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RESEARCH ARTICLE

ACTIVE RESIDUES AND IMMOBILIZATION OF CYANIDE HYDRATASE FROM *CLADOSPORIUM OXYSPORUM*

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Abstract

Cyanide hydratase (EC.4.2.1.66) was purified from *Cladosporium oxysporum* with specific activity of 39.6 units mg^{-1} protein. The enzyme exhibited appreciable stability at both 50 ºC and 60 ºC. Ca^{2+} was the best activator for the enzyme activity where Hg^{2+} was the most potent inhibitor among the various tested ions. On the other hand, K^{+} as monovalent cation did not express any remarkable effect on enzyme activity. N-ethylmaleimide (NEM), diethylpyrocarbonate (DEPC), N-bromosuccinimide (NBS) and phenylglyoxal (PGO) inhibited the enzyme activity revealing necessity of cysteinyl, histidyl, tryptophanyl and arginyl groups, respectively for enzyme catalysis. *O*-Phenanthroline and α-α-dipyridyl inhibited the enzyme activity and the inhibition was concentration-dependent. Cyanide hydratase was immobilized on Ca alginate, chitosan, silica-gel and agar-agar. The chitosan was the best bead for the immobilization. The immobilized cyanide hydratase expressed higher optimal pH and higher optimal temperature compared to the free one. The immobilized enzyme expressed storage stability at 4 ˚C better than that of the free one. Cyanide hydratase was capable to degrade cyanide at 5, 10 and 15 mM particularly within the first ten hours. The results suggests that cyanide hydratase from *C. oxysporum* can be applied in biodegradation of polluted cyanide.

INTRODUCTION

Enzymes are biocatalysts characterized by their specificity and reactivity in biological systems. Enzymes are found in all living cells where they serve to activate and regulate the chemical reactions necessary for the continuing life of the organism (El-Shora et al., 2015a).

Microbial enzymes are better than the plant or animal sources because of their economic production, consistency, modification, optimization and ease of process. They are more stable than plant or animal enzymes.

They exhibit a wider diversity of catalytic activities (Adrio and Demain, 2014). The majority of enzymes currently used in industry are of microbial origin (Nolan et al., 2003; El-Shora et al., 2015b).

Cyanide is used in many of industrial processes including extraction of gold from ore, plastics, electroplating and manufacturing. These types of industrial processes leave large quantities of cyanide-containing waste and these wastes should be detoxified before released into the environment (Dash et al., 2009).

The cyanide ion is a strong inhibitor of growth and cellular metabolism, including nitrogen and phosphate metabolism as well as respiration (Shete and Kapdnis, 2013).

The activity of cyanide hydratase appears to be correlated with the capability of some fungi to infect cyanogenic plants despite the release of hydrogen cyanide into the infection site in toxic concentrations (Ahmed et al., 2006).

Enzymes are localized in or on a variety of insoluble matrices with the concomitant retention of biological activity. Enzymes can be immobilized and among the advantages of enzyme immobilization are: (1) increasing of its stability, (2) the support system can be easily removed from solution without contamination of the contents of the reaction mixture, (3) a single aliquot of enzyme can be used repeatedly to achieve more analyses than using the same amount of enzyme in solution and (4) it is possible to prepare unstable, sensitive or expensive reagents using an immobilized enzyme (Datta et al., 2013; El-Shora et al., 2015a).

Thermal and pH stabilities reflect the ability of the enzyme-support system to resist higher temperatures or pHs at acidic/alkaline sides before occurring of denaturation. Storage stability is identified as the ability of the enzyme preparation to retain its activity under some certain storage conditions and can provide information about the shelf life. The operational stability is not only a function of the enzyme, but also a function for inhibitor concentration in the solution and the carrier durability (Raafat et al., 2011).

Cyanide hydratase was purified from *C. oxysporum* to 39.6 units mg^{-1} protein (El-Shora et al., 2014). Therefore, the present investigation aimed to investigate the kinetics, the active groups taking part in the enzyme catalysis, enzyme immobilization and comparing the stability of free and immobilized enzyme.

Materials and Methods

General culture conditions

The fungus was isolated from tomato. Cultivation of *C. oxysporum* was carried out by submerged fermentation (SmF) in 250 ml shaken Erlenmeyer flasks using conical flasks containing 100 ml of Czapekdox liquid medium with glucose as source of carbon, autoclaved at 121 °C; 15 1bs pressure for 20 min and then inoculated with spore suspension 10^7 spores/ml final concentration. Flasks were incubated in the dark at 28 °C for 7 days.

Extraction of intracellular cyanide hydratase

The culture was filtered by Whatman no. 1 filter paper. The mycelia washed twice by 0.1 % saline solution. The washed mycelia were crushed in 50 mM buffer (pH 7.0) in homogenizer for 30 min and centrifuged at 10,000 rpm for 20 min. The resulting supernatant was collected and kept in refrigerator and used for enzyme assay.

