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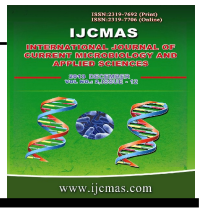

Original Research Article

The influence of heavy metals toxicity on the antioxidant enzyme activities of resistant *E. coli* strains isolated from waste water sites

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K e y w o r d s

Antioxidant enzymes; Heavy metals; Oxidative stress.

A B S T R A C T

The potential of the antioxidant enzyme Catalase (CAT), Peroxidase (POX) and Ascorbate peroxidase (APX) of four *Escherichia coli* resistant strains isolated from a heavy metal-polluted area. Resistance previous strains for heavy metals Cr^{6+}, Cr^{3+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} toxicity has been explored in this study. The key effects of such metals particularly chromium on each enzyme were increased into different categories. Additional studies on isoenzyme patterns of CAT, POX, APOX in addition to Glutathione reductase (GR), Superoxide dismutase (SOD), Lipid peroxidation and Potassium efflux of *E. coli* ASU 7 showed generally an increasing under the exposure effect to hexa- and trivalent chromium and this increase consider as a certain protection against heavy metals. The results of this study showed that antioxidant enzymes can be used as biomarkers of heavy metals pollution

Introduction

Heavy metals are toxic because they interfere with the normal biochemical reactions of the human body. The release of heavy metals into the environment causes an environmental pollution problem because they are nonbiodegradable and hence accumulate in living organisms. Heavy metals, H_2O_2 and other pollutants increase the production of reactive oxygen species (ROS) such as $O_2^{\cdot -}$, $OH^{\cdot}$, H_2O_2 (Abassi, 1998). The importance of antioxidant enzymes is generally emphasized in preventing oxidative stresses by scavenging ROS or reduction of oxidized glutathione to reduced form (Lenártová *et al.*, 1998). Superoxide radicals ($O_2^{\cdot -}$) generated is converted to H_2O_2 by the action of Superoxide dismutase (SOD). The accumulation of H_2O_2 is prevented in the cell by Catalase (CAT), Peroxidase (POX) or by the ascorbate glutathione cycle where Ascorbate Peroxidase (APOX) reduces it to H_2O (Harinasut, 2003). Glutathione reductase is produced by *Streptococcus bovis* and

Selenomonas ruminantium as a result of mercury stress to eliminate the toxic effect of oxidized glutathione (Lenártová *et al.*, 1998). Cadmium and copper increased plasma membrane permeabilization, with associated cellular K^+ efflux has been extensively reported for the yeast *Saccharomyces cerevisiae* (Howlett and Avery, 1997).

The objectives of this work are isolation and identification strains of *Escherichia coli*, testing the resistance of these strains to different heavy metals and the influence of heavy metals particularly chromium on antioxidant defense system, lipid peroxidation and K^+ efflux of *Escherichia coli* resistant strains

Materials and Methods

Isolation and identification of *E. coli* isolates

E. coli was enumerated from wastewater samples by MPN techniques using lactose broth medium at 37 ºC for 48 hours (Mac Faddin, 1985). Positive presumptive tubes showed acid and gas. The purified isolates were confirmed on E.M.B agar medium. Metalic green isolates were selected randomly, preserved on nutrient agar for further studies and identified according to Bergey's manual (Bergey's manual of systematic bacteriology, 2005).

Examination of heavy metal resistance for the potent strains of *E. coli*

Two hundred µl (0.9 at O.D 600 nm) of overnight isolates were growing in tris minimal broth medium and assayed of their resistance on agar plates cotaining the following concentrations of different heavy metal salts Cr^{6+} (1 & 2 ppm), Cr^{3+} (20) ppm, Co^{2+} (20, 25 & 40) ppm, Cu^{2+} (10 & 20) ppm, Ni^{2+} (10, 25 & 50) ppm, Zn^{2+} (100) ppm, Cd^{2+} (50 & 100) ppm, Pb^{2+} (100 & 200) ppm (Mergeay, 1995; Versalovic, 2011) . In the case of lead toxicity test, the amount of sodium β-glycerophosphate was reduced to 0.0125g/l, tris buffer was omitted and pH adjusted to 6.0 to limit metal complexations and precipitations.

Determination of bacterial proteins of *E. coli* ASU 7

Two hundred µl (0.9 at O.D 600 nm) of *E. coli* ASU 7 was cultivated in tris minimal medium containing different concentrations (0 , 1, 5, 10; 0, 20, 30, 40 ppm) Cr^{6+} and Cr^{3+} incubated at 37 °C with agitation at 150 rpm for 12 hr, respectively. The bacterial cells were centrifuged at 10.000 rpm and the pellet was washed three times with saline solution NaCl 0.9% then dried at 50 °C over night. The dried cells (0.05g) were boiled for 1 hour in 10 ml 0.1 N NaOH. After cooling, the total proteins content in the supernatant were determined according to the method adopted by (Lowry *et al.*, 1951). Assay the activity of antioxidant enzymes and their visualization by native PAGE. Two hundred µl (0.9 at O.D 600 nm) of *E. coli* ASU 7 was grown in tris liquid minimal medium for 24 hr at 37 ºC. The individual heavy metal was added after 3 h of incubation. After incubation, about (0.05g) cells were harvested, washed three times with saline solution NaCl 0.9% and ground with mortar & Pestle in liquid nitrogen by using 50 mM Potassium Phosphate buffer (PH 7.5) containing 2 mM EDTA, 0.1% W/V polyvinylpyrrolidone (PVP) and 0.1% W/V ascorbic acid. The extract was centrifuged at 10,000 x g for 10 min at 4°C and the supernatant was used for

measuring the activity of antioxidant enzymes. Native PAGE in 7.5 % gel was carried out by the method of Davis (Davis, 1964). A hundred µg of proteins were loaded. 50 volt was applied during electrophoresis for 8 hours.

A modified method of Abassi (Abassi *et al.*, 1998) was employed for the assay of catalase by monitoring the decrease in absorbance at 240 nm. Using the extinction coefficient (ϵ = 39.4/mM/cm). Isoenzyme gel was stained as described by Woodbury (Woodbury *et al.*, 1971).

Peroxidase (POX) activities were determined by the increase in absorbance at 470 nm using the extinction coefficient (ϵ = 26.6/mM/cm) (Cakmak and Marschner, 1992). Isoenzyme gel was stained as described by Sidhu et al 1984 (Sidhu *et al.*, 1984). Ascorbate peroxidase (APOX) was assayed by the decrease in absorbance at 290 nm (Dalton *et al.*, 1987) using the extinction coefficient (ϵ = 2.8/mM/cm). Isoenzyme gel was stained as described by Mittler and Zilinskas (Mittler and Zilinskas, 1993). Glutathione reductase activity was determined by using the oxidation of NADPH at 340 nm extinction coefficient (ϵ = 6.2 mM^{-1} cm^{-1}) as described by Jiang and Zhang (Jiang and Zhang, 2002). Isoenzyme gel was stained according to the method of Kang et al. (Kang *et al.*, 1999).

