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Enzymatic study of cyanide utilizing *Pseudomonas* species isolated from contaminated soil

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Abstract

Present study deals with understanding the probable metabolic pathway utilized to degrade cyanide present in the bacteria isolated from contaminated soil harboring the loads of industrial effluent mainly rich in cyanide compounds. This was done by performing the enzymatic assay of the enzymes used in the pathway. Present studies were conducted on cyanide utilizing *Pseudomonas* species isolated from previous work of isolation and characterization. It is supposed that the growing *Pseudomonas* species would be able to evolve a mechanism to utilize cyanide present in the soil as sole source of nitrogen and carbon. For the study, cell free extract of isolated bacterial species grown in the presence of potassium cyanide (KCN) was prepared and activities of enzymes having role in cyanide degradation pathway were observed. Literature studies had revealed that mainly four enzymes Cyanide dihydratase, Cyanide oxygenases (mono or dioxygenase), Nitrilase and Cyanase play an important role in different cyanide degrading pathways so activity of these four enzymes was detected on the basis of the measurement of released end products. Results showed the probability of presence of Cyanide dioxygenase enzyme which governs the oxidative pathway of cyanide degradation in bacterium. Studies were conducted to know about the favorable parameters like pH and temperature to get the maximum activity of the enzyme and studies revealed that the enzyme worked best when the growth conditions maintained as temperature 270C and pH 8.5.

Keywords: *Pseudomonas* species, Potassium cyanide, Cell-free extract(s), Cyanide dioxygenase.

Introduction

Cyanide compound contain the cyano-moiety, which consists of the carbon atom triply bonded to the nitrogen atom (-C≡N-). There are inorganic and organic cyanide compounds include simple salts of cyanide with various metals such as sodium cyanide (NaCN), Potassium cyanide (KCN), ferric ferrocyanide etc.[1, 2] Industrially it is used in the processes like gold mining, petrochemical refining, the synthesis of organic chemical and plastics, electroplating, aluminum works, and metal mining.[3-5] Untreated effluent containing cyanide poses great threat to different organism health.[6] Various chemical and physical methods are applied to treat the effluent but they were found to be very expensive and not sophistically operated. As an alternative, now a day, biological treatment using whole cells as well as free/immobilized enzymes are used because of careful operation system and its ecofriendly products.[8] Numerous bacteria like *Pseudomonas* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Burkhoderia cepacia*, *Bacillus pumilus*, *Pseudomonas aeruginosa*, *Pseudomonas flourescens*, *Pseudomonas putida*,

Klebsiella sp., *Escherichia coli* and some fungi like *Fusarium oxysporum* N-10, *Candida guilliermondii*, *Fusarium solani*, *Rhodococcus* sp., *Gloaecocercospora sorghi*, *Stempylium loti*, *Rhizopus oryzae* and only few algae like *Arthrospira maxima*, *Scenedesmus obliqus* and *Chlorella* spp. are able to degrade cyanide and cyanide containing compounds.[9, 10] These microbes possess various enzymes to degrade these compounds and by studying them understanding of the biological pathways operating in the organisms could be gained.[11, 12] Literature survey had revealed that mainly three metabolic pathways Hydrolytic, Oxidative and Reductive were operated for cyanide degradation in different microbes and the end products generated were ammonia, carbon dioxide, formate, formaldehyde and carbonate etc., which are eco-friendly products. Some details about the reactions catalyzed by different enzymes and end products released present in different group of microbial species were depicted in Table 1.[13-20]

Table 1: List of major pathways, their respective enzymes and presence in different microorganisms responsible for cyanide degradation

