Chapter 2

Recent Trends in Phosphatase-Mediated Bioremediation

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Additional information is available at the end of the chapter

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Abstract

Industrial effluents from tanneries and electroplating industries from small- and large-scale sector industrial plants contain substantial amount of toxic heavy metal, which pollutes rivers and lakes, land, air and sea leading to imbalance of ecosystem and certain health issues to humans, animals as well as plants. The worldwide environmental regulations stipulate the reduction of heavy metals in the effluents to permissible levels before discharging into water bodies. Enzyme-mediated precipitation of heavy metals affords a novel eco-friendly method for remediation of toxic heavy metals from various industrial effluents like tannery, electroplating and dye industries. This chapter has paid attention to bacterial alkaline phosphatase (BAP) from *Escherichia coli* C90 and calf-intestinal alkaline phosphatase (CIAP), which catalyses phospho mono- and diesters and produces inorganic phosphate (Pi). The Pi thus generated precipitates the heavy metals as metal-phosphate complexes. The kinetic behaviour of both the enzymes with *para*-nitrophenyl phosphate, ascorbic acid 2-phosphate and α-naphthyl phosphate was investigated at various pH regimes from 8 to 11. The chapter also explains in detail the descriptive information on the capability of BAP- and CIAP-mediated precipitation of heavy metals, which is desirable and convenient method for the toxic heavy metals such as chromium, cadmium, nickel and cobalt.

Keywords: bacterial alkaline phosphatase, calf-intestinal alkaline phosphatase, heavy metals, enzyme kinetics, bioremediation

1. Introduction

Phosphatase-mediated bioremediation of heavy metals plays an important role in the bioremediation of industrial, municipal and nuclear wastewater. Heavy metals, such as Li^{3+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ba^{2+}, Hg^{2+}, Pb^{2+}, $Hg2^{+}$, Ag^{2+}, Cd^{2+}, $A1^{3+}$, Ni^{2+}, Cu^{2+} and Pb^{2+}, are found in various

industrial effluents. High quantity of Cd^{2+}, Ni^{2+}, Co^{2+} and $Cr^{3+/6+}$ was found in tannery and electroplating effluents which creates high risk in the environment which is one of the important issues in Asian countries. The use of alkaline phosphatase offers great promises in vast research field and could be a model to study metal ion-dependent catalysis to address such a kind of problems in the application field, viz., environmental biotechnology, molecular biology and immunodetection. Alkaline phosphatases are commonly found from bacteria to higher mammals, which efficiently hydrolyze several mono- and diesters. They are nonspecific and react with a variety of substrates from natural to synthetic compounds. There has been a considerable effort in recent years towards the application of alkaline phosphatases for bioremediation of heavy metals and radionuclides from industrial and nuclear wastes. In order to extend bioremediation possibilities to heavy metals contaminated wastes, this present research work explores the advantages of using bacterial (*Escherichia coli* C90) and calf-intestinal alkaline phosphatase enzymes with *p*-nitrophenyl phosphate (*p*NPP), 1-naphthyl phosphate monosodium salt monohydrate and L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate as substrate for enhancing bioprecipitation from single-ion metal solutions (Cd^{2+}, Ni^{2+}, Co^{2+}, Cr^{3+} and Cr^{6+}) and effluents from tannery and electroplating industrial units, which contain Cr^{3+}, Cr^{6+} and Cd^{2+}, Ni^{2+}, Co^{2+}, respectively.

2. Phosphatase enzymes

Phosphatase is a hydrolase enzyme. Phosphatases play a very important role in the phosphate metabolism of the organism by hydrolysis of polyphosphates and organic phosphates [1]. The essential feature of phosphatase is that the enzymes have wide specificity. Phosphatases cleave phosphate ester bonds, which play an important role in the hydrolysis of polyphosphates and organic phosphates. They are also responsible for phosphate transport within the cells [2]. Phosphatase enzymes are used by many soil microorganisms to access organically bound phosphate nutrients.

Two types of phosphatase enzymes are as follows:

- Acid phosphatases
- Alkaline phosphatases

2.1. Alkaline phosphatase

Alkaline phosphatases (APase or AP or ALP; orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) are common in a wide variety of bacteria and mammals which efficiently hydrolyse several mono- and diesters [3]. Alkaline phosphatase, which hydrolyses organic phosphate esters and releases inorganic orthophosphate, is a dimeric metallic enzyme [4]. In bacteria, alkaline phosphatase is present in the periplasmic space. The three-dimensional structure of *E. coli* alkaline phosphatase (**Figure 1**), determined by Kim and Wycoff, reported that each of the two identical subunits contain 449 amino acids [5, 6]. Alkaline phosphatase from *E. coli* has been the focus of several studies dealing with molecular properties, subunit composition and catalytic mechanism; however, much less has been done with mammalian

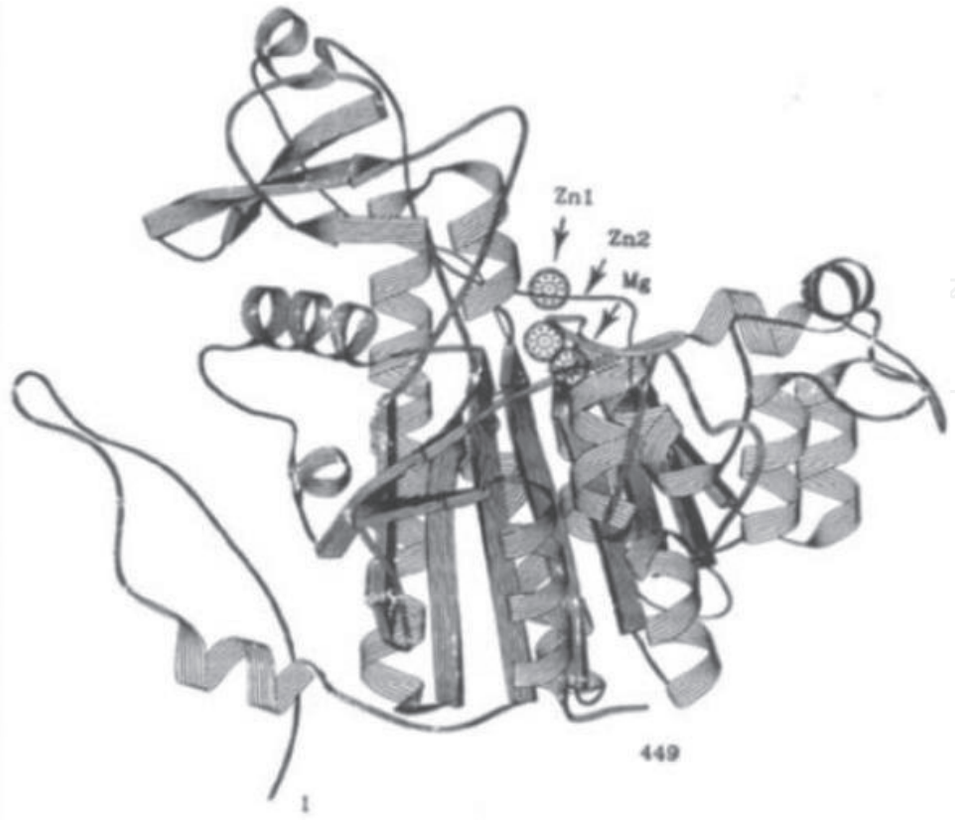

Figure 1. A subunit of *E. coli* alkaline phosphatase shown in ribbon representation. Znl, Zn2 and Mg are indicated as spheres. The monomer shows 10 β-sheets flanked by 15 α-helices of different lengths [12].

alkaline phosphatases. The latter are glycoproteins and exist as different isoenzymes such as placental (PLAP) (**Figure 2**), germ cell (GCAP), intestinal and tissue nonspecific isoforms [3]. The tissue nonspecific isoforms include AP present in bone, liver and kidney and they differ from the other isoforms due to modifications brought about by post-translational events [7]. Mammalian alkaline phosphatase is a very important enzyme physiologically and is an important component of medical diagnosis [8]. ALPs are capable of catalysing the hydrolysis of monoesters of phosphoric acid and also transphosphorylation reaction in the presence of large concentrations of phosphate acceptors. Both bacterial and mammalian ALPs contain two Zn and one Mg on each catalytic site [9] (**Figures 1** and **2**). Both the metal ions are crucial for the enzyme to maintain its structural stability and catalytic activity. About 25–35% similarity was observed in alkaline phosphatase from mammals in the secondary structure and also at the catalytically important residues [9]. The active site residues such as Asp 91, Ser 92, Arg166 and ligands coordinating the divalent metal ions (Zn^{2+} and Mg^{2+}) are all conserved [6]. These structural similarities suggest that mammalian ALPs may catalyse hydrolysis of both pyrophosphate and orthophosphate moieties through a mechanism similar to that of *E. coli* enzyme [10]. Ser-102 residue of the enzyme attacks the phosphoryl group leading to phosphorylated enzyme [11]. The greater advantage of using ALP is its broad substrate specificity.