SOD activity was assayed at 480 nm by determing the autoxidation of epinephrine (adenochrome) as described by Misra and Fridovich (Misra and Fridovich, 1972) using the extinction coefficient of (ϵ = 4020 M^{-1} cm^{-1}). Activity is reported as µmol adenochrome/ min/mg protein. Isoenzyme gel was stained according to the method of Butow et al., 1997 (Butow *et al.*, 1997). All enzymes were calculated according to the Beer Law (Whiteley and Lee, 2006):

$$\text{Activity} = \Delta A \frac{V}{\varepsilon t v}$$

Where ΔA is the change in absorbance, V is the total volume of assay mixture, *v* is the volume of the sample, *ε* the extinction coefficient in ml $\mu mol^{-1}$and *t* the time in min. The units of enzyme activity per mass of protein are referred to as the specific activity and the amount of protein in the biomass.

Determination of Potassium efflux

Asset of Erlenmeyer flask containing 100 ml of tris minimal medium at concentrations (0, 10 ppm and 0, 40 ppm) of Cr^{6+} and Cr^{3+} respectively, were inoculated with 200 µl of preculture of isolate. Erlenmeyer flask was incubated at 37 °C with agitation at 150 rpm for 8 h. 5 ml aliquots were removed and centrifuged for 10 min and then the supernatant was diluted with 4 volumes of distilled deionized water. Extra-cellular K^+ was measured during revealing times 3, 4 and 6 h by Jenway PFP7 flame photometer (Williams, 1960).

Determination of lipid peroxidation

The level of lipid peroxidation was measured by determination of malondialdehyde (MDA) a breakdown product of lipid peroxidation. MDA content was determined with thio-barbituric acid (TBA) reaction. Briefly 0.25 g of fresh cells was homogenized in liquid nitrogen in 5 ml 0.1% Trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 rpm for 5 min. To 1 ml aliquot of the supernatant 4 ml of 20% TCA containing

0.5% TBA were added. The mixture was heated at 95 °C for 15 min and cooled immediately. The developed colour was extracted with 2 ml n-butanol and the absorbance was measured at 532 nm. The level of lipid peroxidation was measured at revealing times 3, 4 and 8 h and expressed as nmol of MDA using an extinction coefficient of 155 mM cm^{-1} (Zhao, 1994).

All stained gels were densitometry scanned and analysis with The LabImage 1D (2006) software produced by KAPELAN (Germany) for molecular weight determination and retention factor.

Statistical analysis

All experiments are repeated three times. Data were analyzed using one way analysis of variance (ANOVA) with differences determined using Duncan´s test by SPSS 10.0 software. Differences were considered to be significant among means ±SE standard error (n=3) at a probability of ($P<0.05$).

Results and Discussion

Heavy metal resistance on *E. coli* isolates

Heavy metal resistance can be defined as the ability of microorganism to withstand the effect of heavy metals. Four resistance *E. coli* isolates ASU 3, 7, 8 and 18 were isolated to determine the CAT, POX and APOX enzymes as described in Table (1); Isolate ASU 7 is the most resistant isolate to heavy metals. It is resistance to 200 ppm of Pb^{2+}, 100 ppm of Zn^{2+} and Cd^{2+}, 25 ppm of Ni^{2+}, 20 ppm of Co^{2+}, Cu^{2+}, $Cr^{3+}$and 1 ppm of Cr^{6+}. The order of toxicity of the latter isolate is $Cr^{6+} > Co^{2+} = Cu^{2+} = Cr^{3+} > Ni^{2+} > Cd^{2+} = Zn^{2+} > Pb^{2+}$. This finding comes in accordance to the results obtained by Mergeay, 1995 and hassen et al., 1998 (Mergeay, 1995; Hassen *et al.*, 1998; Hassen A., 1998).

Effect of Cr^{6+} and Cr^{3+} on protein content of *E. coli* ASU 7

The effect of Cr^{6+} and Cr^{3+} on protein content of *E. coli* ASU 7 was studied as depicted in Fig. (1) The data described that different concentrations of Cr^{6+} were more toxic than Cr^{3+}. The protein content of *E. coli* ASU 7 expressed in (µg/g dry wt) was inhibited by the addition of different concentration of Cr^{6+}. It corresponds to 32.3, 40 and 58.3% at 1, 5 and 10 ppm, while the different concentration of Cr^{3+} reached about 14.9%, 28.46% and 30.46% at 20, 30 and 40 ppm respectively. These results are in agreement with hassen et al. (Hassen *et al.*, 1998) who stated that heavy metals toxicity results mainly from their ability to denaturate protein molecule. Similarly Jianlong (Jianlong *et al.*, 2004) who concluded that concentration of 10 µM chromium had a significant inhibitory effect on protein biosynthesis, the percentage of inhibition reached about 32%, compared with the control experiment and higher chromium concentration resulted in more serious inhibitory effect.

Effects of some heavy metals on the activities of three antioxidant enzymes (Cat, POX, APOX) of *E. coli* strains

Among all the metals, chromium (hexa and trivalent) show the biggest element capable to increase the activities of CAT, POX and APOX than other elements especially in *E. coli* ASU 7. The percentage of enzymes activities were increased and classified into three categories high (>1086), moderate (544 to 1085) and low (106 to 543) under stress of (Cr^{6+}, Cr^{3+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} & Cd^{2+},

Table.1 Response of *E.coli* resistant strains to different concentrations of heavy metals

Strains ASU	Heavy metals Resistance PPM (mM)							
	Ni^{2+}	Co^{2+}	Zn^{2+}	Cd^{2+}	Cu^{2+}	Cr^{6+}	Cr^{3+}	Pb^{2+}
3	50 (0.850)	25 (0.420)	100 (1.52)	100 (0.880)	10 (0.15)	1 (0.019)	25 (0.48)	100 (0.48)
7	25 (0.425)	20(0.336)	100 (1.52)	100 (0.88)	20 (0.3)	1 (0.019)	20 (0.384)	200 (0.96)
8	25 (0.425)	40 (0.672)	100 (1.52)	100 (0.88)	20 (0.3)	1 (0.019)	20 (0.384)	50 (0.24)
18	10 (0.170)	40 (0.672)	100 (1.52)	50 (0.44)	20 (0.3)	2 (0.038)	20 (0.384)	200 (0.96)

Pb^{2+}) as described in Table (2).The activity of enzymes were μmol / min^{-1} / gm protein. Peroxidases are widely accepted as 'stress enzymes' (Gaspar, 1991). Increasing the activity in peroxidase has been documented under a variety of stressful conditions such as Drought-stress (Zhang and Kirkham, 1994) and under toxic levels of Al, Cu, Cd, Zn (Chaoui *et al.*, 1997; Shah *et al.*, 2001; Hussein, 2013).