Sr. No.	Pathways & Enzymes	Example of microorganisms
1.	**Oxidative Pathway**	
	I. <u>Cyanide oxygenase</u> $HCN+O_2+H^++NADPH \rightarrow HOCN+NADP+H_2O$	*Pseudomonas* species, *Pseudomonas flourescens*
	II. <u>Cyanase</u> $HOCN+HCO^{3-}+H_2O \rightarrow 2CO_2+NH_3+OH^-$ Cyanide oxygenase converts cyanide into cyanate which will further catalyse by cyanase into ammonia and carbon dioxide.	*Bacillus cereus, Bacillus pumillus*
	III. <u>Cyanide dioxygenase</u> $HCN+O_2+H^++NADPH \rightarrow CO_2+NH_3+NADH^{+2}$ Dioxygenase directly converts HCN into ammonia and carbon dioxide.	*E. coli, Rhodococcus rhodochrous*
2.	**Hydrolytic Pathway**	
	I. <u>Cyanide hydratase</u> $HCN+H_2O \rightarrow HCONH_2$ Cyanide hydratase catalyzes the hydrolysis of cyanide to formamide.	Pathogenic fungi, *Pseudomonas* species, *Corynebacterium* spp.,
	II. <u>Nitrile hydratase</u> $R\text{-}CN+H_2O \rightarrow RCONH_2$	*Alcaligenes xylosoxidans*

	III. Cyanidase/Cyanide dihydratase $HCN+2H_2O \rightarrow NH_3 + HCOOH$ Cyanidase/Cyanide dihydratase catalysis the hydrolysis of cyanide to ammonia and formate.	*Klebsiella ozaenae, Arthrobacter* species
	IV. Nitrilase $R\text{-}CN+H_2O \rightarrow RCONH_2$ Nitrilase having capacity to degrade nitrile compounds, but having broad substrate specificity to convert it into acids as an end product. Cyanide hydratase and cyanidase have recently been shown to have similarity at both the amino acid and structural levels to nitrilase and nitrile hydratase enzymes.	*Pseudomonas aeruginosa*
3.	**Substitution Pathway**	
	I. Rhodanese $HCN+S_2O_3 \rightarrow HCNS+SO3^{-2}$ Occurs in Rodents, thiocyanate is an end product **II. Cyanoalanine synthase** $Cys+HCN \rightarrow \beta\text{-cyanoalanine}+HS^-$ Majority occurs in thiocyanate degradation, where end products are β-cyanoalanine and sulfide.	*Thiobacillus denitrificans,* *Bacillus subtilits, Bacillus steriothermophilus* *Bacillus megaterium*

In the previous studies, we have isolated microbes from soil contaminated with cyanide, enriched them in media having filtered sterilized KCN and characterized them with biochemical and 16S rDNA techniques.[21] One of the microbes found to degrade cyanide was *Pseudomonas* species which was taken further for studying the preliminary cyanide degradation pathway studies through enzymatic studies. End product released after performing the enzymatic assay was detected and probable pathway of degradation was detected. Optimization of favorable conditions like pH, and temperature for the concerned enzyme was performed. Literature studies had revealed that whole microbial cells or its enzymes were used to treat effluent released from industries. Thus, the present study was carried out with the aim of studying the enzymatic mechanisms for breakdown the cyanide compound and optimization of physic-chemical parameters for making it suitable for bioremediation of cyanide contaminated soils.

Materials and methods

Preparation of Cell-free extract

To prepare cell free extract, 42 hr old grown enriched culture of *Pseudomonas* cells were taken, harvested by centrifugation at 15,000 rpm for 15 minutes and washed twice into cold 50 mM phosphate buffer (pH-7.5). The packed cells (1.0 g wet weight) were suspended in 100 ml

of cold 50 mM potassium phosphate buffer and disrupted by sonication for 10 min. After sonication, the culture was again centrifuged at 15,000rpm for 10 minutes and cell-free supernatant fraction called crude cell extract was taken as a source for crude enzyme activity studies. All above steps were performed at 4^0C temperature.[22] Total protein estimation of the crude extract was determined and same was used to carry out different enzymatic assays.[23]

Enzyme assays

Assays for different cyanide degrading enzymes were carried out to know the presence of specific enzyme as well as probable metabolic pathway of cyanide degradation. The assays were carried out according to the procedures mentioned below.