It can hydrolyse a wide variety of substrates, that is, synthetic as well as natural substrates. The substrates include ATP, ADP, AMP, PPi, glucose-1-phosphate, glucose-6-phosphate,

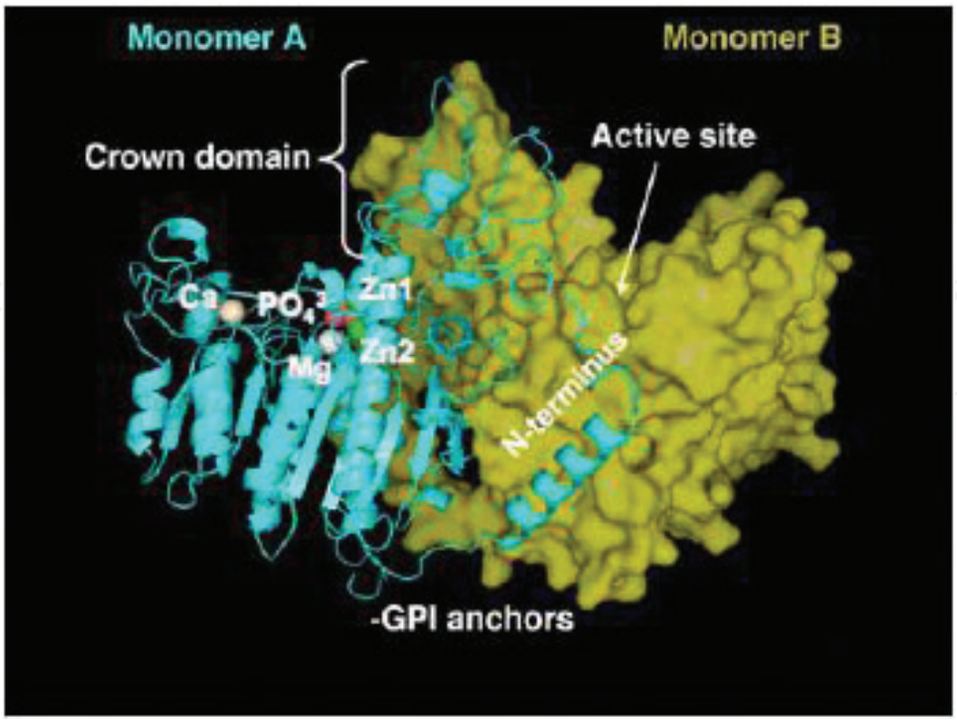

Figure 2. Three-dimensional structure of PLAP. An overview of the structure of human PLAP from the crystallographic coordinates [13]. Ribbon representation of subunit A in cyan and surface representation of subunit B in yellow are shown. Active site metal ions such as Zn1, Zn2 and Mg in addition to the new fourth metal Ca are indicated [9].

fructose-6-phosphate, b-glycerophosphate, bis-(p-nitrophenyl)-phosphate, and so on. [12]. Based on the X-ray crystallography study, a revised mechanism of hydrolysis of a substrate by alkaline phosphatase has been proposed by Stec et al. [14] (**Figure 3**).

This causes a second inversion of configuration at the phosphorus centre. Nucleophilic Ser102 gets regenerated and reprotonation of Ser102 by the Mg-coordinated water molecule may facilitate the departure of the inorganic phosphate product from the non-covalent E.Pi complex. Alternatively, the phosphate group, for its release, may directly get protonated by the Mg-coordinated water molecule [14].

According to this proposed mechanism, the active site of the free enzyme (E) is occupied by three water molecules and the hydroxyl group of S102 forms hydrogen bond with a hydroxide ion which is coordinated by Mg. As a result of the enzyme-substrate complex (E.ROP) formation, the ester oxygen atom coordinates with Zn1 and Zn2 in addition to the guanidinium group of Arg166 [9]. The site opposite to the leaving group gets occupied by Ser102. As a result of binding of the phosphomonoester (ROP) to form the enzyme-substrate complex (E.ROP), the Ser102 O^{γ} becomes fully deprotonated for nucleophilic attack with the consequent transfer of the proton to the Mg-coordinated hydroxide group to form Mg-coordinated water molecule. Coordination of Zn2 stabilizes Ser102 O^{γ} in its nucleophilic state. The activated hydroxyl group of Ser102 attacks the phosphorus centre of the substrate in the enzyme-substrate complex (E-ROP) to form a covalent serine-phosphate intermediate (E-P) resulting in inversion of the phosphorus centre and the loss of the leaving group (RO^{-}). A nucleophilic hydroxide ion coordinated to Zn1 attacks the covalent serine-phosphate intermediate (E-P), resulting in the formation of non-covalent enzyme-phosphate complex [14].

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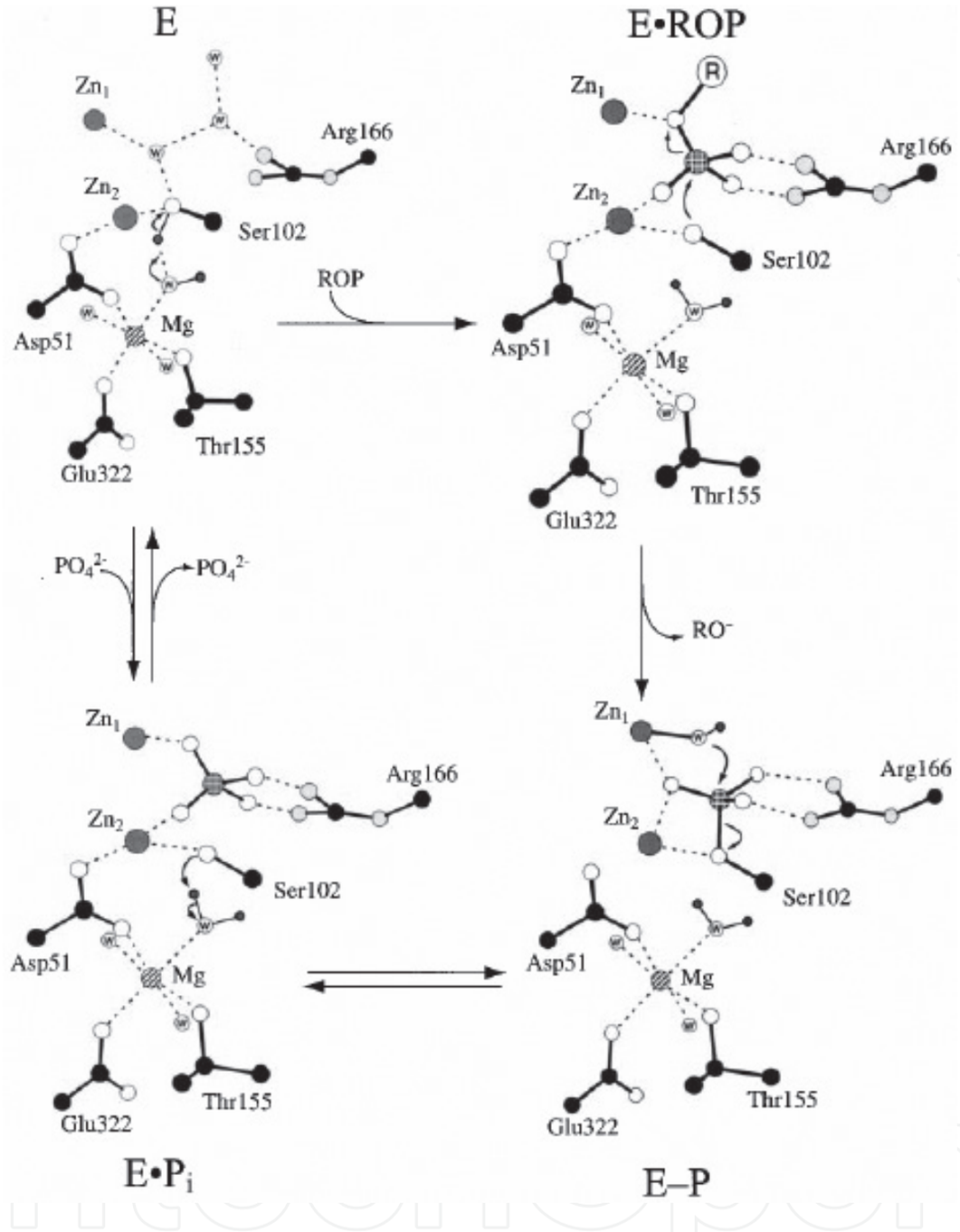