Effect of Cr^{6+} and Cr^{3+} on activities and isoenzyme patterns of CAT, POX, APOX, GR and SOD

Heavy metals such as chromium are subjected to auto-oxidation or enzymatic oxidation, resulting in the formation of reactive intermediates, mainly radicals that react with oxygen, producing the well-known reactive oxygen species (ROS). Antioxidant enzymes play a great role for scavenging of ROS (Kang *et al.*, 1999; Sharma, 2012). Catalases are involved as one of the mechanisms used to protect cells against the damage caused by ROS to cellular components including nucleic acids, lipids and proteins (Imlay, 2002).

FIG (2) described the zygogram of native-PAGE of catalase, The data revealed multiple forms under different stress concentrations of Cr^{6+} (1 and 5 ppm) and Cr^{3+} (20 and 30 ppm). For CAT activity, there was only a single band in control, while two or three bands under stress of 1 or 5 ppm of Cr^{6+}, respectively. In case of Cr^{3+} two isoenzymes were appeared and the intensity of bands was increased in all treatments with 15 to 30 fold more than control. Banjerdkij et al., (Banjerdkij *et al.*, 2005) stated that the exposure of *Xanthomonas campestris* to 75 μM $CdCl_2$ for 30 min increased CAT for 10 fold.

Changes of POX activity and isozyme patterns under environmental stresses have been suggested as indicators for biotic or a biotic stresses (Lee, 1997; PAKNIYAT, 2010; Tian *et al.*, 2012). Fig (3) shows the zygogram of native-PAGE of POX. It revealed multiple forms of peroxidase under different stress concentrations of Cr^{6+} (1, 5 and 10 ppm) and Cr^{3+} (20, 30 and 40 ppm). There was only a single enzyme band of POX III for peroxidase activity in the control, 1ppm of Cr^{6+} and 30 ppm of Cr^{3+} but two isoenzymes of

POX III and POX II appear under stress of 10 ppm of Cr^{6+} and 40 ppm of Cr^{3+} also two isoenzymes of (POX III and POX I) appear under 5 ppm of Cr^{6+}. Intensity of bands was increased in all treatments with half to two and half fold more than control. The level of POX III isozyme patterns in marine alga *Nannochsloropsis oculata* to Cd stress were increased notably by enhanced band intensity (Lee and Shin, 2003). Fig (4) depicts the zygogram of native-PAGE of ascorbate peroxidase under the above stress concentrations of Cr^{6+} and Cr^{3+}. Our result depicted that only a single enzyme band was appeared and its intensity increased in all treatments with two to four fold more than control. (Lee and Shin, 2003) observed that APOX isoenzyme was increased after cadmium treatments in marine alga *Nannochloropsis oculata*. glutathione reductase is needed to maintain a high ratio of reduced glutathione from oxidized glutathione (Kushkevych *et al.*, 2011), as the latter has been shown to be toxic, and this key role has been demonstrated by analysis of fungi mutants lacking the enzyme (Grant *et al.*, 1998). Fig (5) shows the zygogram of native-PAGE of glutathione reductase which catalyses the NADPH-dependent reduction of oxidized glutathione under the above stress concentrations of Cr^{6+}, Cr^{3+}. There were two isoenzymes bands

Figure.1 Toxic effect of Cr^{6+} and Cr^{3+} on the total protein content of *E. coli* ASU7

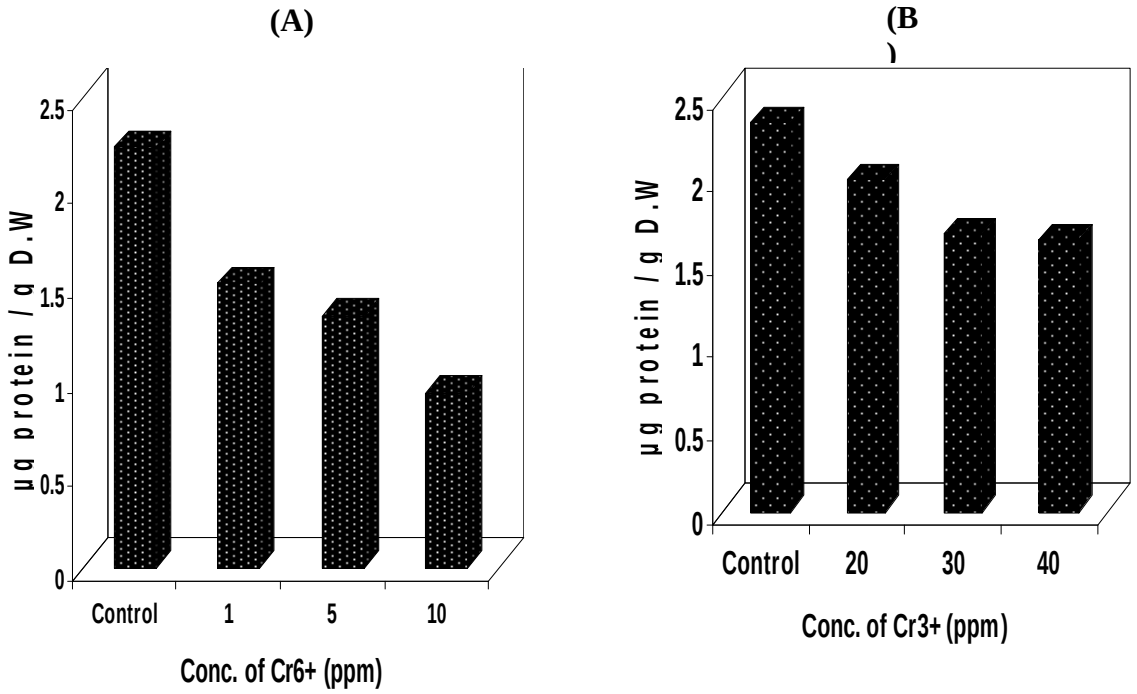


Table.2 The effect of heavy metals (Cr^{6+}, Cr^{3+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} & Pb^{2+}) on the activity of antioxidant enzymes (Cat, POX & APOX) produced by different strains of *E. coli* ASU (3, 7, 8, 18).

