Chapter -8

*In vitro* Cr(VI) reduction by soluble cell free extract (Cytosolic fraction) and comparison with membrane fraction (Sonicated pellet) of *Planococcus* sp. VITP21

8.1 INFLUENCE OF DIFFERENT ELECTRON DONORS ON Cr(VI) REDUCTION

Cr(VI) reduction experiments were conducted in the presence of different electron donors. It was observed that the cell free extract (CFE) readily reduced 78% of 200 mg/l of Cr(VI) even in the absence of external electron donor, possibly due to endogenous electron donors. With the addition of different external electron donors, significant increase (approximately one fold) was observed with almost for all the electron donors used in the study (Figure 8.1a). However, NADH and NADPH reduced Cr(VI) slightly higher than the other electron donors. *Bacillus coagulans* (Philip et al., 1998) and *Acenitobacter haemolyticus* (Pei et al., 2009) and *B. sphaericus* AND 303 (Pal et al., 2005) were reported to reduce Cr(VI) even in the absence of electron donor, due to the presence of endogenous electron reserves. However, as in the present study slight enhancement was also reported with the addition of external electron donor (NADH) (Philip et al., 1998 and Pal et al., 2005). *Pseudomonas putida* (Ishibashi et al., 1990) and *Bacillus* sp (Elangovan et al., 2006) showed effective reduction in the presence of both NADH and NADPH, whereas other soluble reductases shown dependent on either NADH or NADPH (Focardi et al., 2012 and Sarangi et al., 2008). In the present study, though the crude membrane fraction (Figure 8.1b) showed negligible reduction in the absence of electron donor, 3.6 to 4 fold increase occurred in the presence of different electron donor, yet maximum of 84 - 85% (200 mg/l of Cr(VI)) reduction observed in the presence of dextrose, NADH and NADPH which is lesser than the reduction efficiency by soluble CFE.
Figure 8.1a: Cr(VI) reduction by soluble CFE of strain VITP21 in the presence of different electron donor [1mM electron donor, initial Cr(VI) = 200 mg/l, incubation time = 20 min, 7 pH and 35°C]

Figure 8.1b: Cr(VI) reduction by membrane fraction of strain VITP21 in the presence of different electron donor [1mM electron donor, initial Cr(VI) = 200 mg/l, incubation time = 20 min, 7 pH and 35°C]
8.2 EFFECT OF pH ON Cr(VI) REDUCTION

Figure 8.2a: Effect of different pH on Cr(VI) reduction by soluble CFE of strain VITP21 [1mM NADH, initial Cr (VI) = 200 mg/l, incubation time = 20 min and 35ºC]

Figure 8.2b : Effect of different pH on Cr(VI) reduction by membrane fraction of strain VITP21 [1 mM NADH, initial Cr(VI) = 200 mg/l, incubation time = 20 min and 35ºC]
The effect of pH on chromium reduction by CFE was studied in the pH range of 5 to 9 pH (Figure 8.2a). The soluble CFE exhibited Cr(VI) reduction in a wider pH range (pH 5 to pH 9) with optimum at 7 (92 % of 200 mg/l). At lower (pH 5) and higher pH (pH 9) values, reduction efficiency reduced slightly to 70% and 60 % respectively. With pH 6 and pH 8 greater than 85 % of 200 mg/l of Cr(VI) was reduced. *Pseudomonas ambigua* G-1 (Suzuki et al., 1992) showed reductase activity in a wider pH range with 6-9 pH, *Arthrobacter rhambi* – RE (Elangovan et al., 2010) showed optimum at 5.5 when tested in the range of pH 4.5 to 8. *Enterobacter* strain showed an optimum range from 6.5 to 8.5 (Wang et al., 1990). *O. intermedium* BCR400 activity was in the range of pH 5 to 9 with optimum at 7 pH (Kavita and Keharia, 2012). A moderate halophilic bacteria, *Halomonas* sp. TA-04 (Focardi et al., 2012) showed reduction activity with a narrow pH range (5- 6.5) with an optimum at 6.5 pH. In the present study, the crude membrane fraction (Figure 8.2b) also exhibited similar trend in Cr(VI) reduction with optimal condition as pH 7, but lesser reduction efficiency on comparison with soluble CFE.

