genera such as *Pseudomonas*, *Mycobacterium*, *Agro-bacterium* and *Arthrobacter* were found to have plant growth-promoting characteristics that can potentially support heavy metal uptake and reduce stress symptoms in plants (Dell'Amico *et al*., 2005). He *et al*., (2009) observed an increase in root growth and Cd contents in above ground tissues of hyperaccumulator tomato grown in Cd conta-minated soil when inoculated with two metal-resistant bacteria; *Pseudomonas* sp. and *Bacillus* sp. Both the bacteria were indole acetic acid and aminocyclopropane-1-carboxylate deaminase producers. Thus proliferation of root growth in metal contaminated soil either by presence of indole acetic acid or aminocyclopropane-1-carboxylate deaminase enzyme could lead to enhanced uptake of heavy metals in hyperaccumulator plants which could help in bioremediation.

Conclusion

Bacteria isolated from heavy metal-contaminated peri urban areas of Gujranwala have the ability to tolerate higher concentrations of Cd. Cadmium resistance potential and root growth promoting activity of these isolates demonstrated that, these bacteria could be used as a potential candidate in the bioremediation of Cd contaminated wastewater and soil.

Acknowledgement

The research work was financially supported by the Pakistan Agricultural Research Council through the 'Research for Agricultural Development Programme'; We thank Ghulam Haider, Riaz Ahmad and Ishfaq Ahmad for assistance in laboratory and field work.

References

Akhtar, S., Mahmood-ul-Hassan, M., Ahmad, R., Suthor, V., Yasin, M. 2013. Metal tolerance potential of filamentous fungi isolated from soils irrigated with untreated municipal effluents. *Soil & Environment,* **32:** 55-62.

Ansari, M.I., Malik, A. 2007. Biosorption of nickel and cadmium by metal resistant bacterial isolates from agricultural soil irrigated with industrial wastewater. *Bioresource Technology*, **98:** 3149-3153.

Atkinson, B.W., Bux, F., Kasan, H.C. 1998. Considerations for application of biosorption technology to remediate metal-contaminated industrial effluents. *Water Safety*, **24:** 129-35.

Belimov, A.A., Hontzeas, N., Safronova, V.I., Demchinskaya, S.V., Piluzza, G., Bullitta, S., Glick, B.R. 2005. Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). *Soil Biology and Biochemistry*, **37:** 241-250.

Benson, H.J. 1994. Microbiological Applications. *Laboratory Manual in General Microbiology*. W.C. Brown Publishers, Dubuque.

Brick, J. M., Bostock, R.M., Silverstone, S.E. 1991. Rapid in situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. *Applied and Environmental Microbiology*, **57:** 535-538.

Bueno, B.Y.M., Torem, M.L., Molina, F., de Mesquita, L.M.S. 2008. Biosorption of lead (II), chromium (III) and copper(II) by *R. opacus*: Equilibrium and kinetic studies. *Mineral Engineering*, **21:** 65-75.

Burnett, P.G., Handley, K., Peak, D., Daughney, C.J. 2007. Divalent metal adsorption by the thermophile *Anoxybacillus flavithermus* in single and multi-metal systems. *Chemistry and Geology*, **244:** 493-506.

Chibuike, G.U., Obiora, S.C. 2014. Heavy metal polluted soils: Effect on plants and bioremediation methods. *Applied and Environmental Soil Science*, **2014:** 1-12.

Ciba, J., Kolewicz, T., Turek, M. 1999. The occurrence of metals in composted municipal wastes and their removal. *Water Air and Soil Pollution*, **111:** 159-170.

Congeevaram, S., Dhanarani, S., Park, J., Dexilin, M., Thamaraiselvi, K. 2007. Biosorption of chromium and nickel by heavy metal resistant fungal and bacterial isolates. *Journal of Hazardous Material*, **146:** 270-277.

Dell'Amico, E., Cavalca, L., Andreoni, V. 2005. Analysis of rhizobacterial communities in perennial Graminaceae from polluted water meadow soil, and screening of metal-resistant, potentially plant growth-promoting bacteria. *FEMS Microbiology Ecology*, **52:** 153-162.

Dhaliwal, G.S., Kukal, S.S. 2005. *Essentials of Environmental Scienc*e. 369 pp., Kalyani Publishers, New Delhi, India.