Strain No.	Metals ppm	Metals mM	Catalase †	%	Peroxidase †	%	Ascorbate Peroxidase †	%
ASU 3		Contr	0.584a	(100)	1.449a	(100)	47.33a ±0.6	(100)
	Cr^{6+} (1)	0.019	3.620b	(619.8**	2.8b +0.13	(194*)	534.9c +0.23	(1130*
	Cr^{3+}(25	0.48	1.907a	(326*)	1.77a +0.02	(119.6*	102.3c +2.06	(215*)
	Co^{2+}(2	0.42	6.928d	(1186***	4.839c +0.2	(334*)	259.5b +3	(548**
	Cu^{2+}(1	0.15	6.140cd	(1051**)	0.898a +0.1	●[61.9]	666.3d +62	(1407*
	Ni^{2+}	0.85	5.650c	(967**)	2.71b +0.1	(187*)	122a +48	(257*)
	Zn^{2+}(1	1.52	1.415a	(242*)	0.398a	●	20.5a +2.6	●
	Cd^{2+}(1	0.88	0.543	● [92.9]	0.912a	●	24.6a +3.4	●
	Pb^{2+}(1	0.48	1.002a	(171*)	1.67a +0.03	(115.8*	37.9a +1.9	● [80]
ASU 7		Contr	0.488a	(100)	0.257c	(100)	18.967a +2	(100)
	Cr^{6+}(1)	0.019	2.318b	(408*)	2.82a +0.1	(1097*	194.99d +8.1	(1028*
	Cr^{6+}(5)	0.096	6.648d	(1134**	6.52a +0.5	(2540*	134.6bc +19	(709**
	Cr^{6+}(10	0.19	-	-	3.75a +0.03	(1463*	583.33e +60	(3086*
	Cr^{3+}(20	0.38	2.77c +0.2	(570**)	0.75a	(294*)	29.83a +0.7	(192.9*
	Cr^{3+}(30	0.57	11.6e +0.6	(1968**	1.317a	(512*)	129.2bc +4	(657**
	Cr^{3+}(40	0.76	-	-	6.87a +0.5	(2675*	169.41cd +7	(866**
	Co^{2+}(2	0.33	6.15c +0.5	(1276**	0.47a +0.01	(186*)	27.42a +0.2	(144*)
	CU^{2+}(2	0.31	1.05a +0.3	(215*)	1.94d +0.2	(757**	170.42cd	(898**
	Ni	0.42	3.91d	(801**)	1.33b +0.16	(521*)	103.37b +6.8	(544**
	Zn^{2+}(1	0.31	2.01ab	(411*)	0.337a	(134*)	26.86a +0.2	(141*)
	Cd^{2+}(1	0.88	1.98ab	(405*)	0.59b +0.03	(233*)	106.49b +1.7	(561**
	Pb^{2+}(2	0.96	1.61ab	(329*)	0.407c	(161*)	128.29bc	(676**
ASU 8		Contr	0.613a	(100)	0.478a	(100)	65.133ab	● (100)
	Cr^{6+}(1)	0.019	3.463b	(564.9**	9.52b +1	(1993*	253.93d +7	(389.9*
	Cr^{3+}(20	0.38	5.373c	(877**)	0.355a	●	19.46a +0.8	●[30.1
	Co^{2+}(4	0.67	1.323a	(215*)	0.509a	(106*)	102.63ab	(157*)
	CU^{2+}(2	0.31	1.427a	(323*)	0.351a	●	123.23c +2.8	(189*)
	Ni^{2+}(25	0.42	12.76d	(2082**	0.872a	(182*)	105.93b +0.6	(162*)
	Zn^{2+}(1	1.52	1.483a	(241*)	1.243a	(260*)	17a +0.3	●[26.1]
	Cd^{2+}(1	0.88	2.637a	(430*)	1.607a	(336*)	65.133b +5.8	(157*)
	Pb^{2+}(5	0.24	1.479a	(241*)	1.723a	(360*)	83.83a +1.1	(128*)
ASU 18		Control	0.775a	(100)	0.381a	(100)	122.133a +3	(100)
	Cr^{6+}(2)	0.038	3.57a +0.2	(461.2*)	6.54c +0.2	(1818*	273.03b +3.2	(223.5*
	Cr^{3+}(20	0.38	1.89c	(245*)	0.101a	(126*)	109.86a +4.1	●
	Co^{2+}(4	0.67	0.04	● [5.93]	0.391a	(202*)	94.96a +2.4	[77.75]
	CU^{2+}(2	0.31	1.73a +0.1	(223*)	0.828b	(317*)	395.16c	(291*)
	Ni^{2+}(10	0.17	2.51b	(325*)	0.919b	(341*)	318.43b	(260*)
	Zn^{2+}(1	1.52	0.41a	● [52.9]	0.27a +0.03	●	28.43a +0.8	●
	Cd^{2+}(5	0.44	2.76b	(356*)	0.08a	(120*)	78.33a +3	●
	Pb^{2+}(2	0.96	2.7b	(349*)	0.11a	(128*)	31.65a +0.92	●

The ANOVA was carried out by using SPSS 10.0 comparisons among means ±SE standard error (n=3), different letters show significance according to Duncan test (P<0.05).

*** High activity % (>1086) ** Moderate activity % (544 to 1085) * Low activity % (106 to 543). - No activity ● [ ] Data represent activity less than control [< 100].

Data in Parenthesis represent % of Catalase, Peroxidase and A scorbate Peroxidase.

Figure.2 Percentage of catalase under stress of 1, 5 ppm Cr^{6+} and 20, 30 ppm Cr^{3+} **(A)** Native PAGE (7.5 %) of catalase activity of *E. coli* ASU 7, (1) Control; (2) 1 ppm Cr^{6+}; (3) 5 ppm Cr^{6+}; (4) 20 ppm Cr^{3+}; (5) 30 ppm Cr^{3+}; respectively **(B)**. (►) Arrowheads point at some of the new bands that appeared during stress of hexa- or trivalent chromium.

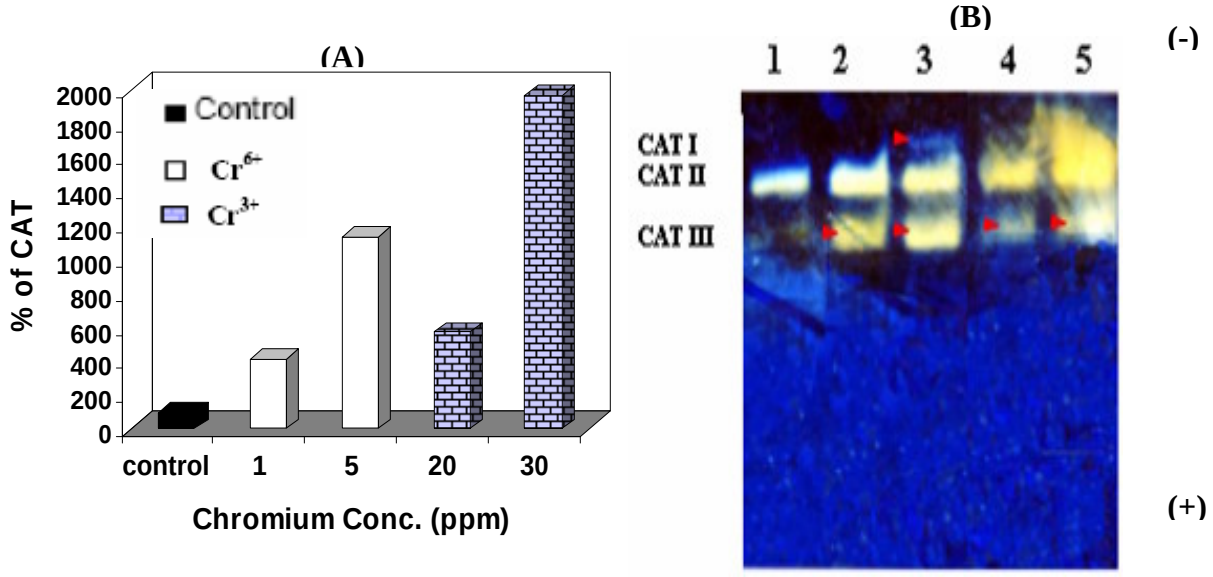


Figure.3 Percentage of peroxidase under stress of 1, 5, 10 ppm Cr^{6+} and 20, 30, 40 ppm Cr^{3+} **(A)** Native PAGE (7.5 %) of peroxidase activity of *E. coli* ASU 7, (1) Control; (2) 1 ppm Cr^{6+}; (3) 5 ppm Cr^{6+}; (4) 10 ppm Cr^{6+}; (5)20 ppm Cr^{3+}; (6) 30 ppm Cr^{3+}; (7) 40 ppm Cr^{3+}, respectively **(B)**. (►) Arrowheads point at some of the new bands that appeared during stress of hexa- or trivalent chromium.