Assay of enzyme activity

The enzyme activity was assayed according to the method of Watanabe et al. (1998). Enzymatic activity was assayed in 1.4 ml 50 mM phosphate buffer (pH 8.0) containing 10 mM KCN as the substrate. The reaction was carried out at 45 ˚C for 40 min. The amount of ammonia produced in the reaction mixture was estimated. One unit of the enzyme is defined as the amount needed to catalyze the formation of 1 µmol ammonia from cyanide min^{-1} under the above conditions.

Purification of cyanide hydratase

Cyanide hydratase was purified from *C. oxysporum* to 39.6 units mg^{-1} protein (El-Shora et al., 2014).

Estimation of ammonia-N

The method used for estimation of ammonia-N was that of Delroy (1949) using Nessler´s reagent as modified by Naguib (1964). An aliquot of that tissue extract was mixed with 1 ml of 1N-NaOH and 0.5 ml of 0.5% $ZnSO_4$. The mixture was made up to 14 ml with distilled water before 1 ml of Nessler´s reagent was added, shaken well and allowed to stand for 5 min. The optical density was then measured at 450 nm. Ammonia concentration was determined from a standard curve using ammonium sulphate.

Estimation of cyanide

Cyanide estimation was done by pyridine-barbituric acid method (Shigeru, 1984). Aliquot of 4ml from the fermented broth was taken and 2 ml chloramine-T was added. Immediately after this 5 ml pyridine-barbituric acid reagent was added with constant gentle swirling. The mixture was then diluted to 50 ml with bi-distilled water. CN^- at alkaline pH gets converted to CNCl with chloramines –T, which forms reddish blue colour on reaction with

pyridine-barbituric acid reagent. The absorbance was recorded at 578 nm after 8 min but within 15 min. A standard graph was prepared using potassium cyanide.

Thermal stability assay

The denaturation property of the cyanide hydratase was determined by measuring enzyme activity at different temperatures in the range 50 °C – 60 °C for 1 h duration at various intervals using cyanide. In order to examine the thermal stability of the enzyme solution an aliquot was withdrawn at appropriate time intervals for measuring of the enzyme activity using the assay method of the enzyme. The residual activity was expressed as a percentage relative to the initial enzyme activity as calculated from the following equation:

% Remaining activity = (Enzyme activity at time t x100) / (Enzyme activity at time t_0).

Effect of metal cations on cyanide hydratase activity

The effect of divalent cations Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Al^{3+}, Cu^{2+} and Hg^{2+} (in the form of their chlorides) at 5 mM and 10mM on the activity of cyanide hydratase was studied by including each individual cation in the standard reaction mixture of the enzyme. The control was done without metals (100 %).

Cyanide hydratase immobilization

1- Immobilization of cyanide hydratase on Ca-alginate

Sodium alginate solution (5 % w/v) in 100 mM sodium phosphate buffer (pH 8.0) was prepared by warming at 50 ºC, cooled down to room temperature followed by mixing the enzyme solution with sodium alginate solution in total volume 10 ml. The mixture was taken into a syringe, and beads were formed by dropping the solution into 100 mM calcium chloride solution with gentle stirring for 2 h at 4 ºC. The beads were filtered and thoroughly washed with distilled water, dried using filter paper (Whatman no.1) followed by exposure to the open air for 1 h before use (Ertanet el al., 2007). The filtered calcium chloride solution was collected for enzyme activity determination.

2- Immobilization of cyanide hydratase on agar-agar

The solution of agar was prepared in potassium phosphate buffer, 20 mM, pH 8.0 by warming at 50 ºC, cooled down to room temperature. The enzyme (containing 0.5 mg protein / ml) was mixed with the matrix solution in a total volume of 10 ml and immediately casted on preassembled glass plates. After solidification at room temperature, agar-agar gel was cut into small chips of 5 × 5 mm size and washed several times before use to remove any enzyme attached to the gel surface. The immobilized chips were stored in extraction buffer in refrigerator. The activity of these chips was assayed spectrophotometrically as described before.

3- ***Immobilization of cyanide hydratase on silica gel.***

Silica gel (1 g) was mixed with the enzyme solution and stored at 4 ºC overnight. The unbound enzyme was removed from the carriers by washing several times with 10 mM phosphate buffer (pH 8.0). Protein content and cyanide hydratase activity were estimated in washed solution (Abdel-Naby, 1993).