**8.3 EFFECT OF TEMPERATURE ON Cr(VI) REDUCTION**

Effect of temperature on Cr(VI) reduction by soluble CFE was studied in the temperature range of 30 to 45°C at pH 7 for the initial Cr(VI) concentration of 200 mg/l. The optimum reduction occurred at 35°C (91 %), however greater than 88 % was observed at 30°C and 40°C (Figure 8.3a). A slight decrease in percent reduction was observed at 45°C (78%). *Pseudomonas* sp. G1DM21 though reported for optimal temperature to be at 30°C, the reduction efficiency was not affected considerably till the temperature of 50°C (Desai et al.,2012b). The soluble reductases of *Bacillus* sp. KCH3 and *Exiguobacterium* sp.KCH5 were reported to exhibit optimum at 35°C whereas *Leucobacter* sp.KCH4 at 30°C (Sarangi and Krishnan,2008). *Halomonas* sp. TA-04 (Focardi et al., 2012) exhibited optimum temperature at 28°C. Though many reductases in literature were reported for optimum temperature in the range of 30 – 40°C (Megharaj et al., 2003; Desai et al., 2008a,b; Park et al., 2000; Pal et al., 2005; Kavita and Keharia, 2012; Xu et al., 2012), few reductases were reported to have optimum temperature at 65°C (*Thermus scotoductus* SA-01 (Opperman et al., 2008)). The temperature range for crude membrane fraction (Figure 8.3b), in the present study exhibited optimum temperature at 35°C with 84 % reduction of Cr(VI).
Figure 8.3a: Effect of different temperature on Cr(VI) reduction by CFE of strain VITP21 [1mM NADH, initial Cr (VI) = 200 mg/l, incubation time = 20 min and 7 pH]

Figure 8.3b: Effect of different temperature on Cr(VI) reduction by membrane fraction of strain VITP21 [1mM NADH, initial Cr(VI) = 200 mg/l, incubation time = 20 min, 35°C and 7 pH]
8.4 EFFECT OF DIFFERENT METAL ION ON Cr(VI) REDUCTION

Figure 8.4a: Effect of different divalent metal ion (1 mM) on Cr (VI) reduction by CFE of strain VITP21 [100 % relative Cr(VI) reduction = 91%, 1mM NADH, initial Cr(VI) = 200mg/l, incubation time = 20 min, 35ºC and 7pH ]