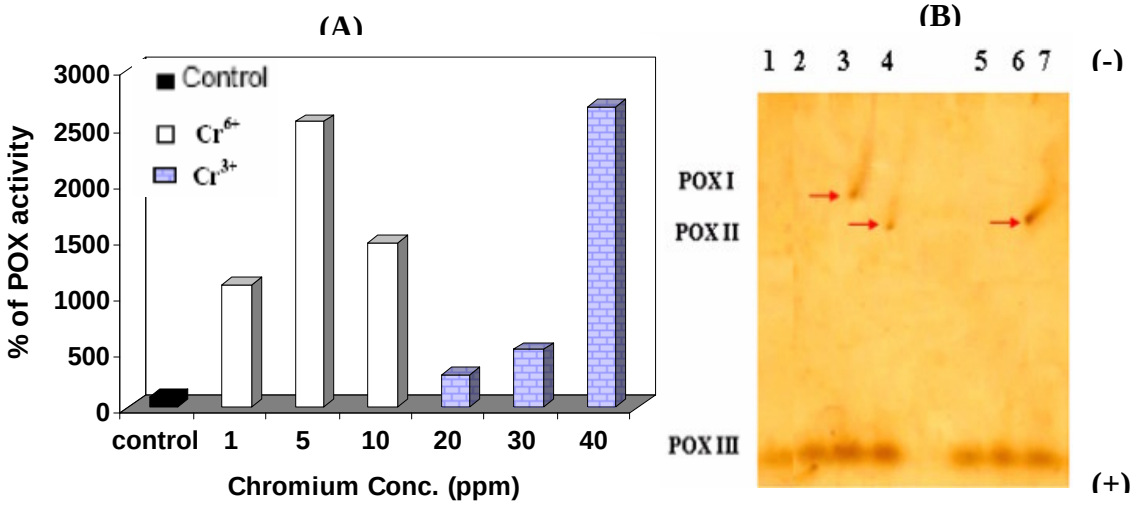


Figure.4 Percentage of ascorbate peroxidase under stress of 1, 5 and 10 ppm Cr^{6+} and 20, 30 and 40 ppm Cr^{3+} **(A)**. Native PAGE (7.5 %) of ascorbate peroxidase activity of *E. coli* ASU 7. (1) Control ; (2) 1 ppm Cr^{6+}; (3) 5 ppm Cr^{6+}; (4) 10 Cr^{6+} ppm; (5) 20 ppm Cr^{3+}; (6) 30 ppm Cr^{3+}; (7) 40 ppm Cr^{3+}; respectively **(B)**. (►) Arrowheads point at some of the new bands that appeared during stress of hexavlent or trivalent Chromium.

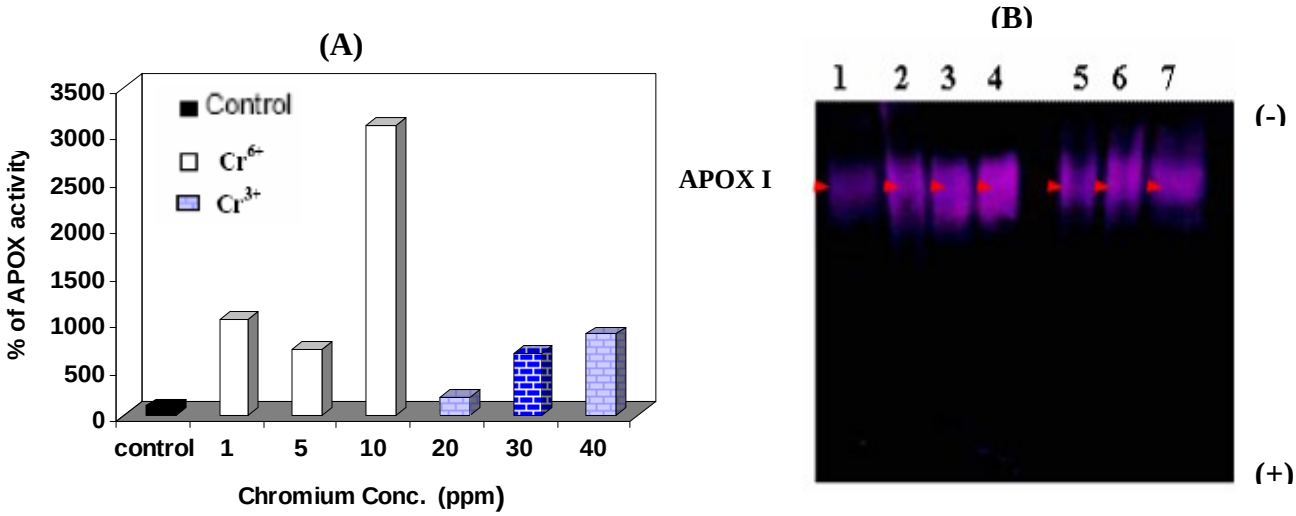


Figure.5 Percentage of glutathione reductase under stress of 1, 5 and 10 ppm Cr^{6+} and 20, 30 and 40 ppm Cr^{3+} **(A)**. Native PAGE (7.5 %) of glutathione reductase activity of *E. coli* ASU 7. (1) Control ; (2) 1 ppm Cr^{6+}; (3) 5 ppm Cr^{6+}; (4) 10 ppm Cr^{6+}; (5) 20 ppm Cr^{3+}; (6) 30 ppm Cr^{3+}; (7) 40 ppm Cr^{3+}, respectively **(B)**. (►) Arrowheads point at some of the new bands that appeared during stress of hexa- or trivalent chromium.