For the detection of oxidative pathway presence of all three enzymes viz. Cyanide dioxygenase, Cyanide oxygenase and Cyanase were performed as follows:

Cyanide dioxygenase: Cyanide dioxygenase enzyme was assayed according to the protocol given by Kunz *et al*., 1998. The reaction mixture for this enzyme was prepared by taking 200 µl of crude cell extract, 5 µl of 2.0 mM KCN, 10 µl of 4 mM NADH and 35 µl of 50mM sodium-potassium buffer pH 7.0. Final volume of the reaction mixture was made up to 250 µl and reaction was allowed to take place at 27^0C. End product released in the reaction catalysed by cyanide dioxygenase was ammonia and carbon –di-oxide. Formation of ammonia was detected for the presence of activity of this enzyme.

Cyanide oxygenase: The enzymatic assay protocol for detection of cyanide oxygenase was same as performed for cyanide dioxygenase mentioned above but the end product of the reaction was different. Here cyanate was formed as end product which is difficult to estimate. Other possibility was detection of cyanase as it could convert cyanate into ammonia so cyanase activity was detected to confirm the activity of cyanide oxygenase.

Cyanase: Presence of Cyanase activity was determined according to assay protocol given by Anderson, 1980.[24] 5 ml of the reaction product of the above reaction was taken and assay was done by adding 0.04 ml of sodium bicarbonate and 0.04 ml of 50 mM potassium phosphate buffer pH 7.6. Final volume of the reaction mixture was made up to 2.0 ml and reaction was allowed to occur at 27^0C. It was assumed that if there would be any formation of cyanate by the activity of cyanide oxygenase then another pathway catalysed by Cyanase would get operated. Release of ammonia was detected as end product.

Presence of hydrolytic pathway was determined by performing the assay for the presence of two enzymes Cyanide dihydratse and Nitrilase mentioned as follows:

Cyanide dihydratase: An assay was performed by mixing 2.0 ml of crude extract, 1.0 ml of 2.0 mM KCN and 2.0 ml of 50 mM phosphate buffer pH 8.0. Final volume of the mixture was maintained to 5.0 ml at 27^0C.[25] Quantitative measurement of ammonia was carried out to check the activity of cyanide dihydratase enzyme.

Nitrilase: According to literature survey, nitrilase was also one of the enzymes which could degrade cyanide owing to broad substrate specificity. The assay of nitrilase was carried out by taking 80 µl of crude extract and 20 µl of 2.0 mM buffered KCN, pH 8.0 and finally maintaining the volume to 100 µl at 27^0C.[26] Estimation of formamide as end product was performed to know the activity of this enzyme.

Ammonia & Formamide detection

Enzymatic activity was determined in terms of ammonia production which by detected according to Phenate method.[27] 1.0ml of the sample was taken and final volume was made upto 25ml by using distilled water. 1.0ml of 1% phenol, 0.5% sodium nitroprusside and 2.5ml of oxidizing agent was added and mixture was incubated for one hour in dark. After one hour the mixture was properly mixed and OD was taken at 640nm.

Qualitative estimation of formamide was carried out by Snell and Snell method.[28] It was performed by mixing 1.0 ml of crude extract and 1.0ml of 1:1 hydroxylamine hydrochloride and sodium hydroxide solution. Mixture was kept at 60^0C temperature for 10 minutes which leads to formation of hydroxamic acid. After that 1.0ml of hydrochloric acid and ferric chloride solution was added and color change from yellow to deep red was observed. Deep red color indicates the presence of formamide in the mixture.