Figure 8.4b: Effect of different divalent metal ion (1mM) on Cr(VI) reduction by membrane fraction of strain VITP21 [100 % relative Cr(VI) reduction = 84 %, 1mM NADH, initial Cr(VI) = 200mg/l, incubation time = 20 min, 35ºC and 7pH ]
The effect of different divalent metal ions ($Cu^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Mn^{2+}$, $Pd^{2+}$, $Zn^{2+}$, $Cd^{2+}$) on Cr(VI) reduction by crude CFE (pH 7, 35°C and 1mM NADH) with initial Cr(VI) concentration of 200 mg/l was investigated (Figure 8.4a). Of these, $Cu^{2+}$ showed enhancement of 8%, whereas divalent metal ions like $Mn^{2+}$, $Pd^{2+}$, and $Zn^{2+}$ showed slight inhibitory effect (< 5 %). Other metals like $Ni^{2+}$, $Co^{2+}$, $Cd^{2+}$ did not exhibit any significant effect on Cr(VI) reduction. The influence of copper ion on Cr (VI) reduction has been reported by many soluble reductases by different genera of bacteria $Bacillus$ sp. ES 29 (Camargo et al., 2003) $Pseudomonas$ sp. G1DM21 (Desai et al., 2012b), $Halomonas$ sp. TA-04 (Focardi et al., 2012), $A. crystallopoietes$ ES 32 (Camargo et al., 2004) and $P. phragmitetus$ LSSE-09 (Xu et al., 2011). Amphibacillus sp. KSUCr3 (Ibrahim et al., 2012) reported copper dependent membrane associated chromium reduction enhancement. However some reports indicate inhibitory effect by copper ion on Cr(VI) reduction by soluble chromium reductases (Park et al., 2000; Pal et al., 2005). $Zn^{2+}$ inhibition on Cr(VI) reduction in $Bacillus$ sp. (Elangovan et al., 2006) and $Zn^{2+}$ and $Pd^{2+}$ inhibition on Cr(VI) reduction in $Ochrobacterium intermedium$ SDCr-5 (Sultan and Hasnain, 2007) was reported. Soluble reductase of $Pseudomonas$ sp. G1DM21 did show any effect on Cr(VI) reduction in the presence of $Ni^{2+}$ and $Co^{2+}$, whereas exhibited inhibition in the presence of $Cd^{2+}$ (Desai et al., 2012b). In $Bacillus$ sp. ES 29 (Camargo et al., 2003), $Mn^{2+}$ and $Co^{2+}$ showed slight stimulatory effect whereas $Mn^{2+},Ni^{2+},Cd^{2+},Pb^{2+}$ inhibited Cr(VI) reduction in $Arthrobacter rhombi$-RE (Elangovan et al., 2010). Soluble CFE from moderately halophilic bacteria, $Halomonas$ sp. TA-04 showed stimulatory effect and inhibition in the presence of $Ni^{2+}$ and $Zn^{2+}$ respectively, whereas $Cd^{2+}$ did not show any effect (Focardi et al., 2012). $Mn^{2+}$ and $Cd^{2+}$ inhibition was observed in $P. phragmitetus$ LSSE-09 (Xu et al., 2012). However, in the present study, Cr(VI) reduction by membrane fraction exhibited similar effect on Cr(VI) reduction in the presence of $Cu^{2+}$ and little or no effect in the presence of $Zn^{2+}, Ni^{2+}, Pb^{2+}, Cd^{2+}$ whereas $Mn^{2+}, Co^{2+}$ showed slight inhibition on Cr (VI) reduction (Figure 8.4b).
8.5 EFFECT OF EDTA, SODIUM AZIDE AND DNP ON Cr(VI) REDUCTION

Figure 8.5a: Effect of EDTA, Sodium azide and DNP (1 mM) on Cr(VI) reduction by CFE of strain VITP21 [100 % relative Cr(VI) reduction = 91 %, 1mM NADH, initial Cr(VI) = 200 mg/l, incubation time = 20 min, 35°C and 7 pH]

Figure 8.5b: Effect of EDTA, Sodium azide and DNP (1 mM) on Cr(VI) reduction by membrane fraction of strain VITP21 [100 % relative Cr(VI) reduction = 84 %, 1mM NADH, initial Cr(VI) = 200 mg/l, incubation time = 20 min, 35°C and 7 pH]
The influence of inhibitors (1mM) on Cr(VI) by CFE of VITP21 was investigated (Figure 8.5a). 2,4 DNP, a decoupler of oxidative phosphorylation and sodium azide, a respiratory system inhibitor did not show significant effect on Cr(VI) reduction (less than 5% inhibition), indicating the non association of respiratory system. These results are similar to the reports by cell free reductases of Bacillus sp. ES 29 (Camargo et al., 2003), A.crystallopoietes ES 32 (Camargo et al., 2004), B.coagulans (Philip et al., 1998) and Pseudomonas sp. G1DM21 (Desai et al., 2012) where partial or no inhibition was observed, whereas membrane associated chromium reductase of Amphibacillus sp. KSUCr3 exhibited inhibition as reported by Ibrahim et al (2012). However EDTA, an ion chelating agent exhibited 20% of inhibition probably suggesting that the Cr(VI) reduction by the soluble CFE could be mediated by a metalloenzyme as reported in Halomonas sp. (Murugavel and Mohanty, 2012). Similar pattern of result was exhibited by membrane fraction (Figure 8.5b) which confirmed that the membrane reductases does not contribute for chromium reduction as in they are not strongly inhibited by respiratory inhibitors (Amphibacillus sp. KSUCr3 (Ibrahim et al., 2012)).