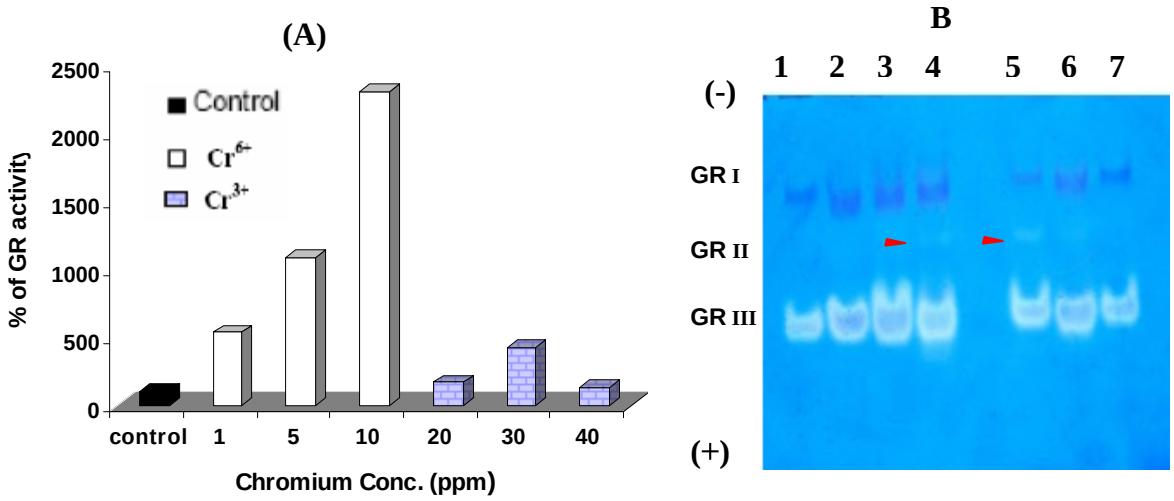


Figure.6 Percentage of superoxide dismutase activities under stress of 20, 30 and 40 ppm Cr^{3+} **(A)**. Native PAGE (7.5 %) of superoxide dismutase activity of *E. coli* ASU 7. Lane 1 Control ; Lane 2, 1 ppm Cr^{6+}; Lane 3, 5 ppm Cr^{6+}; Lane 4, 10 ppm Cr^{6+}; Lane 5, 20 ppm Cr^{3+}; Lane 6, 30 ppm Cr^{3+}; Lane 7, 40 ppm Cr^{3+}, respectively **(B)**.

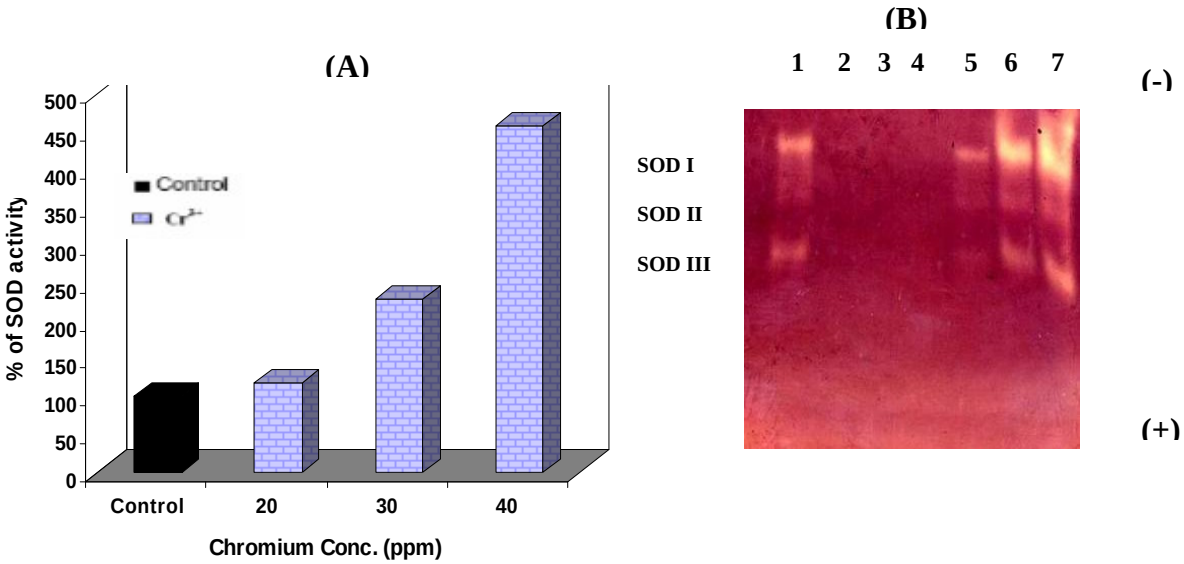
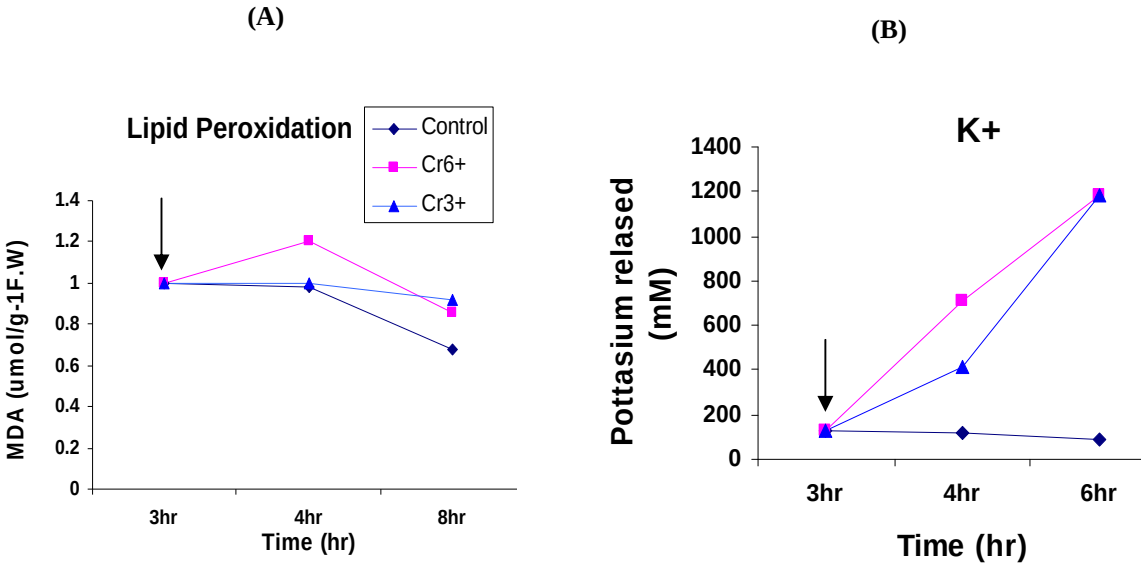


Figure.7 Lipid peroxidation (A) and Potassium Efflux (B) under Cr^{6+} and Cr^{3+} stress of 40 and 10 ppm, respectively, at different times.



GR I and GR III represent in control, 1, 5 ppm of Cr^{6+} and 30, 40 ppm of Cr^{3+} but three isoenzymes GR I, GR II and GR III bands were appeared under stress of 10, 20 ppm of Cr^{6+}, Cr^{3+}, respectively. The intensity of enzyme bands increased with 10 to 30 fold more than control. Lenartova et al., (Lenártová *et al.*, 1998) reported that glutathione reductase was produced by *Streptococcus bovis* and *Selenomonas ruminantium* under mercury stress.