Dupree, M.R., Wilcox, G. 1977. Bromothymol blue and carbohydrate sensitive plating media. *Journal of Clinical Microbiology*, **6:** 343-347.

El-komy, H.M. A. 2005. Coimmobilization of *Azospirillum lipoferum* and *Bacillus megaterium* for successful phosphorus and nitrogen nutrition of wheat plants. *Food Technology and Biotechnology*, **43:** 19-27.

Ezzouhri, L., Castro, E., Moya, M., Espinola, F., Lairini, K. 2009. Heavy metal tolerance of filamentous fungi isolated from polluted sites in Tangier, Morocco. *African Journal of Microbiology Research,* **3:** 35-48.

FAO/WHO, 2001. *Codex alimentarius commission. Food additives and contaminants.* Joint FAO/WHO Food Standards Program 2001. FAO Viale delle Terme di Caracalla, Rome, Italy.

Hamzah, W.N., Arifin, W.M., Khoo, K.S., Lee, L.J. Sarmani, S.B. 2009. Screening of biosorption bacteria tolerance towards copper and cadmium from oil sludge pond. *Journal of Radioanalytical and Nuclear Chemistry*, **281:** 295-298.

He, L.Y., Chen, Z.J., Ren, G.D., Zhang, Y.F., Qian, M., Sheng, X.F. 2009. Increased cadmium and lead uptake of a cadmium hyperaccumulator tomato by cadmium-resistant bacteria. *Ecotoxicology and Environmental Safety*, **72:** 1343-1348.

Hussain, I. 2006. Investigation of heavy metals Cu, Pb, Cd, Mn, Cr, Fe and Ni in *Ammi visnaga. Journal of Chemical Society of Pakistan,* **28:** 241-245.

Kalantri, N. 2008. Evaluation of toxicity of iron, chromium and cadmium on *Bacillus cereus* growth. *Iranian Journal of Basic Medical Sciences,* **10:** 222-228.

Khan, A., Javed, S., Muhmood, A., Majeed, T., Niaz, A., Majeed, A. 2013. Heavy metal status of soil and vegetables grown on peri urban area of Lahore district. *Soil & Environment*, **32:** 49-54.

Khan, M.S., Zaidi, A., Wani, P.A., Oves, M. 2009. Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. *Environmental Chemistry Letters*, **7:** 1-19.

Kotrba, P., Mackova, M., Macek, T. 2011. *Microbial Biosorption of Metals*. Springer, New York, USA.

Laddaga, R.A., Silver, S. 1985. Cadmium uptake in *Escherichia coli* K-12. *Journal of Bacteriology*, **162:** 1100-1105.

Lorenz, N., Hintemann, T., Kramarewa, T., Katayama, A., Yasuta, T., Marschner, P., Kandeler, E. 2006. Response of microbial activity and microbial community composition in soils to longterm arsenic and cadmium exposure. *Soil Biology and Biochemistry*, **38:** 1430-1437.

Mahmood-ul-Hassan, M., Suthar, V., Rafique, E., Ahmad, R., Yasin, M. 2012. Metal contamination of vegetables grown on soils irrigated with untreated municipal effluent. *Bulletin of Environmental Contamination and Toxicology*, **88:** 204-209.

McGrath, S.P. 1995. Long term effects of metals in sewage on soils, microorganisms and plants. *Journal of Industrial Microbiology,* **14:** 94-104.

Pepper, I.L., Gerba, C.P. 2004. Dilution and plating of bacteria and growth curves. In: *Environmental Microbiology; A Laboratory Manual*. pp. 3-10. Elsevier Academic Press, USA.

Piotrowska-Seget, Z., Cycon, M., Kozdroj, J. 2005. Metal-tolerant bacteria occurring in heavily polluted soil and mine spoil. *Applied Soil Ecology*, **28:** 237-246.

Sanita di Toppi, L., Gabrielli, R. 1999. Response to cadmium in higher plants. *Environmental and Experimental Botany*, **41:** 105-130.

Singh, G., Bhati, M. 2005. Growth of *Dalbergia sissoo* in desert regions of western India using municipal effluent and the subsequent changes in soil and plant chemistry. *Bioresource Technology*, **9:** 1019-1028.

Sheng, X.F., Xia, J.J. 2006. Improvement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. *Chemosphere*, **64:** 1036-1042.