For each enzyme activity study, samples from reaction mixture were withdrawn at 15, 30 and 45 minutes time intervals to check the time for maximum activity. The reaction for each enzyme was terminated by keeping it at 65^0C for 5 minutes. Each experiment carried out in triplicate and control sample was kept by using distilled water instead of crude extract of enzyme. Optimization

studies to determine the appropriate temperature and pH values at which the above mentioned enzymes showed the maximum activity were conducted by growing the *Pseudomonas* at different pH like 7.5, 8.5 and 9.5 and temperatures 15, 27 and 47^{0}C as referred from literature studies.

Results and discussion

Enzymatic studies from cell-free extract(s)

In nature, some microbe uses cyanide as nitrogen source only while some as carbon and nitrogen both. Thus, for the isolation of cyanide utilizing microbial flora, researchers had used various medium for growth. Babu *et.al.,* 1996 had isolated *Pseudomonas putida* using sterile phosphate buffer medium amended with sodium cyanide. Here, sodium cyanide acted as sole source of carbon and nitrogen.Another group Kao *et al.,* 2006 had used sterile nitrogen free glucose medium in which cyanide and nitrile compounds act as nitrogen source for the *Klebsiella oxytoca*. In our experiment, the organisms were grown in Bushnell-Haas medium which lacks carbon and nitrogen source in it. Potassium cyanide was added in the media as substrate which acts as a sole source of carbon and nitrogen. Growth of microbes in this simulated medium indicates that organism was capable to breakdown the cyanide. It is well known that all the metabolic pathways operate with the help of specific enzymes. Thus, growth of organisms in the media depicted that isolated *Pseudomonas* sp. posses the necessary enzymatic mechanism to metabolize cyanide. Enzymatic studies thus performed to know about the specific enzymes present in the microbes. Activity of different enzymes with effect of different factors was carried out by measuring its end product. Fernandz *et.al*.,2004 worked on various enzymes like Cyanide oxygenases, Cyanase, Cyanidase, Formamide dehyrolase to detect the specific pathway of cyanide biodegradation. Literature survey studies on different pathways operating in different microbes mentioned in table 1 reveals that in majority of reactions the final product formed was ammonia with or without intermediates. In the present studies we have detected the presence of ammonia which demonstrates that cell-free extract(s) obtained from *Pseudomonas* species would be able to metabolize potassium cyanide which we have added in our media as substrate. Studies on activities of enzymes like Cyanide dioxygenase, Nitrilase, Cyanide dihyratase and Cyanase were performed to know the presence of specific enzyme.[29] Cell free extract of *Pseudomonas* was prepared and estimation of protein content in the extract was done by Lowry *et al*., 1951. The amount of protein was found 80μg/ml. It used further to calculate the activity of enzymes.

Detection of enzymatic activity of major cyanide degrading pathways

Conversion of cyanide to ammonia has been found to be via different ways in different microbes. Fry and Miller, 1972, White *et al*., 1988 had demonstrated hydrolytic pathway leading to formation of formamide or formate and ammonia. *Pseudomonas fluorescens* NCIMB 11764 was able to catabolise cyanide into formamide, formate, ammonia and carbon dioxide in the experimental work of Kunz *et al*., 1992.[30-32] Dorr and Knowles, 1989 had proposed two step mechanisms of oxygenases that is conversion of cyanide into cyanate with the help of primary enzyme cyanide monoxygenase, which was further catabolized into ammonia with cyanase.[33] Raybuck, 1992 has found that cyanase generally give protection against cyanate poisoning by convert it into biocarbonate/carbond dioxide and ammonia and majority it is found in bacteria, fungi, animals and plants. Enzymatic studies on five different enzymes catalyzing mainly two pathways oxidative and hydrolytic were performed and results were shown in table 2 and figure 1. According to that, maximum activity of Cyanide dioxygenase of oxidative pathway while minimum with Cyanide dihydratase was obtained within 15 minutes of incubation in *Pseudomonas* species. Dubey and Homes, 1995 suggested that Cyanase converts cyanate into ammonia and carbon dioxide.[34] Cyanate would be available if there would activity of Cyanide oxygenase so in our studies no activity was found with Cyanase, when the assay for theses two enzymes was conducted in consecutive manner. This evidence suggested that *Pseudomonas* sp. might use oxidative pathway by direct conversion of KCN into ammonia and carbon dioxide in presence of cyanide dioxygnease.[35-38] Cyanide is present as numerous complex compounds in nature like thiocyanate, selenocyanate etc. Essentially a second pathway for the degradation was proposed leads to theory of dioxygenases which convert cyanide directly into ammonia and carbon dioxide.[39] Wang et al., 1996 had used radio-labeled oxygen and proposed the theory of incorporation of two molecules of oxygen into cyanide with the help of dioxygenase.[40] This leads to formation of carbon dioxide and ammonia without any intermediates.