8.6 EFFECT OF OXYANIONS ON Cr(VI) REDUCTION

Cr(VI) reduction by soluble CFE was tested in the presence of different oxyanions (Figure 8.6a) like tellurite, tungstate, selenate, selenite, arsenate, molybdate, nitrate and sulphate (1 mM). It was observed that nitrate and sulphate slightly enhanced Cr(VI) reduction (≤2%) whereas tellurite did not show any effect. Nitrate and sulphate ions can act as competitive electron acceptors thereby reducing the rate of Cr(VI) reduction (Xu et al., 2011), however in the present study Cr(VI) reduction was not affected in the presence of nitrate and sulphate. The other acceptors exhibited inhibition in the following order: Arsenate > Selenite> Tungstate> Selenate> Molybdate. The higher inhibition on Cr(VI) reduction was in the presence of arsenate (15%) followed by Selenite and Tungstate (≈8%). Selenate and Molybdate exhibited ≤3% of inhibition on Cr(VI) reduction by soluble cell free extract.
Figure 8.6a: Effect of different oxyanions (1 mM) on Cr(VI) reduction by soluble CFE of strain VITP21 [100 % relative Cr (VI) reduction = 91%, 1mM NADH, initial Cr(VI) = 200mg/l, incubation time = 20 min, 35°C and 7 pH]

Figure 8.6b: Effect of different oxyanions (1 mM) on Cr(VI) reduction by membrane fraction of strain VITP21 [100 % relative Cr (VI) reduction = 84 %, 1mM NADH, initial Cr(VI) = 200mg/l, incubation time = 20 min, 35°C and 7 pH]
The inhibition on Cr(VI) by different oxyanions/electron acceptor might be due to wider substrate preference or competitive inhibition (Cheung and Gu, 2005 and Amoozegar et al., 2007). In the presence of selenite and tellurite, in addition to Cr (VI) reduction, orange - red and black precipitates appeared in soluble cell free extract which indicate the co-reduction of selenite and tellurite. Cell free extracts of *Thermus thermophilus* HB8 reported for NADH-dependent tellurite reduction in addition to selenite and sodium sulfite reduction (Chiong et al., 1998) but Cr(VI) reduction by the same was not reported. The Cr(VI) reduction efficiency by membrane fraction varied slightly than that of soluble cell free extract (Figure 8.6b). Enhancement of Cr(VI) reduction occurred in the presence of sulphate (12%) and followed by nitrate (7%). Arsenate enhanced Cr(VI) reduction with less than 2%, whereas Molybdate neither inhibited nor enhanced. The other electron acceptors like tellurite, tungstate and selenate showed approximately 17 % of inhibition and selenite exhibited 11 % of inhibition.

8.7 EFFECT OF DIFFERENT CONCENTRATION OF NaCl ON Cr(VI) REDUCTION

The effect of different concentrations of NaCl (0, 2, 3, 4 and 5 M) on Cr(VI) reduction by soluble cell free extract was investigated for 10 different initial concentration of Cr(VI) (50 to 500mg/l). Figure 8.7a shows the effect of NaCl concentration on Cr(VI) reduction represented as % Cr(VI) remaining in the graph. In the absence of NaCl, greater than 90 % reduction was observed for initial Cr(VI) concentration ranging from 50 to 250 mg/l, whereas for the remaining Cr(VI) concentration (300 to 500 mg/l) >80 % of reduction was observed. On an average 88% of Cr(VI) reduction was observed for 50 to 500 mg/l of Cr(VI) concentration. At 2 M NaCl, slight increase in the reduction efficiency occurred with >95 % and ≥ 90 % of reduction for Cr(VI) concentration of 50 to 200 mg/l and 250 to 400 mg/l respectively. For the other concentrations (450 and 500 mg/l) ≈ 88 % of reduction was observed. Similar trend with trivial increase was observed in the presence of 3 M NaCl. On an average for the range of 50 to 500 mg/l, 94 % of Cr(VI) reduction was observed (3M) which was almost similar to 2 M NaCl with 93 % of average reduction.
Figure 8.7a: Effect of different NaCl concentration on Cr(VI) reduction by soluble CFE of strain VITP21 [1mM NADH, incubation time= 20 min, 35°C and 7 pH]