Figure 3. Proposed mechanism of hydrolysis of substrate by alkaline phosphatase [14].

Alkaline phosphatase catalyses the cleavage of phosphate group from *p*-nitrophenyl phosphate and liberates *p*-nitrophenol (PNP) and inorganic phosphate (Pi) [14]. The liberated inorganic phosphate interacts with metal ion or radionuclide and precipitates as metal phosphate or radionuclide phosphate as follows:

$$\text{p-nitrophenyl phosphate} + \text{AP} \rightarrow \text{p-nitrophenol} + \text{phosphate} \quad (1)$$

When reacting with heavy metal:

p-nitrophenyl phosphate + AP + heavy metal → *p*-nitrophenol + heavy-metal phosphate

3. Kinetic behaviour of calf-intestinal and bacterial alkaline phosphatase with pNPP

3.1. Effect of pH and substrate concentration on hydrolysis of CIAP with pNPP

The effects of pH on the kinetics of CIAP-catalysed pNPP hydrolysis reaction were determined in Tris-HCl buffer. CIAP activity was highest at pH 11 in Tris-HCl buffer, whereas in glycine-NaOH buffer the highest activity was recorded at pH 9.5. The rate-determining step at pH 8 was attributed to the non-dissociation of phosphate from the non-covalent enzyme-phosphate complex. At pH 8, the rate of hydrolysis of 4-nitrophenyl phosphate was increased, in the presence of Tris-Cl [15] and also with the substrate 4-methylumbelliferyl [16]. Experimental data show that even at pH 11, Tris buffer was not inhibited. Despite the increase in substrate concentration at pH 11, no reduction in the enzyme activity was observed; by contrast, the activity observed was much higher than that of the activity observed between pH 8.5 and 11 (**Figure 4**). This states that the enzyme exhibits functionally stable conformations at pH 8–8.5 and at pH 11. In the mean time, at a wide substrate concentration between 0.2 and 4 mM the enzyme showed a relatively stable catalytic activity. Similar increase in the pH to 9 was reported previously with ALPs from dog's intestine, kidney and liver [17]. Moreover, at pH 11, the enzyme also showed an optimum temperature at 45°C instead of 37°C. Meanwhile, the enzyme ceased its activity upon incubation at 45°C beyond 60 min, whereas at 37°C the activity was recorded till 2h. This states that CIAP at alkaline pH from 8 to 11 undergoes slight conformational changes. The enzyme might be attaining kinetically stable conformations at pH 8.5 and 11, as evident from the experimental findings [18].

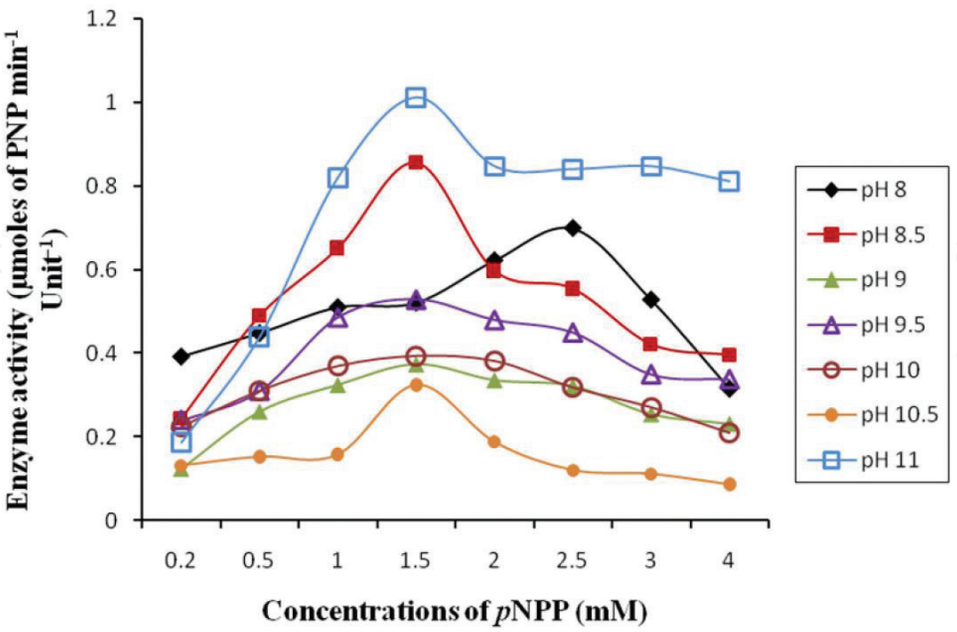

Figure 4. Hydrolysis of pNPP by CIAP under different concentrations of pNPP and varying pH regimes in Tris-HCl buffer.

The effect of substrate concentration on the CIAP activity across a wide range of pH values between 8 and 11 was observed. The observations were in sharp contrast to the observations made by Fernley and Walker [19], who reported inhibition of the enzyme at 0.2–4 mM concentrations of the substrate. From the enzyme-substrate saturation kinetics, it was noted that the saturation of the enzyme for substrate binding occurred at 1.5 mM, regardless of the pH 8.5–11 in Tris-HCl buffer. Inhibition in enzyme activity was observed at increasing substrate concentrations above1.5 mM pNPP at all pH range except pH 11 where stable activity was noticed. At pH 11, dissociation of pNP from pNPP or transfer of the enzyme to another quaternary state might be highly facilitated [18].

3.2. Determination of V_{max}, K_m and K_{cat} at different pH for CIAP activity with pNPP

Over a pH range of 8–11, V_{max} and K_m have been derived from Lineweaver-Burk plot. It was observed that V_{max} and K_m varied along with the pH. The lower the K_m value it indicates the stronger the affinity between enzyme and substrate, whereas the higher the K_m value that reflects the weaker the affinity between them [20]. At pH 11, the V_{max} and K_m of CIAP were determined to be 3.12 μmol min^{-1} $unit^{-1}$ and 7.6×10^{-4} M, respectively. This shows that even the rate of hydrolysis was higher at pH 11, least affinity of the enzyme towards the substrate was observed, which shows the possibility of slight conformational changes in the enzyme [21]. CIAP is reported to have K_m value of 9.6×10^{-4} M for phenyl phosphate [17] while it is found to be 3×10^{-2} M for β-glycerophosphate for rat intestinal-mucosal ALP [22]. By contrast, the K_m values were several orders of magnitude lesser than what is reported earlier and the higher affinity reported here could be partly due to the purified enzyme used [15, 22]. The highest turnover number was found to be 82.98 s^{-1} at pH 11.