Fig (6) shows the zygogram of native-PAGE of Superoxide dismutase under the above stress concentrations of Cr^{6+} and Cr^{3+}. In case of Cr^{6+}, SOD was inhibited by all concentrations, this agreed with Banjerdkij et al., (2005) who stated that cadmium failed to increase the level of total SOD activity in *Xanthomonas campestris*. There were three isoenzymes band of SOD I, II, III in all treatments of Cr^{3+}. The intensity of enzyme bands increased with two to 10% fold more than control. This agreed with (Lenártová *et al.*, 1998) who considered that SOD is the first line of defense against the generation of toxic oxygen species activity. He shows that *Streptococococcus bovis* increased when incubated with 5 µg Hg^{2+} ml^{-1} in anaerobic or aerobic conditions.

Hexa-and trivalent chromium increase lipid peroxidation and K^+ efflux in *E. coli* ASU7. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) upon decomposition. Measurement of malondialdehyde and 4-hydroxyalkenals has been used as an indicator of lipid peroxidation (van Ginkel and Sevanian, 1994). Lipid peroxidation was evaluated as MDA production. The concentrations of (10 ppm) and (40 ppm) of Cr^{6+} and Cr^{3+}, respectively increased MDA production during different time 3, 4 and 8 h (Fig. 7A). Fig (7B) shows that the release k^+ was markedly higher in Cr^{6+} (10 ppm) more than Cr^{3+} (40 ppm) compared with control in different times 3, 4 and 6 h. (Halliwell and Gutteridge, 1991) stated that toxicity of heavy metals to higher organisms has been inferred from observations of elevated levels of lipid peroxidation in metal exposed organism. These results are in agreement with our previous work (Hassan *et al.*, 2008) in which, Heavy metals affect lipid peroxidation and plasma membrane permeabilization, leading to an increase in MDA. (Howlett and Avery, 1997) reported that the toxicity of transition metals caused lipid peroxidation and cellular K^+ efflux in *Saccharomyces cerevisiae* resulting in loss of function and membrane integridity.

The present study suggests that heavy metals cause oxidative stress in *E. coli* and antioxidant enzymes appear to play a pivotal role in combating oxidative stress that provide a protection to *E. coli*. Also antioxidant enzymes, lipid peroxidation and potassium efflux used as a biological monitor for heavy metals pollution.

References

Abassi, N.A., M.M. Kushad and A.G. Endress, 1998. Active oxygen-scavenging enzymes activities in developing apple flowers and fruits. Scientia Horticulturae, 74(3):183-194. Available from http://www.ingentaconnect.com/content/els/03044238/1998/00000074/00000003/art00077 http://dx.doi.org/10. 1016/ S0304-238(98)00077-6. DOI 10.1016/s0304-4238(98)00077-6.

Abassi, N.A., Kushad, M. M. and Endress, A. G., 1998. Active oxygen-scavenging

enzymes activities in developing apple flowers and fruits. Sci. Horti. , 74(3): 183-194. Available from http://www.sciencedirect.com/science/article/pii/S0885576500903138. DOI http://dx.doi.org/10.1006/pmpp.2000.0313.

Banjerdkij, P., P. Vattanaviboon and S. Mongkolsuk, 2005. Exposure to cadmium elevates expression of genes in the oxyr and ohrr regulons and induces cross-resistance to peroxide killing treatment in Xanthomonas campestris. Applied and Environmental Microbiology, 71(4): 1843-1849. Available from http://europepmc.org/abstract/MED/15812010.

Bergey's manual of systematic bacteriology, 2005. The proteobacteria, part a, introductory essays Second Edition, Volume 2, G.M. Garrity, ed.-in-chief, Springer-Verlag, New York, Berlin, Heidelberg.

Butow, B.J., D. Wynne and E. Tel-Or, 1997. Superoxide dismutase activity in peridinium gatunense in lake kinneret: Effect of light regime and carbon dioxide concentration1. Journal of Phycology, 33(5): 787-793. Available from http://dx.doi.org/10.1111/j.0022-3646.1997.00787.x. DOI 10.1111/j.0022-3646.1997.00787.x.

Cakmak, I. and H. Marschner, 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant physiology, 98(4): 1222-1227. Available from http://europepmc.org/abstract/MED/16668779.

Chaoui, A., S. Mazhoudi, M.H. Ghorbal and E. El Ferjani, 1997. Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (phaseolus vulgaris l.). Plant Science, 127(2): 139-147. Available from http://www.sciencedirect.com/science/article/pii/S0168945297001155. DOI http://dx.doi.org/10.1016/S0168-9452(97)00115-5.

Dalton, D.A., F.J. Hanus, S.A. Russell and H.J. Evans, 1987. Purification, properties, and distribution of ascorbate peroxidase in legume root nodules. Plant physiology, 83(4): 789-794. Available from http://europepmc.org/abstract/MED/16665340.

Davis, B.J., 1964. Disc electrophoresis – ii method and application to human serum proteins*. Annals of the New York Academy of Sciences, 121(2): 404-427. Available from http://dx.doi.org/10.1111/j.1749-6632.1964.tb14213.x. DOI 10.1111/j.1749-6632.1964.tb14213.x.

Gaspar; H Greppin; J Lobarzewski; C Penel, 1991. Biochemical, molecular, and physiological aspects of plantperoxidases. Genève: Université de Genève.

Grant, C.M., G. Perrone and I.W. Dawes, 1998. Glutathione and catalase provide overlapping defenses for protection against hydrogen peroxide in the yeast Saccharomyces cerevisiae. Biochemical and Biophysical Research Communications, 253(3): 893-898. Available from http://europepmc.org/abstract/MED/9918826.

Halliwell, B. and J.M.C. Gutteridge, 1991. Free radicals in biology and medicine, second edition: 540 pp. 1989. Free Radical Biology and Medicine, 10(6): 449-450. Available from http://www.sciencedirect.com/science/article/pii/0891584991900558. DOI http://dx.doi.org/10.1016/0891-5849(91)90055-8.

Harinasut, P., Poonsopa, D., Roengmongkol, K., & Charoensataporn, R. , 2003. Salinity effects on antioxidant enzymes in mulberry cultivator. Science Asia, 29: 109-113.

Hassan, S.H., R.N. Abskharon, S.M. El-Rab and A.A. Shoreit, 2008. Isolation, characterization of heavy metal resistant strain of Pseudomonas aeruginosa isolated from polluted sites in assiut city, Egypt. Journal of Basic Microbiology, 48(3): 168-176. Available from http://europepmc.org/abstract/MED/18506899.

Hassen, A., N. Saidi, M. Cherif and A. Boudabous, 1998. Resistance of environmental bacteria to heavy metals. Bioresource Technology, 64(1): 7-15. Available from http://www.sciencedirect.com/science/artic

le/pii/S0960852497001612. DOI http://dx.doi.org/10.1016/S0960-8524(97)00161-2.

Hassen A., S.N., Cherif M., Boudabous A. , 1998. Resistance of environmental bacteria to heavy metals. Bioresour Technol, 64: 7-15.