4- Immobilization of cyanide hydratase on chitosan beads.

i) Preparation of chitosan beads

Chitosan powder (3 g) was suspended into 99 ml of distilled water by stirring for 10 min. Glacial acetic acid (1 ml) was then added and stirring was allowed for 3 h at room temperature. The solution was filtered and dried and some NaOH aqueous solution (2%) was added to neutralize the acetic acid in the chitosan film. The films were repeatedly washed with deionized water and finally dried again.

ii) ***Reinforcement of chitosan beads by glutaraldehyde treatment***

The cross-linking was carried out by adding the dried chitosan into 0.05 % (w/v) glutaraldehyde solution in 50 mM phosphate buffer (pH 8.0) for 1 h. The brownish reinforced beads were washed several times by 50 mM cold phosphate buffer (pH 8.0) to remove the excess of glutaraldehyde.

iii) Immobilization of cyanide hydratase on chitosan beads

Chitosan beads were mixed with 2 mg/ml cyanide hydratase solution in 50 mM phosphate buffer (pH 8.0) for 3 h with slight stirring and washed at 4 ºC followed by drying at room temperature and storing at 4 ºC. The amount of immobilized enzyme was estimated by subtracting the amount of protein determined in the supernatant after immobilization from the amount of protein added for immobilization.

Assay of immobilized cyanide hydratase

Approximately 100 mg of cyanide hydratase-immobilized chitosan beads were used for enzyme assay as described earlier for the free enzyme.

Immobilization yield (%) = (Activity of immobilized enzyme / Activity added for immobilization) x 100

Statistical analysis

All the data in the present study are expressed as mean ± SE obtained from the measurements.

Experimental Results

Thermostability of cyanide hydratase at 50 ˚C and 60 ˚C at various time intervals (10 – 60 min) (Fig.1) showed that the enzyme activity decreased gradually with increasing the time of incubation at 50 ˚C and 60 ˚C. It is noticed that the enzyme activity at 60 ˚C decreased remarkably than that subjected to 50 ˚C throughout the experimental periods.

Studying the effect of K^+, Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Al^{3+}, Cu^{2+} and Hg^{2+} at 5 and 10 mM as chloride salts showed that Ca^{2+} was the best activator for cyanide hydratase at both tested concentrations followed by Mg^{2+} (Fig. 2).The other remaining divalent cations Co^{2+}, Zn^{2+}, Al^{3+}, Cu^{2+} and Hg^{2+} inhibited the enzyme activity with various rates particularly Hg^{2+} and Cu^{2+}. On the other hand, K^+ as monovalent did not exhibit any effect on cyanide hydratase activity.

NEM was used as reagent for -SH residue (El-Shora and Metwally, 2009) and DEPC was used as reagent for histidyl residues (El-Shora et al., 2008) in protein of enzymes Cyanide hydratase was inhibited by NEM and DEPC (Fig. 3) and DEPC was stronger inhibitor than NEM. Also, NBS was used as reagent for tryptophanyl reagent (El-Shora, 2001a) and PGO was used as arginyl reagent in enzyme protein (El-Shora and Youssef, 2008). Cyanide hydratase was inhibited by these two compounds particularly NBS (Fig. 4). The inhibition was concentration-dependent. It should be stressed that the inhibition was more apparent in case of 20 mM NBS.

Cyanide hydratase was inhibited by *o*-phenanthroline and *α*-*α*-dipyridyl (Fig. 5) and the enzyme was also dependent on the concentration. *O*-phenanthroline was stronger inhibitor than *α*-*α*-dipyridyl.

Cyanide hydratase was immobilized on agar-agar, chitosan, calcium alginate and silica-gel (Table 1). The yield of immobilization was 40.3 and 60.7 %, 35% and 23.8%, respectively for the above materials. Thus, the results show that chitosan was the best bead and therefore it is used in the future experiments.

Studying the effect of immobilization time on chitosan (Fig. 6) showed an increase in the immobilization yield at the 2nd and 3rd hours followed by continuous decline at the 4th, 5th and 6th hours.

The effect of glutaraldehyde concentration (2, 4, 6, 8 and 10 % v/v) on the immobilization yield of cyanide hydratase from *C. oxysporum* was investigated and the results are shown in Fig. 7. The results reveal that the optimal concentration was 2% (v/v) after which the enzyme activity decreased gradually with increasing glutaraldehyde concentration.

The results in Fig. 8 indicate that the pH of the immobilized enzyme was shifted to pH 9.0 which is higher than observed pH 8.0 for the free enzyme.

Also, the optimal temperature of the immobilized enzyme was shifted to 50 ˚C, which is higher than 45˚C recorded for the free enzyme (Fig. 9).

The immobilized enzyme exhibited longer storage stability than the free enzyme throughout 11 weeks. It was noticed that the free enzyme lost its activity after 8 weeks whereas the immobilized one retained 17 % of its activity after 10 weeks (Fig. 10).

Cyanide hydratase was applied to various cyanide concentrations (5, 10 and 15 mM). The results in Fig. 11 demonstrate that the percentage of cyanide degradation at each tested concentration was increased with time throughout 30 h and the optimal degradation time was 10 hr.