Figure 8.7b: Effect of different NaCl concentration on Cr (VI) reduction by membrane fraction of strain VITP21 [1mM NADH, incubation time= 20 min, 35°C and 7 pH]
At higher NaCl concentration no substantial decrease in Cr(VI) reduction efficiency occurred compared to 3 M NaCl. The average Cr(VI) reduction for the range of 50 to 500 mg/l was ≈92 % for 4 and 5 M NaCl. The effect of NaCl on Cr (VI) reduction was optimally observed with enhancement of 5 to 6 % in the range of 2 to 3 M NaCl compared with absence of NaCl. The enhancement of Cr(VI) reduction by soluble cell free extract was reported in the presence of 1mM sodium ions by moderately halophilic bacteria, Halomonas sp. TA-04 (Focardi et al., 2012), A. crystallopoietes ES 32 (Camargo et al.,2004), Pseudomonas sp. G1DM21 (Desai et al., 2008b) and Bacillus sp. ES 29.(Camargo et al.,2003), however Arthrobacter rhombi-RE (Elangovan et al.,2010) and Halomonas sp. (Murugavelh and Mohanty, 2012) exhibited inhibition in the presence of NaCl. In the present study, reduction rate was not affected in the presence of NaCl (2 M to 5 M) compared to absence of NaCl. This infact is the first report on the effect of high salt concentration of NaCl on Cr(VI) reduction by cell free extract which illustrate its bioremediation potential under high salt condition. This assumes importance of owing the high NaCl concentration of salt present in the tannery effluents.

The influence of NaCl on Cr(VI) reduction by membrane fraction was higher compared to cell free extract (Figure 8.7b represented as % Cr(VI) remaining in the graph). In the absence of NaCl, the average Cr(VI) reduction was observed to be ≈76 %, whereas it exhibited with an increase of ≈ 90% of Cr(VI) reduction in the presence of 2 M NaCl for the Cr(VI) concentration (50 to 500 mg/l). In the presence of 3 – 5 M NaCl, average Cr(VI) reduction was almost same with ≈ 90% for Cr(VI) concentration (50 to 500 mg/l), however it was lesser compared with cell free extract.

### 8.8 EFFECT OF DIFFERENT CONCENTRATION OF DIVALENT COPPER ION ON Cr(VI) REDUCTION

The effect of different concentrations of copper ion (0.5 to 3 mM) was studied on Cr(VI) reduction by cell free extract (Figure 8.8a represented as % Cr(VI) remaining in the graph). It was observed that there was 4 fold increase of Cr(VI) reduction for the range of initial Cr(VI) concentration (100 to 500 mg/l) used in the study. The reduction was observed to be 88 to 92 % in the presence of 0 and 0.5 mM copper ion respectively. But beyond 0.5 mM copper ion concentration (1 to 3 mM),...
there was a positive change in the Cr(VI) reduction efficiency, from 4 fold (0.5 mM) to 5 fold (1mM to 3 mM), thus ensuring that even low concentration of copper ion could enhance the Cr(VI) reduction.

For the range of copper ion concentration used (1 to 3 mM), the average Cr(VI) reduction for different Cr(VI) concentration remained ≈ 93 % with no significant enhancement/ inhibition. The enhancement on Cr(VI) reduction in the presence of copper ion was first reported in cell free extract of Bacillus sp. ES 29 by Camargo et al (2003). Copper enhanced Cr(VI) reduction by cell free extract have been reported in Leucobacter sp. KCH4 (Sarangi and Krishnan, 2008), Halomonas sp. TA-04 (Focardi et al., 2012) and A. crystallopoietes ES32 (Camargo et al., 2004), however effect of different concentration of copper ion on different Cr(VI) concentration have not been reported. The stimulatory mechanism by Cu$^{2+}$ on Cr(VI) reduction was reported to be related to electron transport protection or acting as electron redox centre and, in some cases, as a shuttle for electrons between protein or prosthetic group for many reductase enzyme (Sultan and Hasnain, 2007 and Camargo et al., 2003).