Howlett, N.G. and S.V. Avery, 1997. Induction of lipid peroxidation during heavy metal stress in Saccharomyces cerevisiae and influence of plasma membrane fatty acid unsaturation. Applied and Environmental Microbiology, 63(8): 2971-2976. Available from http://europepmc.org/abstract/MED/9251184.

Hussein, K. A. and J. H. Joo, 2013. Heavy metal resistance of bacteria and its impact on the production of antioxidant enzymes. African Journal of Microbiology Research, Vol. 7(20): 2288-2296.

Imlay, J.A., 2002. How oxygen damages microbes: Oxygen tolerance and obligate anaerobiosis. In: Advances in microbial physiology. Academic Press: pp: 111-153.

Jiang, M. and J. Zhang, 2002. Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. Journal of Experimental Botany, 53(379): 2401-2410. Available from http://europepmc.org/abstract/MED/12432032.

Jianlong, W., M. Zeyu and Z. Xuan, 2004. Response of Saccharomyces cerevisiae to chromium stress. Process Biochemistry, 39(10): 1231-1235. Available from http://www.sciencedirect.com/science/article/pii/S0032959203002553. DOI http://dx.doi.org/10.1016/S0032-9592(03)00255-3.

Kang, K.-S., C.-J. Lim, T.-J. Han, J.-C. Kim and C.-D. Jin, 1999. Changes in the isozyme composition of antioxidant enzymes in response to aminotriazole in leaves ofarabidopsis thaliana. J. Plant Biol., 42(3): 187-193. Available from http://dx.doi.org/10.1007/BF03030477. DOI 10.1007/bf03030477.

Kushkevych, I.V., S.O. Hnatush and H.V. Mutenko, 2011. Glutathione level of desulfovibrio desulfuricans imv k-6 under the influence of heavy metal salts. Ukrainskii biokhimicheskii zhurnal, 83(6): 104-109. Available from http://europepmc.org/abstract/MED/22364026.

Lee, M. and H. Shin, 2003. Cadmium-induced changes in antioxidant enzymes from the marine alga nannochloropsis oculata. Journal of Applied Phycology, 15(1): 13-19. Available from http://dx.doi.org/10.1023/A%3A1022903602365. DOI 10.1023/a:1022903602365.

Lee, M.Y., 1997. The changes of peroxidase activity and isoperoxidase patterns from pine needles under the salinary stress The Korean Journal of Ecology 20 (5): 315-321.

Lenártová, V., K.N. Holovská and P. Javorský, 1998. The influence of mercury on the antioxidant enzyme activity of rumen bacteria Streptococcus bovis and Selenomonas ruminantium. FEMS Microbiology Ecology, 27(4): 319-325. Available from http://www.sciencedirect.com/science/article/pii/S0168649698000774. DOI http://dx.doi.org/10.1016/S0168-6496(98)00077-4.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. The Journal of Biological Chemistry, 193(1): 265-275. Available from http://europepmc.org/abstract/MED/14907713.

Mac Faddin, J.F., 1985 Media for isolation-cultivation-identification-maintenance of medical bacteria. Vol.1. Baltimore, MD.: Williams & Wilkins.

Mergeay, M., 1995. Heavy metal resistances in microbial ecosystems. In: Molecular Microbial Ecology Manual, A. L. AkkermansJ. Elsas and F. Bruijn, (Eds.). Springer Netherlands: pp: 439-455.

Misra, H.P. and I. Fridovich, 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. The Journal of Biological Chemistry, 247(10): 3170-3175. Available from http://europepmc.org/abstract/MED/4623845.

Mittler, R. and B.A. Zilinskas, 1993. Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium. Analytical Biochemistry, 212(2): 540-546. Available from http://www.sciencedirect.com/science/article/pii/S0003269783713667. DOI http://dx.doi.org/10.1006/abio.1993.1366.

ABEDI T.and H. PAKNIYAT, 2010. Antioxidant enzyme changes in response to drought stress in ten cultivars of oilseed rape (Brassica napus l.) Czech J. Genet. Plant Breed., , 46((1)): 27-34.

Shah, K., R.G. Kumar, S. Verma and R.S. Dubey, 2001. Effect of cadmium on lipid peroxidation, superoxide anion generation and activities of antioxidant enzymes in growing rice seedlings. Plant Science, 161(6): 1135-1144. Available from http://www.sciencedirect.com/science/article/pii/S0168945201005179. DOI http://dx.doi.org/10.1016/S0168-9452(01)00517-9.

Sharma, P.J., A.B. Dubey, R.S. and Pessarakli M. , 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany, 2012: 26. Available from http://dx.doi.org/10.1155/2012/217037. DOI 10.1155/2012/217037.

Sidhu, J.S., J.S. Ravi and J.L. Minocha, 1984. Peroxidase isozyme patterns in primary trisomics of pearl millet. Theoret. Appl. Genetics, 68(1-2): 179-182. Available from http://dx.doi.org/10.1007/BF00252335. DOI 10.1007/bf00252335.

Tian, Z., F. Wang, W. Zhang, C. Liu and X. Zhao, 2012. Antioxidant mechanism and lipid peroxidation patterns in leaves and petals of marigold in response to drought stress. Hortic. Environ. Biotechnol., 53(3): 183-192. Available from http://dx.doi.org/10.1007/s13580-012-0069-4. DOI 10.1007/s13580-012-0069-4.

van Ginkel, G. and A. Sevanian, 1994. lipid peroxidation-induced membrane structural alterations. In: Methods in Enzymology, P. Lester, (Ed.). Academic Press: pp: 273-288.

Versalovic, J.; American Society for Microbiology, 2011. Manual of clinical microbiology. Washington, DC: ASM Press.

Whiteley, C.G. and D.J. Lee, 2006. Enzyme technology and biological remediation. Enzyme and Microbial Technology, 38(3–4): 291-316. Available from http://www.sciencedirect.com/science/article/pii/S0141022905004540. DOI http://dx.doi.org/10.1016/j.enzmictec.2005.10.010.

Williams, V.A.T., S. , 1960. Flame photometric method for sodium, potassium and calcium. In: Peach, k. And m.V. Tracey (ed.): Modern Methods of Plant Analysis. . 5: 3-5. Springer Verlag, Berlin.

Woodbury, W., A.K. Spencer and M.A. Stahmann, 1971. An improved procedure using ferricyanide for detecting catalase isozymes. Analytical Biochemistry, 44(1): 301-305. Available from http://www.sciencedirect.com/science/article/pii/0003269771903757. DOI http://dx.doi.org/10.1016/0003-2697(71)90375-7.

Zhang, J. and M.B. Kirkham, 1994. Drought-stress-induced changes in activities of superoxide dismutase, catalase, and peroxidase in wheat species. Plant and Cell Physiology, 35(5): 785-791. Available from http://pcp.oxfordjournals.org/content/35/5/785.abstract.

Zhao, S.J., Xu, C.C., Zou, Q., Meng, Q.W. , 1994. [improvements of method for measurement of malondialdehyde in plant tissues.] -. Plant Physiol. Commun. , 30: 207-210.