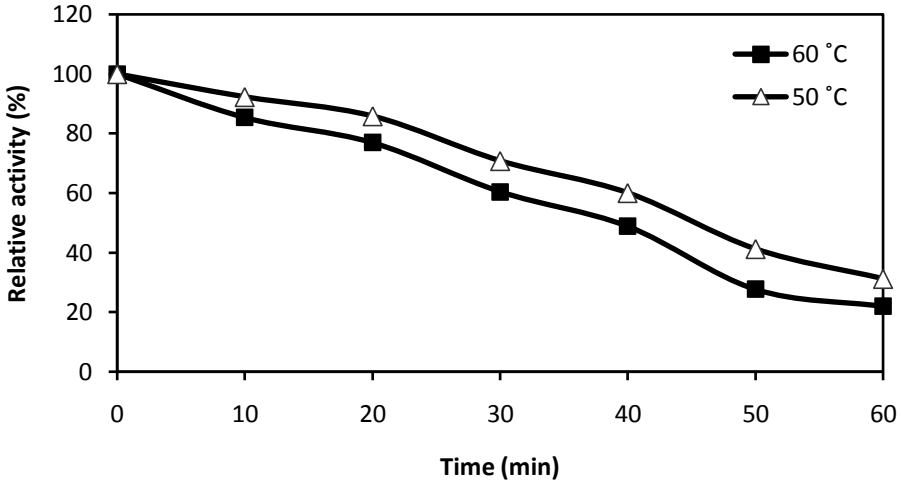

Fig. 1: Thermal stability of cyanide hydratase purified at 50 ˚C and 60 ˚C.

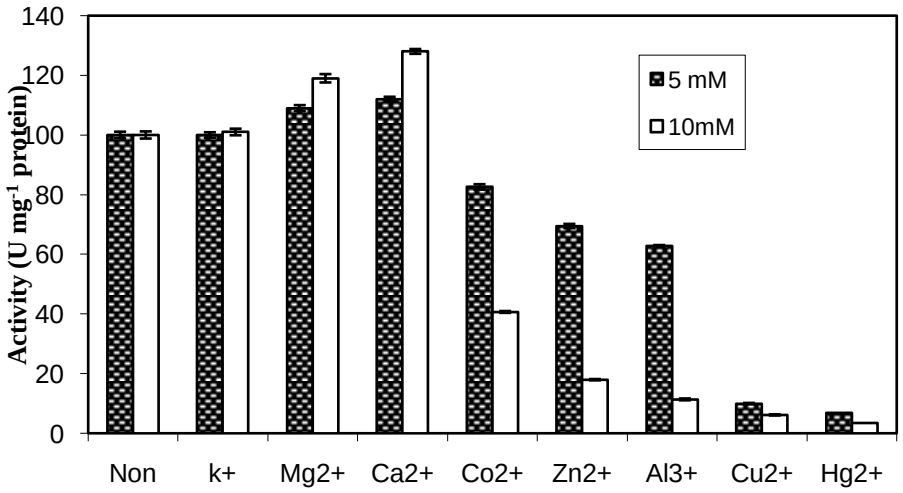

Fig. 2: Effect of metal cations on cyanide hydratase activity.

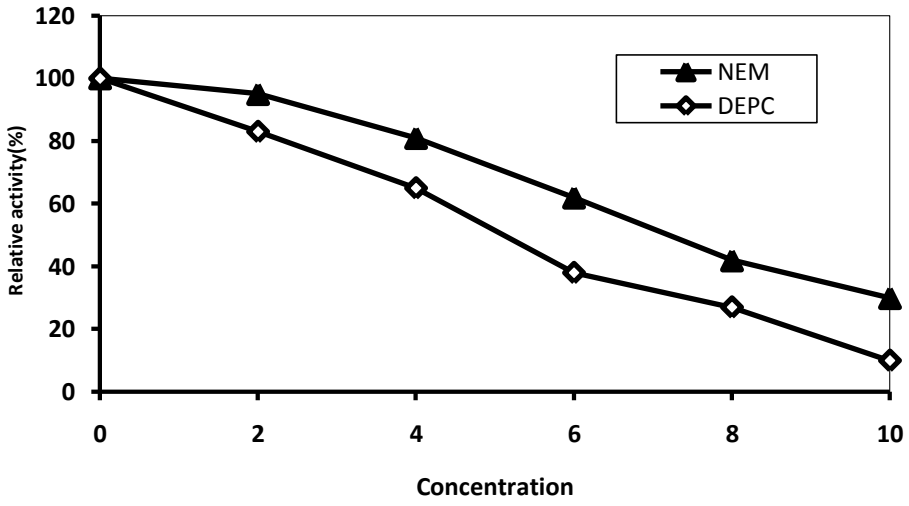

Fig. 3: Effect of NEM and DEPC on cyanide hydratase activity.