3.3. Effect of pH and substrate concentration on BAP activity with pNPP as substrate in Tris-HCl buffer

Buffers play a vital role in the catalytic activity of alkaline phosphatase [23]. In the presence of Tris during the hydrolysis of 4-nitrophenyl phosphate by alkaline phosphatase at pH 8.0, the rate of 4-nitrophenol liberation was increased with concomitant phosphorylation of Tris [24]. On the contrary, it has been discussed that the dephosphorylation of the enzyme would take place at a slower pace under alkaline pH than the catalysis of the substrate. It has also been noted that the activity of BAP, like other enzymes, is pH dependent. BAP upon hydrolysis in Tris-HCl buffer resulted in higher activity at pH 8.5 till pH 10 at 37°C. The activity of enzyme increased linearly with the increase in substrate concentration [18] (**Figure 5**).

3.4. Determination of V_{max}, K_m and K_{cat} at different pH for BAP activity with pNPP

The Michaelis-Menten (K_m) varies from enzyme to enzyme, and also for the same enzyme with different substrates. **Table 1** shows the various kinetic parameters for catalysis of pNPP by BAP in Tris-HCl buffer under different pH. The optimum pH for BAP falls at 8.5 and the V_{max} is 0.82 μmol min^{-1} $unit^{-1}$, and the K_m is 1.5×10^{-3} M. Irrespective of the low rate of hydrolysis, the affinity of the enzyme BAP for pNPP was maximum at pH 8.5. When the K_m for

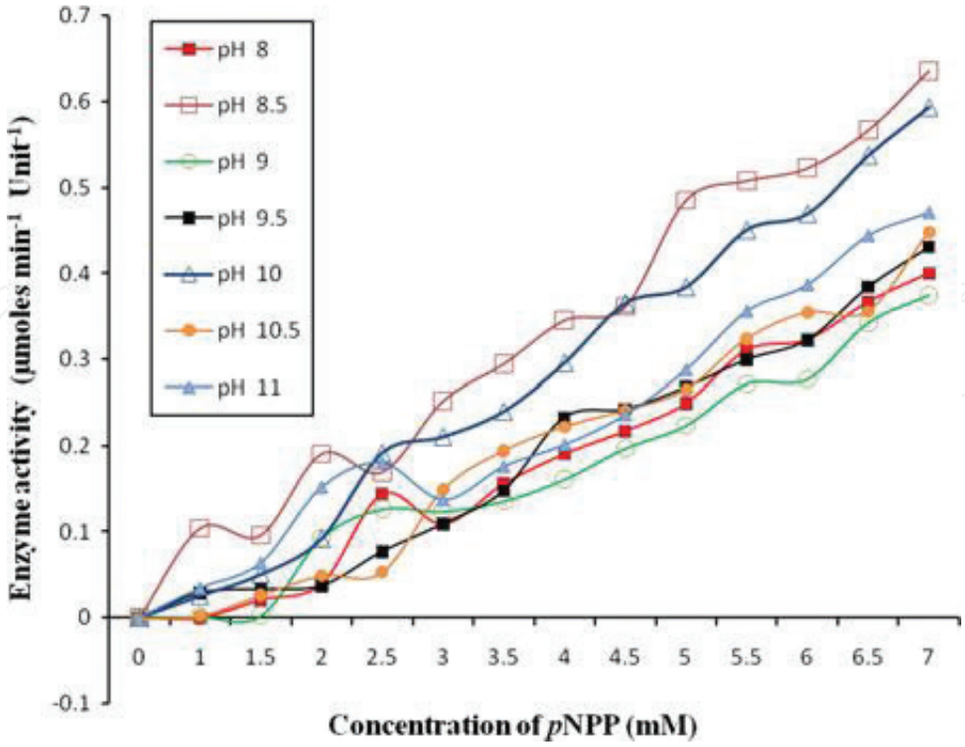

Figure 5. Hydrolysis of pNPP by BAP under different concentrations of substrate and varying pH regimes in Tris-HCl buffer.

pH	V_{max} (µmol min^{-1} unit^{-1})	K_m (M)
8	0.73	7.4×10^{-3}
8.5	0.82	1.5×10^{-3}
9	1.48	3.5×10^{-3}
9.5	0.33	4.66×10^{-3}
10	1.33	1.67×10^{-3}
10.5	0.83	1.75×10^{-3}
11	0.73	7.5×10^{-3}

Table 1. The V_{max} and K_m values obtained with BAP in 50 mM Tris-HCl buffer.

enzyme catalytic reaction is higher, affinity of the enzyme for its substrate is lower and vice versa. Results showed that the catalytic efficiency at pH 8 is 18,220 ± 2 M^{-1} s^{-1} [18].

4. Bioprecipitation studies using calf-intestine alkaline phosphatase

Kinetic parameters obtained for BAP were analysed under standard conditions which showed comparable V_{max} at pH 8, 8.5 and 11, while the values at pH 9 and 10 were alike. The activity

of the enzyme was quite complex, and possibly all the parameters during enzymatic precipitation studies with respect to incubation time, temperature, pH and substrate concentration have to be studied [18].

4.1. Average pattern of precipitation of Cr^{6+}, Cr^{3+}, Ni^{2+}, Cd^{2+} and Co^{2+} from single-ion solutions

A comparison of the average graphs for the precipitation of each heavy metal across the two pH regimes 8 and 11 and the concentrations employed, viz., 250 and 1000 ppm, shows that the efficiency of the enzyme-derived reactions is in the order of Cd^{2+}> Ni^{2+}> Cr^{3+}> Co^{2+}> Cr^{6+} (**Figure 6**).

4.2. Bioprecipitation of Cr^{3+} and Cr^{6+} from tannery effluent

The enzyme CIAP was able to precipitate 35.10% of Cr^{6+} and 38.39% of Cr^{3+} from the original concentration of 600 and 350 ppm, respectively, present in the tannery effluent. These precipitations were obtained at 120 min and at pH 11. Further, the percentage of precipitation of Cr^{6+} in these reactions is also comparable to the precipitation observed with Cr^{6+} in single-ion solution earlier (250 ppm, pH 11). This is attributed to the fact that only in the presence of CIAP the precipitation of metal ions could take place.

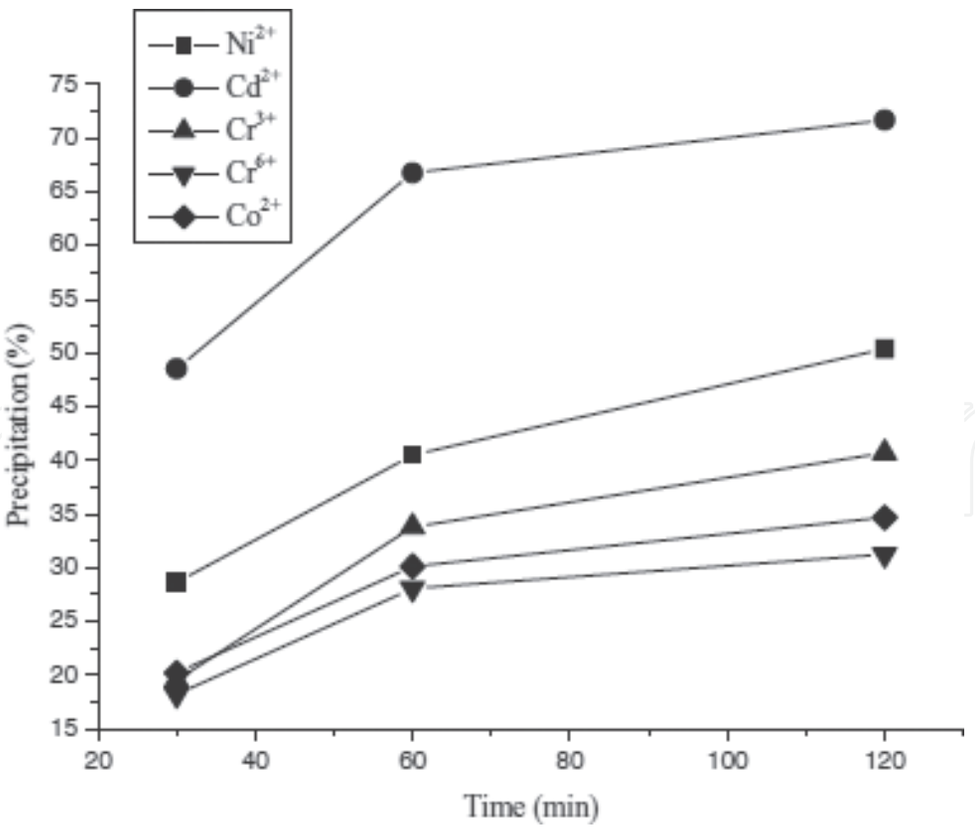

Figure 6. Graphical representation of the average pattern of precipitation of heavy metals from single-ion solution by CIAP and *p*NPP.