![Figure 8.8a: Effect of different copper ion concentration on Cr(VI) reduction by soluble CFE of strain VITP21 [1mM NADH, incubation time = 20 min, 35°C and 7 pH]](image)

Figure 8.8a: Effect of different copper ion concentration on Cr(VI) reduction by soluble CFE of strain VITP21 [1mM NADH, incubation time = 20 min, 35°C and 7 pH]
Figure 8.8b: Effect of different copper ion on Cr(VI) reduction by membrane fraction of strain VITP21 [1mM NADH, incubation time = 20 min, 35°C and 7 pH]

The effect of copper ion on membrane fraction was studied in the similar way as it was studied for CFE (Figure 8.8b represented as % Cr(VI) remaining in the graph). The enhancement on Cr(VI) reduction was found to be higher in case of membrane fraction with an average of 10 fold increase for different Cr(VI) concentration in the presence of 0.5 mM copper ion. Upon increasing the copper ion concentration from 1 to 3 mM, to the effect was observed to be 19 fold. Irrespective of enhancement observed for Cr(VI) reduction capacity by membrane fraction, the average reduction for different Cr(VI) concentration was higher for CFE (≈ 93 %) compared with membrane fraction (≈ 83 %).

8.9 COMBINED EFFECT OF DIVALENT COPPER ION AND NaCl ON Cr(VI) REDUCTION

The effect of different NaCl concentration in the presence of 1mM copper ion on Cr(VI) reduction for different Cr(VI) concentration was studied (Figure 8.9a). The combined effect on Cr(VI) reduction was dependent on Cr(VI) concentration. With lower Cr(VI) concentration ranging from 50 to 300 mg/l, the enhancement on Cr(VI) reduction was observed to be insignificant with approximately 2 fold increase (2 to 5 M NaCl) compared to 0 M NaCl. But for the higher concentration (350 to 500 mg/l),
the enhancement increased from 10 to 14 fold for 2 to 5 M NaCl. In the absence of copper ion, the enhancement of NaCl for higher concentration (350 to 500 mg/l) was observed to be only 7 fold increase (optimum 3M NaCl) ensuring that the presence of copper ions under salt conditions enhances Cr(VI) reduction capacity even in the presence of high salt concentration (5 M). Enhancement of NaCl and copper ion has been reported separately on Cr (VI) reduction by CFE (Focardi et al., 2012; Camargo et al., 2003; Sarangi and Krishnan, 2008). However enhancement on Cr(VI) reduction by NaCl in the presence of copper ion has not been reported to the best of our knowledge.

The Cr(VI) reduction by membrane fraction enhanced higher Cr(VI) reduction than the CFE (Figure 8.9b represented as % Cr(VI) remaining in the graph). For initial Cr(VI) concentration ranging from 50 to 300 mg/l, the average fold increase was observed to be in the range of 2 to 7 for 2 M to 5 M NaCl respectively, whereas for the higher concentration (350 to 500 mg/l) it exhibited 15 (2M NaCl ) to 26 (5M NaCl) fold increase. On comparison with CFE, at lower NaCl concentration difference in Cr(VI) reduction capacity was significant whereas at higher NaCl concentration membrane fraction exhibited similar reduction capacity which needs further investigation.

Figure 8.9a: Combined effect of different NaCl concentration and copper ion (1mM) on Cr(VI) reduction by soluble CFE of strain VITP21 [1mM NADH, incubation time = 20 min, 35°C and 7 pH]
Figure 8.9b: Combined effect of different NaCl concentration and copper ion (1mM) on Cr(VI) reduction by membrane fraction of strain VITP21 [1mM NADH, incubation time =-20 min, 35°C and 7 pH]