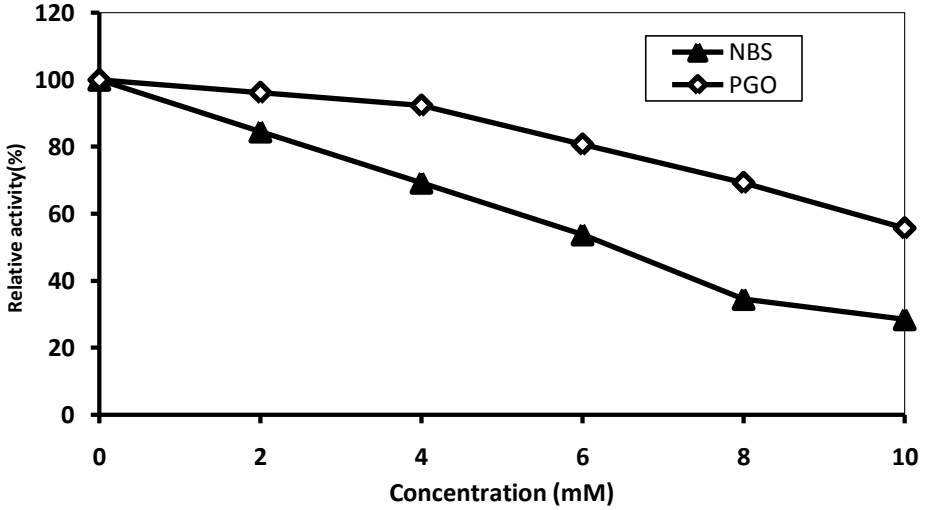

Fig. 4: Effect of NBS and PGO on cyanide hydratase activity.

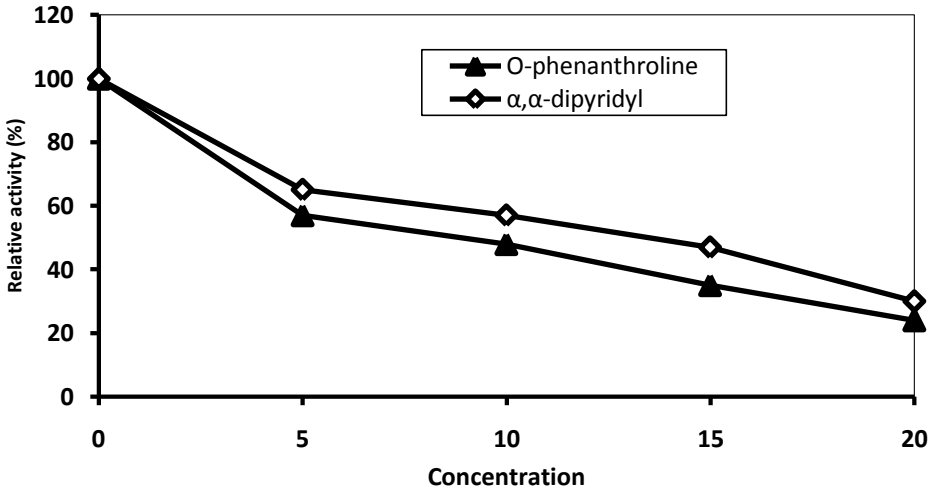

Fig. 5: Effect of *o*-phenanthroline and α,α-dipyridyl on cyanide hydratase activity.

Table 1: Immobilization of cyanide hydratase

Support	% Immobilization yield
Agar-agar	40.3 ± 0.5
Chitosan	60.7 ± 0.9
Calcium alginate	35.0 ± 0.5
Silica-gel	23.8 ± 0.4

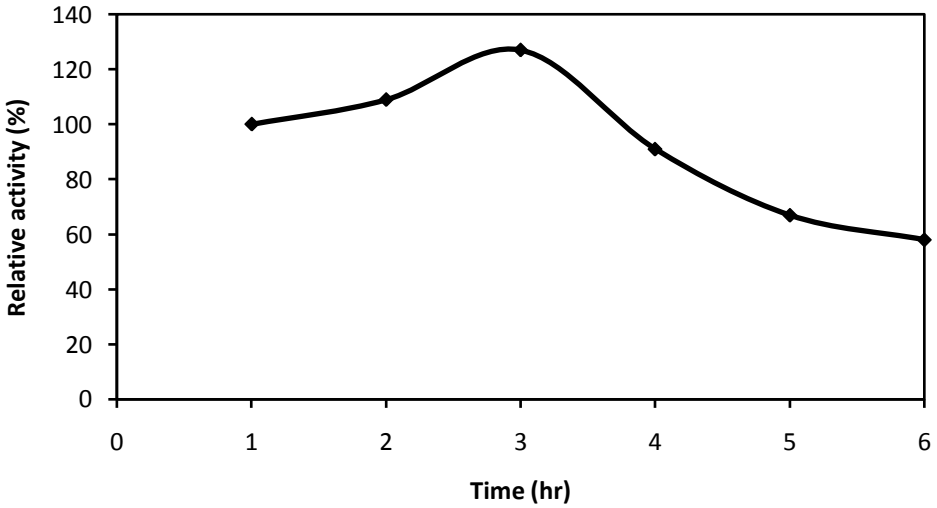

Fig. 6: Effect of time of immobilization on the activity of cyanide hydratase.