Nitrilase converts organic nitriles to corresponding acid and amide. Cyanide hyratase and cyanidase have

significant structural similarity at the amino acid and protein level to nitrilase and nitrile hydratase enzyme. The diversity of nitrilases superfamily and their variation in catalytic activity and substrate specificities presents opportunities for future bioremediation program. Kao *et al.*, 2006 have reported nitrilase compound activity on various nitrile compounds. Because of this unique characteristic, in our isolate, an attempt was made to check the activity of nitrilase. Nitrilase of hydrolytic pathway break C-N bond in nitrile compounds and reported to be present in some cyanide utilizing microbes[41], but its activity was found to be less than the Cyanide dioxygenase. Cyanide dihydratase hydrolysis cyanide into ammonia and formate but absence of ammonia during Cyanide dihyratase assay indicates the absence of this enzyme in whole cell lysate(s).

Table 2: Activity of different enzymes obtained during different reactions

Sr. No.	Enzymes	Activity of enzymes (µmol/minutes)
1.	Cyanide dioxygenase	0.37
2.	Nitrilase	0.22
3.	Cyanase	0.05
4.	Cyanide dihydratase	0.02

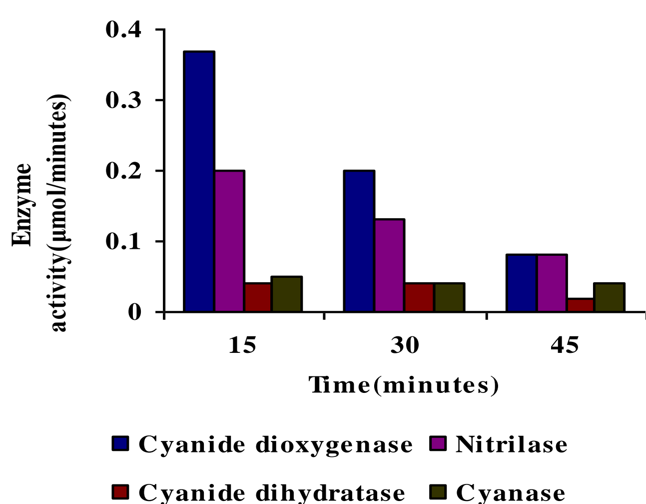

Figure 1: Graphical data had shown the activities of different enzymes in *Pseudomonas* species after incubation period of 15, 30 and 45 minutes

Effect of temperature & pH

The ability to investigate enzyme activities in whole cell lysate(s) or crude extract(s) is important for the initial identification of a particular activity of specific enzyme present in it while effect of different factors gives information on enzyme function in an environment close to the cellular milieu.[42] Kunz and his coworkers in 1996 studied effect of substrates, pH, temperature etc. on cell free extract(s) of *Pseudomonas putida*. They found that enzymatic activity had been found higher with the pH 7.5 and 9.5 at 25^0C temperature with different cyanides, cyanates and thiocyanate compounds. In our studies we had tested the effect of different temperatures and pH on the activity of the enzymes.