4.3. Bioprecipitation of Ni^{2+}, Cd^{2+} and Co^{2+} from electroplating effluent

During experiments with electroplating effluent, it was observed that CIAP was able to precipitate 52.28% of Cd^{2+} from initial concentration of 934 ppm, 31.34% of Co^{2+} from 878 ppm and 30.34% of Ni^{2+} from750 ppm at 120 min at pH 11. The percentage of precipitation of each heavy metal was quite comparable to the observations made with single-ion solutions. However, values obtained were somewhat lesser than what was seen in the former reactions. It could be attributed to the fact that the Pi generated was competed by all the three metal ions simultaneously for the formation of respective metal-phosphate precipitate.

5. Bioprecipitation studies using bacterial alkaline phosphatase

Bacterial alkaline phosphatase precipitated Cr^{6+} and Cr^{3+} from the two different concentrations such as 250 and 1000 ppm at various pH from 8.5 to 10. At 250 ppm, 42 and 52.81% of precipitation for Cr^{6+} and Cr^{3+} were obtained at pH 8.5 during 300 min of incubation. Analysis of the precipitation obtained with BAP under the pH 8.5 and 10, at 250 and 1000 ppm, resulted in differences in the pattern of precipitation. Higher precipitation could be achieved with 250 ppm of Cr^{6+} with 1000 ppm, since the pH seems to play a less important role in determining the precipitation levels. Whereas with Cr^{3+}, the pH influences the precipitation patterns and at both concentrations higher precipitation was obtained at pH 8.5 and 10 [18]. This is because of the dynamics of Cr^{3+} and Cr^{6+} ionization and dissociation as pH dependent rather than the inherent capability of the enzyme in releasing product from pNPP. By determining the statistical data, a significant difference ($p < 0.05$) was observed with a 95% confidence interval, between the two concentrations and pH [18].

5.1. Average pattern of precipitation of Ni^{2+}, Cd^{2+}, Cr^{6+}, Cr^{3+} and Co^{2+} from ion solutions

The average precipitation for every heavy metal between pH 8.5 and 10 at two concentrations 250 and 1000 ppm was compared and is shown in **Figure 7**. The efficiency of the enzyme for the precipitation of different metal ions is in the order of Cd^{2+}>Ni^{2+}>Cr^{3+}>Cr^{6+}>Co^{2+}. No scavenging of inorganic phosphate is expected since the precipitations were carried out in ionic solutions, and in addition the amount of inorganic phosphate available for metal phosphate formation should remain constant. Hence, the variation in the precipitation pattern with different ions might be due to the difference in kinetics of metal phosphate complex formation and also because of the radical effect of metal ions present in the backbone of the enzyme [18].

5.2. Enzymatic precipitation of Cr^{6+}, Ni^{2+} and Cd^{2+} from tannery and electroplating industry effluents

The applicability of the enzymatic precipitation of heavy metals from actual industrial effluent was validated by processing the effluent with BAP and pNPP. About 621 ppm of Cr^{6+} was present in tannery effluent. Upon treatment with the enzyme and substrate pNPP for 300 min, the amount of Cr^{6+} present in the supernatant was 403 ppm, resulting in 35.1% of

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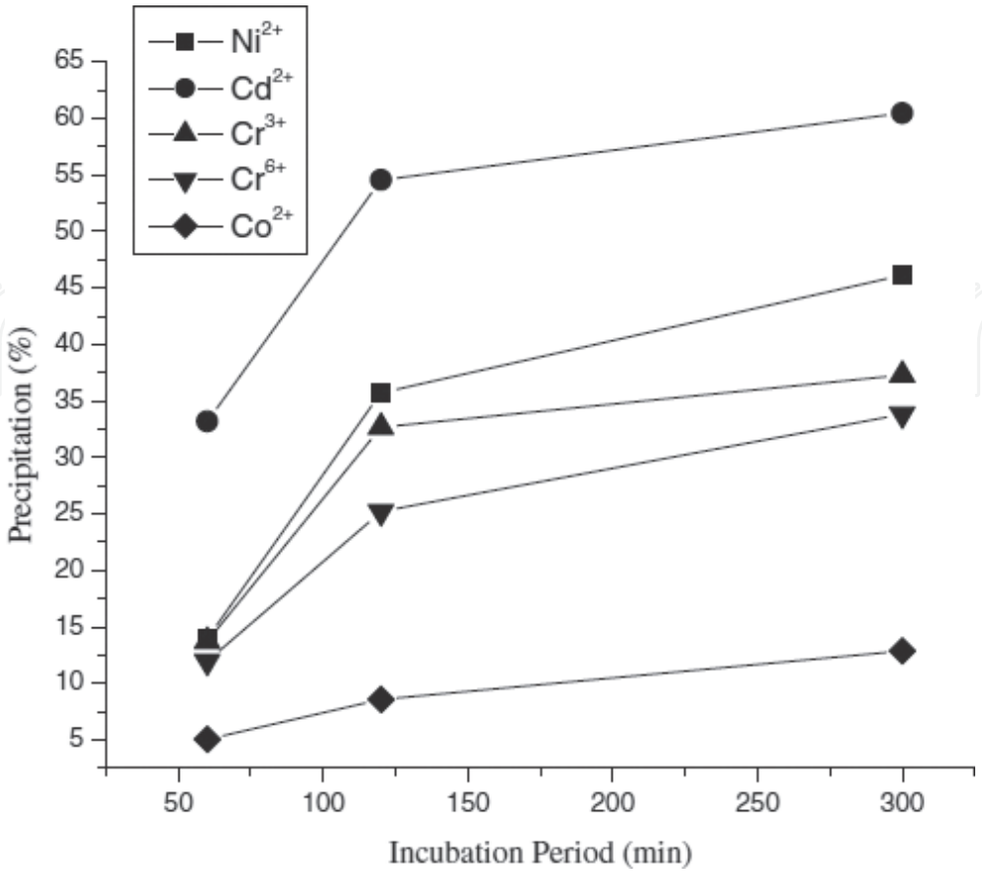

Figure 7. Graphical representation of the average pattern of precipitation of heavy metals from single-ion solutions with BAP using pNPP as substrate.

precipitation. On the other hand, 97 ppm of Ni^{2+} and 122 ppm of Cd^{2+} were also present in the electroplating effluent. Final concentration of 21.43 and 51.95 ppm of Ni^{2+} and Cd^{2+} was present in the supernatant after treating with the enzyme, representing a 77.8 and 57.42% precipitation of the metals, respectively. From the results, it is evident that the two metal ions competed simultaneously for the inorganic phosphate generated by the enzyme. Moreover, Cr^{6+} precipitation from tannery effluent (35.1%) was compared with the values observed in ion solution (42.3%) [18].