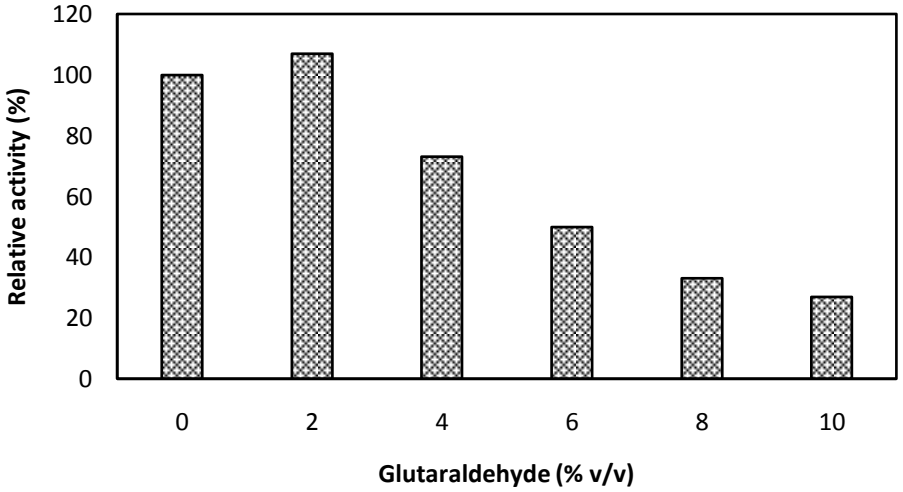

Fig.7: Effect of glutaraldehyde concentration on the activity of cyanide hydratase.

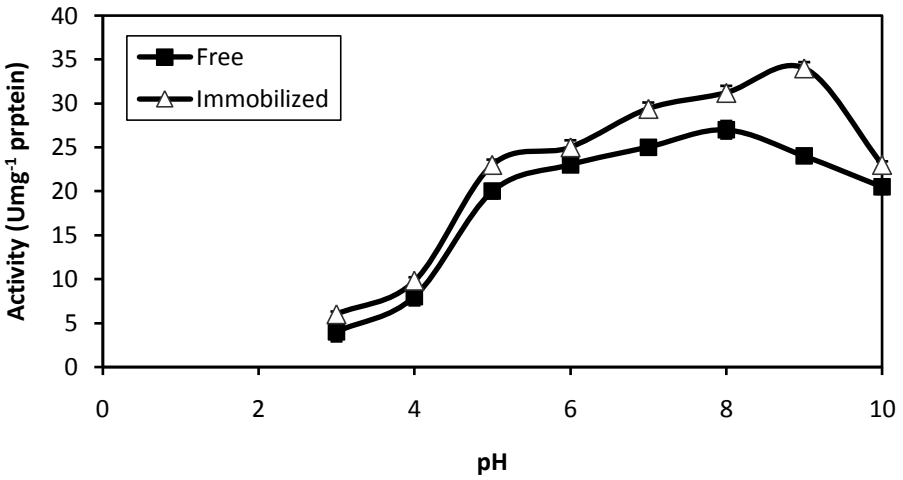

Fig. 8: Effect of pH on the activity of free and immobilized cyanide hydratase.

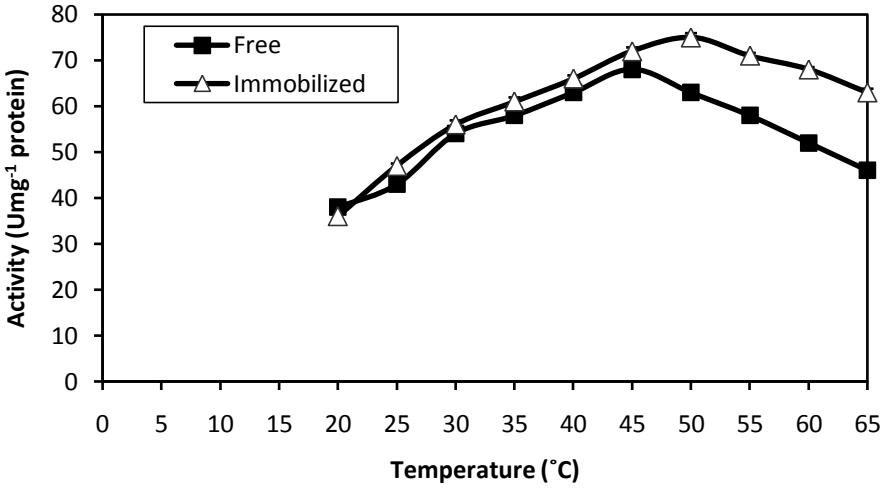

Fig. 9: Effect of temperature on the activity of free and immobilized cyanide hydratase.