Results obtained from different enzymatic assay studies had revealed that the probability of presence of oxidative pathway catalyzed by Cyanide dioxygenase in our isolated *Pseudomonas* species would be there. Thus the optimal conditions for enzyme activity were determined for Cyanide dioxygenase only. The activity of this enzyme at different temperatures and pH were shown in figure 2 (A) and (B). Figure 2(A) indicates that maximum activity of enzyme was observed with the temperature 27^0C within 15 minutes and minimum at 15^0C while moderate at 37^0C. Results obtained from the studies of different pH indicated that maximum activity of cyanide dioxygenase found at pH 8.5 and minimum at 9.5 while intermediate at 7.5.

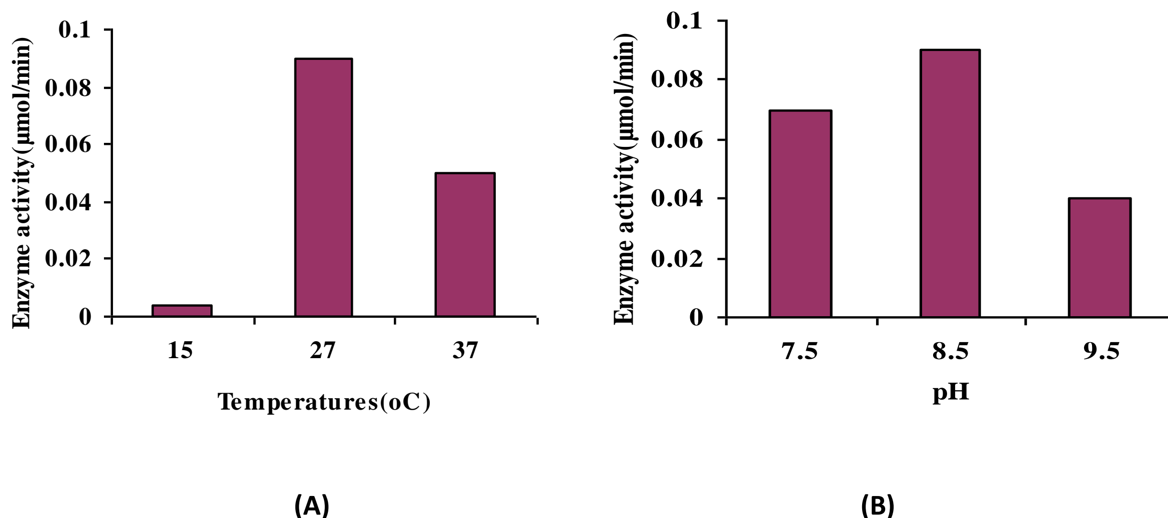

Figure 2: (A) and (B) Graphical data of effect of different temperatures and pH on cyanide dioxygenase

Conclusion

Cyanide is a very toxic compound which exists in different forms via natural as well as manmade activities. Currently, chemical and physical methods are used to remove cyanide containing compounds which are highly expensive, utilizing hazardous chemical compounds. As an alternative, now-a-days, biological treatments using whole cells or its enzyme might advantageous to remove complex and toxic compounds from an environment. With present study it can be concluded that *Pseudomonas* species isolated in our study from the cyanide contaminated soil possessed ability to degrade cyanide with the activity of Cyanide dioxygenase enzyme utilizing oxidative pathway best at temperature 27^0C and pH 8.5and thus can be used in bioremediation of cyanide contaminated sites. Though the activity shown by Nitrilase was less than Cyanide dioxygenase but it was not as low as the activity showed by other two enzymes Cyanase and Cyanide dihydratase. Literature studies had revealed that Nitrilase had broad substrate affinity and also found to be present in *Pseudomonas aeruginosa* so more studies on this enzyme is required to be performed as there might be the possibility of presence of another pathway for degradation of cyanide.

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Conflict of Interest

There is no conflict of interest among the authors regarding the work presented in the manuscript.

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