The application of BAP and CIAP for the precipitation of heavy metals such as Ni^{2+}, $Cd^{2+,}$ $Cr^{3+/6+}$ and Co^{2+} (from single-ion solutions as well as tannery and electroplating industrial effluents) under alkaline pH was studied using ascorbic acid 2-phosphate, a natural substrate. To determine the potential of the enzyme to remain stable at certain environmental conditions, the kinetic characteristics of BAP and CIAP at various pH 8–11 were also studied. Higher activity of the enzyme was obtained at pH 9.5 and 10 for BAP and CIAP, respectively. The average precipitation pattern of metal ions from single-ion solutions by BAP and CIAP occurred in the order of $Cd^{2+} > Ni^{2+} > Co^{2+} > Cr^{3+} > Cr^{6+}$ and $Co^{2+} > Cd^{2+} > Ni^{2+} > Cr^{6+} > Cr^{3+}$, respectively. Results state that the precipitation of Cr^{6+} from tannery effluent by BAP was 15.57% after 300 min of incubation while CIAP resulted in 71.47% at 120 min incubation. By contrast, BAP precipitated Cd^{2+} at much higher percentage than CIAP from electroplating

effluents with the percentage of 94.6 and 66, respectively. Since ascorbic acid 2-phosphate is a natural and biodegradable substrate, this study offers an eco-friendly approach for a sustainable environment [25].

6. Kinetic behaviour of BAP (*E. coli*C90) and CIAP with α-naphthyl phosphate

6.1. Effect of pH and substrate concentration on BAP (*E. coli*C90) and CIAP activity with α-naphthyl phosphate as substrate

The investigation revealed that there is an immense effect of pH as well as the substrate α-naphthyl phosphate concentration on the kinetics of BAP- and CIAP-catalysed α-naphthyl phosphate hydrolysis reaction. The maximum activity of CIAP was found to be at pH 11 (**Figure 8a**) followed by pH 9.5. The second highest pH value after pH 11 was found to be pH 9.5. Experimental data showed that among all pH studied, the highest activity for BAP was found at pH 8.5 (**Figure 8b**). The second highest pH value after pH 8.5 was found to be pH 9. It was observed that with an increase in the substrate concentration, the activity was also being increased. Kinetic parameters such as V_{max} and K_m were calculated for both enzymes. For CIAP, the V_{max} and K_m at pH 11 were 4 μmol min^{-1} $unit^{-1}$ and 2.2×10^{-3} M, respectively. On the other hand, for BAP at pH 8.5 the V_{max} and K_m were found to be 1.25 μmol min^{-1} $unit^{-1}$ and 0.014 M, respectively.

6.2. Average pattern of precipitation of Cr^{6+}, Cr^{3+}, Ni^{2+}, Cd^{2+} and Co^{2+} from single-ion solutions by CIAP and BAP with α-naphthyl phosphate

A comparative analysis of the average graph, obtained on CIAP- and α-naphthyl phosphate-mediated precipitation, was done in order to get a clear picture of the pattern of precipitation. The average precipitation of metals was found to be in the following order: $Co^{2+} > Ni^{2+} > Cd^{2+} > Cr^{3+} > Cr^{6+}$ (**Figure 9a**).

Average graphs for the precipitation of Cr^{3+}, Ni^{2+}, Cd^{2+},Co^{2+} and Cr^{6+} across the two concentrations (250 and 1000 ppm) and the two pH regimes (pH 8.5 and pH 9) are represented in **Figure 9b**. It can be inferred from the comparative analysis that BAP- and α-naphthyl phosphate-mediated heavy-metal precipitation were in the following order: $Ni^{2+} > Co^{2+} > Cr^{6+} > Cr^{3+} > Cd^{2+}$.

6.3. Bioprecipitation of heavy metals from electroplating industrial effluents by CIAP and BAP with α-naphthyl phosphate

CIAP-mediated investigation was performed at pH 11. In electroplating effluent, heavy metals present were Ni^{2+} (initial concentration was 134 ppm), Cd^{2+} (initial concentration was 734 ppm) and Co^{2+} (initial concentration was 278 ppm). The precipitations of Ni^{2+}, Cd^{2+} and Co^{2+} were 66.17, 66.93 and 49.07%, respectively.

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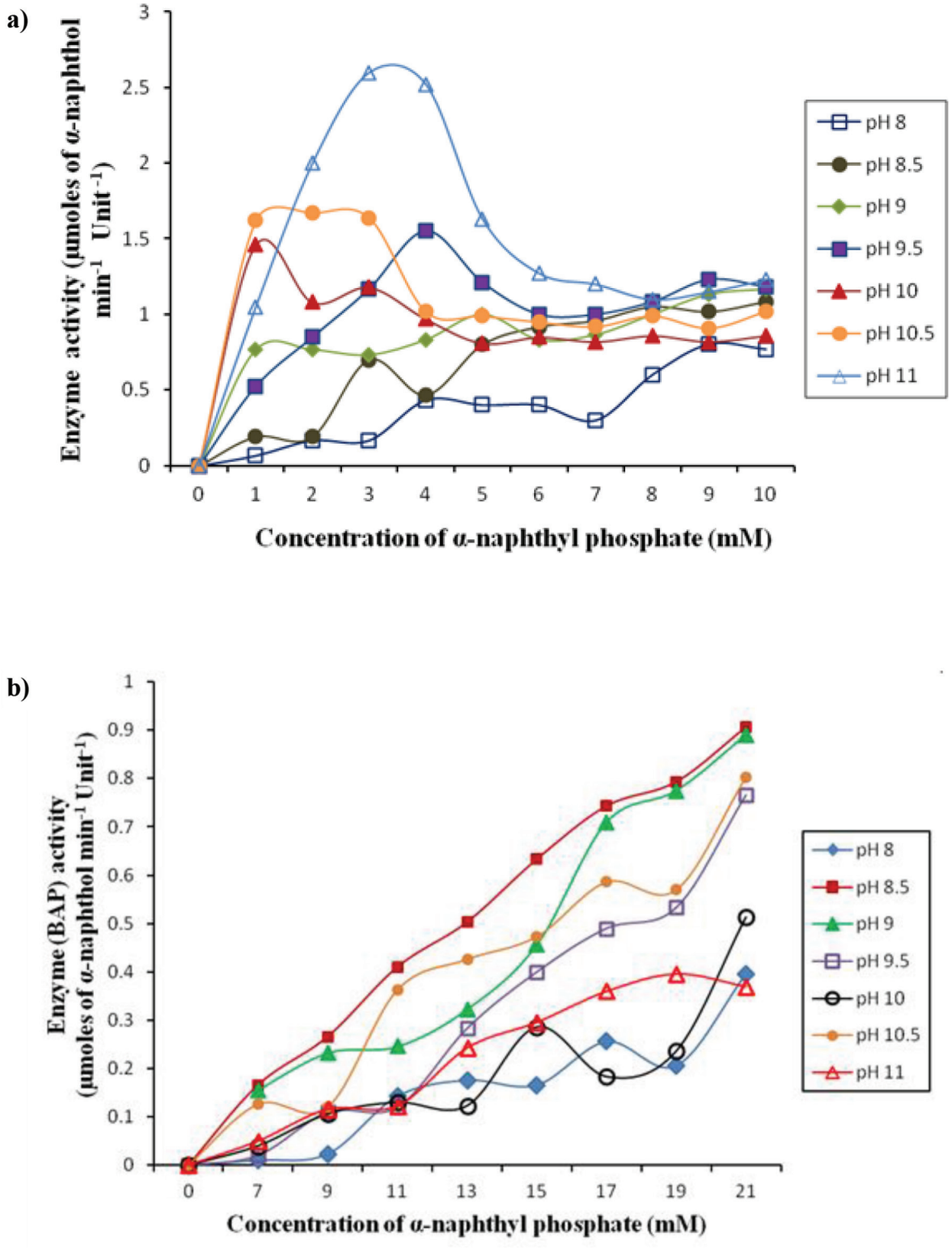


Figure 8. Hydrolysis of α-naphthyl phosphate by (a) CIAP and (b) BIAP under different concentrations of substrates and varying pH regimes.