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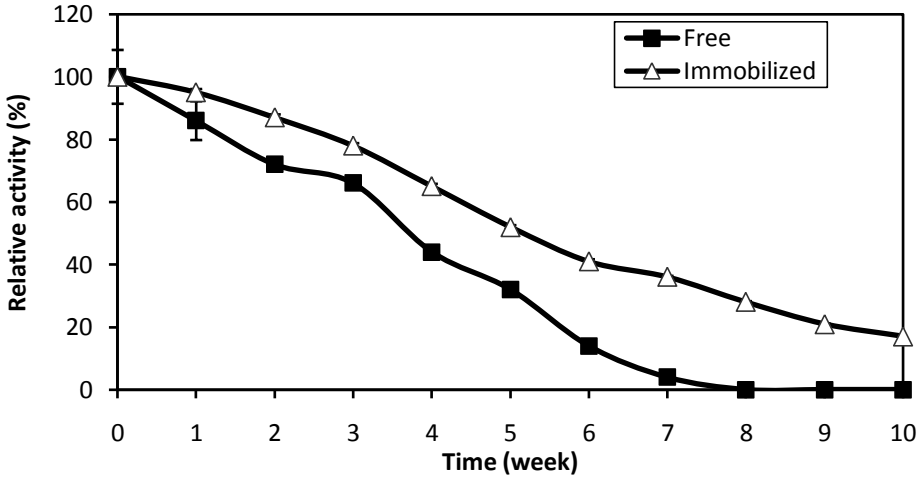

Fig. 10: Storage stability of free and immobilized cyanide hydrates at 4 ^{o}C.

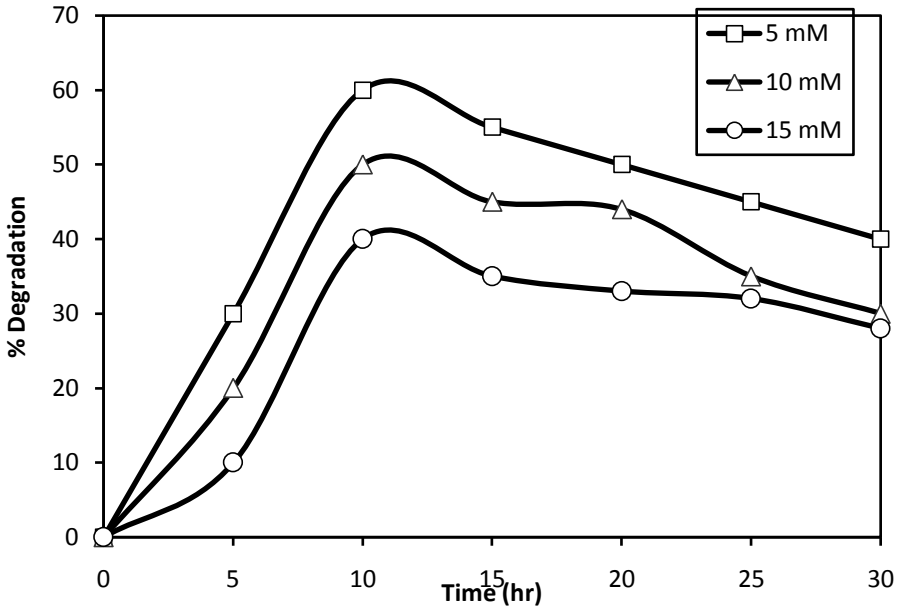

Fig. 11: Cyanide degradation by immobilized cyanide hydratase.

Discussion

Heat inactivation of cyanide hydratase indicated that the enzyme activity from *C. oxysporum* was reduced continuously at 60 ˚C throughout the experimental time than at 50 ˚C. The thermal inactivation is the most important mode of enzyme inactivation and this type of inactivation takes place at elevated temperatures. Enzymes are usually quite stable at the temperature ambient for the organism from which they are isolated and when the temperature is increased to a significantly higher level they lose their activity (El-Shora et al., 2014).

There is still uncertainty about the mechanism of thermal inactivation of enzymes. The first step in enzyme thermal inactivation is partial unfolding of the molecule (Becker et al., 1997). Under normal conditions, the native catalytically active structure of the enzyme is maintained by a delicate balance of different monovalent ionic forces e.g. hydrogen and hydrophobic interaction (Becker et al., 1997). Under the in temperature all of these forces (except for hydrophobic interactions which are significant up to 60 ˚C) diminish and the protein macromolecule unfolds.

The present work showed that Mg^{2+} and Ca^{2+} were activators for cyanide hydratase. Ca^{2+} activated other enzymes such as phytase and Ca^{2+} serves in modulation of some hydrolytic enzymes (El-Shora and Abo-Kassem, 2000; El-Shora et al., 2015b). The activation of the enzymes by Ca^{2+} could be attributed to strengthening of interactions inside the enzyme molecules or by the binding of Ca^{2+} to the autolysis site (Harris and Davidson, 1994; El-Shora et al., 2014b). On the other hand, cyanide hydratase was inhibited by the other tested divalent cations particularly Hg^{2+}. The inhibition of the enzyme by Hg^{2+}reveals the essentiality of –SH group for cyanide hydratase catalysis. Hg^{2+} is well known as toxic metal for biological molecules (Du et al., 2012; El-Shora et al., 2014b).

NEM, DEPC, NBS and PGO are used as reagents for active groups in enzyme protein (El-Shora, 1995; El-Shora, 2001a; El-Shora, 2002; El-Shora and Youssef, 2008). These compounds inhibited the activity of cyanide hydratase enzyme in concentration dependent-manner. This inhibition indicates the necessity of –SH, histidyl, treptophanyl and arginyl groups for enzyme catalysis.

Generally, it is remarkable that the chemical modification studies could selectively target the residues specifically at the active site, demonstrated by substrate protection. In fact, these results prove the usefulness of such studies in the preliminary identification of groups in enzymes responsible for catalysis.

The results showed that both o-phenanthroline and *α*-*α*-dipyridyl inhibited cyanide hydratase activity and particularly O-phenanthroline in concentration-dependent manner. This inhibition reveals that cyanide hydratase is a metalloenzyme. These results are in harmony with those of El-Shora and El-Naqeeb (2014) for L-glutaminase.

These authors observed that L-glutaminase was inhibited strongly by *O*-phenanthroline compared with *α*-*α*-dipyridyl.

Adding glutaraldehyde as a bi-functional reagent at higher concentrations beyond 2 % (v/v) reduced the enzyme activity. This is possibly due to the reaction of the aldehyde groups with the amine groups of cyanide hydratase promoted cross-linking of the protein chains leading to blocking the active site of the enzyme and results in enzyme inactivation during the stabilization process.

The immobilization process showed 60.7 % immobilization yield. The results have significant importance for industrial use of the enzyme. It is important to consider the enzyme stability with respect to various parameters such as temperature, storage stability and reusability. The soluble enzyme cannot be recovered from the reaction mixture and therefore cannot be used to catalyze more reactions, but the process of immobilization can make it feasible.

One of the important goals of enzyme immobilization is the anticipated increase in its stability to different deactivating forces due to restricted conformational mobility of the molecules following immobilization. Thus, the immobilized enzyme could work in harsh environmental conditions with less activity loss compared to the free one(Arica et al., 1999; El-Shora, 2001b; Bai et al., 2006; El-Shora et al., 2015).

The optimum pH of the free purified cyanide hydratase from *C. oxysporum* was 8.0. However, the chitosan-immobilized cyanide hydratase exhibited pH value of 9.0. In general, the behavior of an enzyme molecule may be modified by its immediate microenvironment. The enzyme in solution may have different pH optimum from the same enzyme when immobilized on a solid matrix. Depending on the surface, residual changes on the solid matrix and the nature of the bound enzyme, the pH of the enzyme molecule may change causing a shift in the pH optimum of the enzyme activity (El-Shora, 2001; El-Shora et al., 2014b; El-Shora et al., 2015).

The immobilized cyanide hydratase possessed an optimal temperature at 50°C which is higher than that of free enzyme (45 ˚C). The higher heat resistance of immobilized cyanide hydratase could be attributed to the protecting effect of the immobilization support at high temperatures where deactivation occurs. The conformational flexibility of the enzyme is affected by immobilization. The immobilization of enzyme causes an increase in enzyme rigidity, which is commonly reflected by an increase in stability towards denaturation by raising the temperature (Abdel-Naby, 1993; Chang and Juang, 2004; El-Shora et al., 2015). Immobilization of the enzyme on chitosan is supposed to preserve tertiary structure of enzyme from conformational changes.

The immobilized enzyme expressed better storage stability than the free one throughout the experimental period. Also, the enzyme exhibited appreciable capacity to degrade cyanide.

Conclusion

The present work showed that cyanide hydratase from *C. oxysporum* has appreciable thermal stability and storage stability. Also, the enzyme was immobilized successfully on chitosan and was able to degrade cyanide. This suggests possible use of immobilized cyanide hydratase from *C. oxysporum in* industry and removing of cyanide from polluted waters.

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