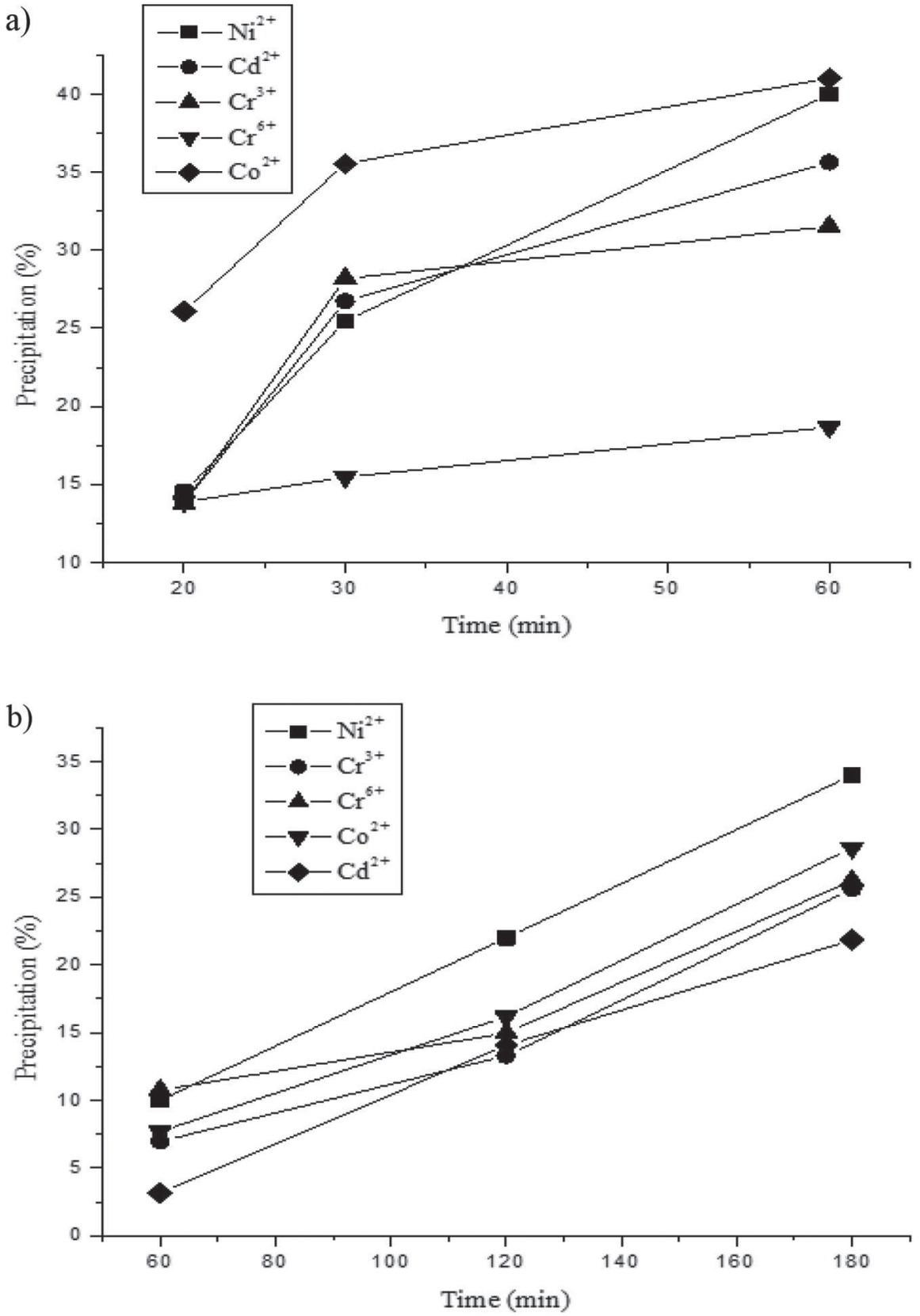


Figure 9. Graphical representation of the average pattern of precipitation of heavy metals from single-ion solution by (a) CIAP and (b) BAP with α-naphthyl phosphate.

On the other hand, the observations showed that BAP-mediated precipitation of Cd^{2+}, Ni^{2+} and Co^{2+} from electroplating effluent was 20.37% (initial concentration was 734 ppm), 57.93% (initial concentration was 134 ppm) and 34.4% (initial concentration was 278 ppm). CIAP with α-naphthyl phosphate-mediated precipitation was greater than that of BAP and α-naphthyl phosphate. This could be attributed to the fact that more P_i had been generated and bound to the heavy metals in the case of CIAP than that of BAP.

7. Kinetic study of BAP (*E. coli*C90) and CIAP with L-ascorbic acid 2-phosphate

7.1. Effect of pH and substrate concentration on BAP (*E. coli*C90) and CIAP activity with L-ascorbic acid 2-phosphate as substrate

The capacity of BAP and CIAP along with ascorbic acid 2-phosphate is being reported as new heavy-metal-remediating tool, the effect of pH and substrate concentration on BAP and CIAP activity was studied at the range of pH values from 8 to 11 and at a range of substrate concentrations from 1 to 17 mM. Generally, tannery and electroplating effluents will have pH of alkaline range. The maximum activity of BAP was observed at pH 9.5 (**Figure 10a**), whereas for CIAP it was at pH 10 (**Figure 10b**).

7.2. Average precipitation of heavy metals from single-ion solution with BAP (*E. coli*C90) and CIAP with ascorbic acid 2-phosphate

A comparative analysis of the average graphs for the precipitation of each heavy metal (Cd^{2+}, Co^{2+}, Ni^{2+}, Cr^{3+} and Cr^{6+}) across the two pH values (pH 9.5 and 10.5 in case of BAP; pH 8 and pH 10 in case of CIAP) and the two concentrations, that is, 250 and 1000 ppm, was employed. The comparative analysis reveals the effect of BAP (*E. coli* C90) on the precipitation of heavy metals which are in the order of $Cd^{2+} > Ni^{2+} > Co^{2+} > Cr^{3+} > Cr^{6+}$ (**Figure 11a**). Similarly in **Figure 11b**, the average graph shows the efficiency of CIAP for heavy-metals precipitation which are in the order of $Co^{2+} > Cd^{2+} > Ni^{2+} > Cr^{6+} > Cr^{3+}$. But at 120 min, the order was different, for example, $Cr^{6+} > Co^{2+} > Cd^{2+} > Ni^{2+} > Cr^{3+}$. The precipitations were carried out in single-ion solutions. Therefore, no scavenging of P_i is expected and the amount of inorganic phosphate liberated for metal-phosphate formation should be constant. In addition, the variation in the precipitation with respect to different ions might be because of the difference in kinetics of metal-phosphate formation and also due to the radical effect of metal ions present in the enzyme [25].

7.3. Bioprecipitation of heavy metals from real-time effluent

The efficiency of BAP and CIAP in precipitating heavy metals from real-time effluents was studied by treating the effluents along with the substrate. The concentration of Cd^{2+} in electroplating effluent initially was found to be 734 ppm. After enzymatic treatment with BAP for 60, 120 and 300 min at pH 9.5, the percentage precipitations obtained were 81.94, 91.39 and 94.62%, respectively, for each time. Likewise, treatment using CIAP for 30, 60 and 120

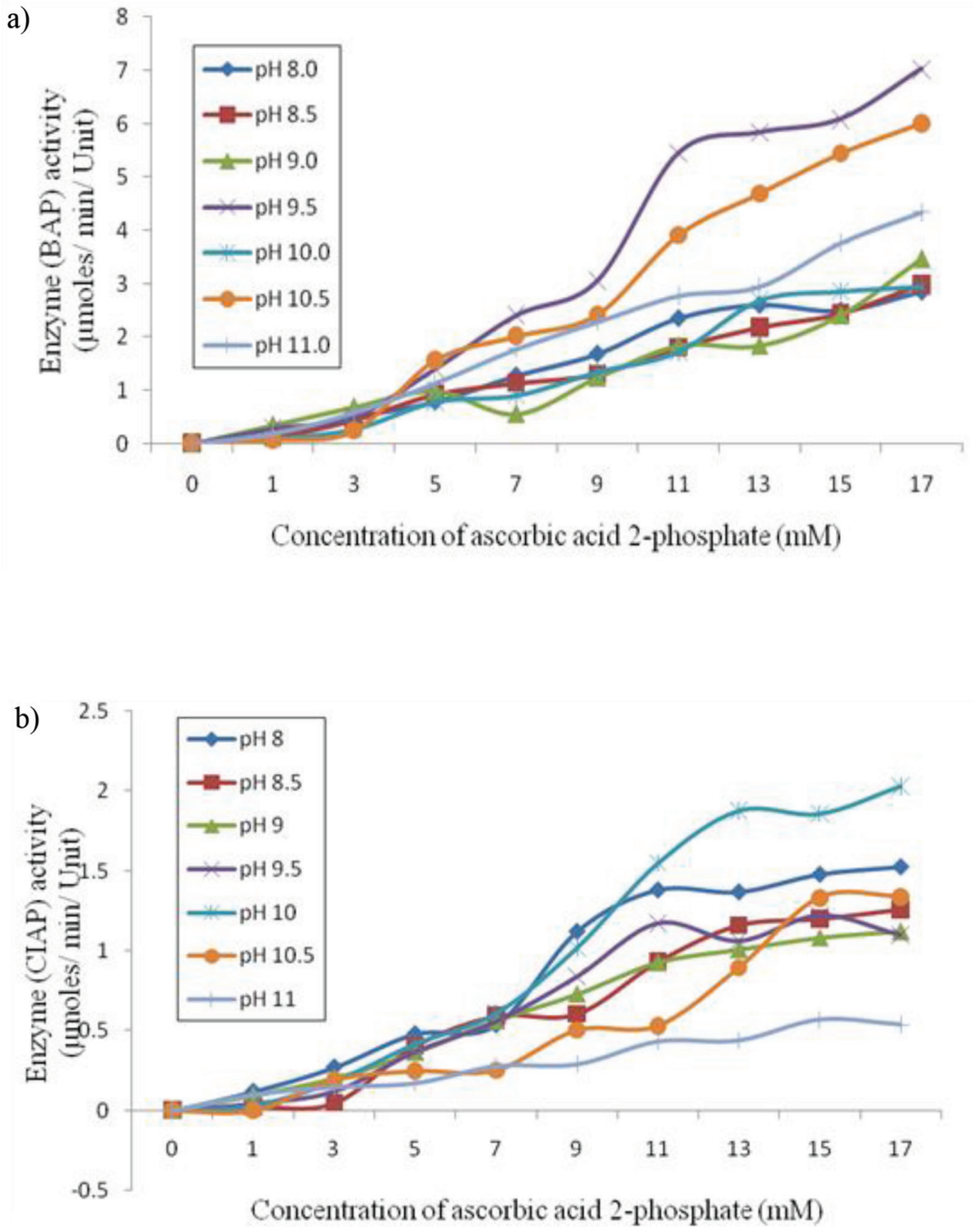
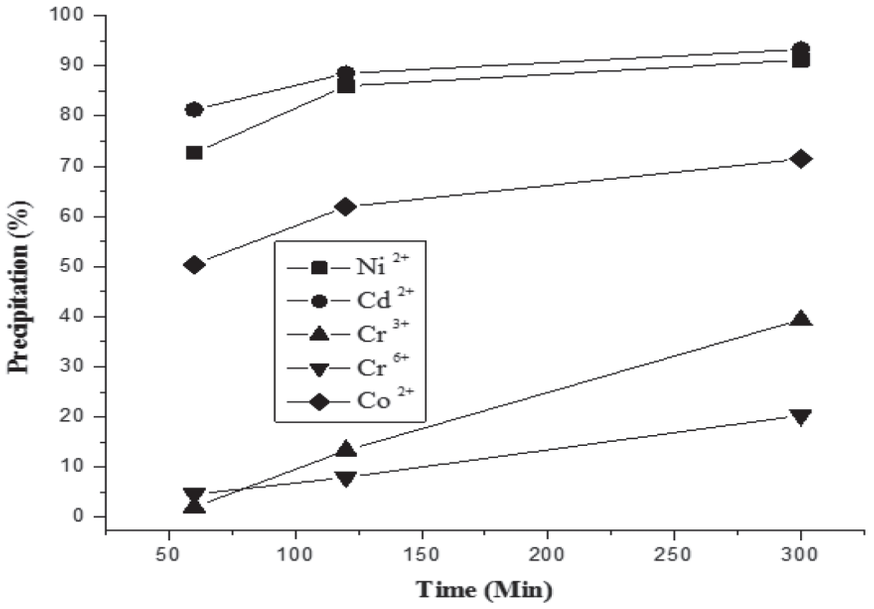


Figure 10. Hydrolysis of L-ascorbic acid 2-phosphate by (a) BAP and (b) CIAP under different concentrations of substrates and varying pH regimes. Reaction conditions: incubation period = 60 min, temperature = 37°C, buffer = 50 mM Tris-HCl.

a)



b)

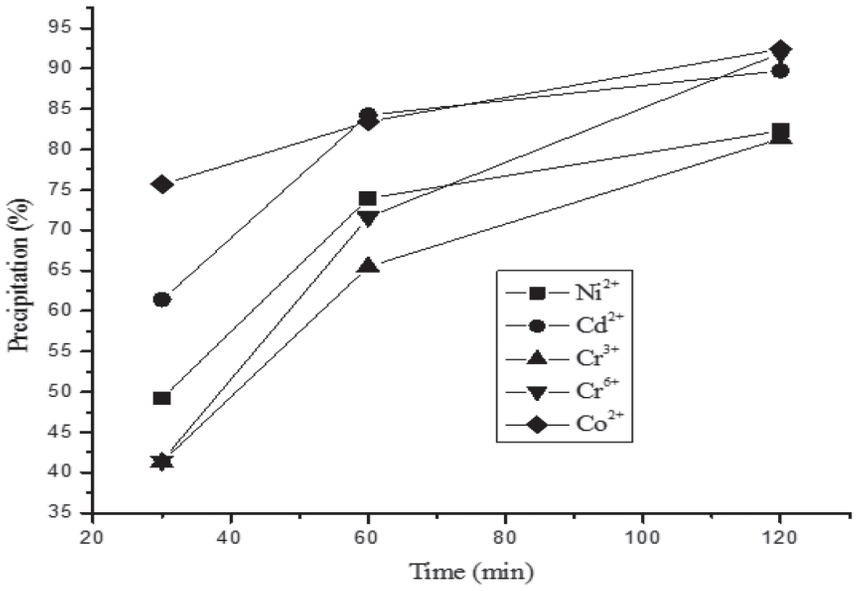


Figure 11. Graphical representation of the average pattern of precipitation of heavy metals from single-ion solution with (a) BAP *(E. coli* C90) and (b) CIAP with ascorbic acid 2-phosphate.

min at pH 10 resulted in 32.75, 53.75 and 65.89% of precipitation, respectively. The removal of Cr^{6+} from tannery effluent at an initial concentration of 560 ppm was analysed by treating with BAP and CIAP using ascorbic acid 2-phosphate as substrate. When compared to CIAP, BAP showed reduced levels of precipitation. Moreover, at pH 9.5 for 60, 120 and 300 min, the percentages of precipitation of Cr^{6+} by BAP were 0, 5.61 and 15.57%, respectively. Whereas at pH 10 for 30, 60 and 120 min, the percentage of removal of Cr^{6+} were 38.32, 64.55 and 71.47%, respectively [25].

8. Conclusion

Bioprecipitation study with BAP and CIAP along with the substrate ascorbic acid 2-phosphate is a much safe and convenient method for the removal of heavy metals such as cobalt, nickel, chromium, cadmium, and so on. The comparative studies have given a clear picture of both the enzymes' kinetic behaviour and bioprecipitation efficiency at different pH, different metal concentration and at different time. Based on this information, further studies can be conducted with other metals which have not been studied in the present work. Immobilization of these enzymes also can be done for large-scale application in industries.

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