Research Article

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CYANIDE HYDRATASE PRODUCTION USING ACCLIMATIZED STRAIN OF *STREPTOMYCES PHAEOVIRIDAE* AND ITS CHARACTERIZATION

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Abstract: Cyanide and cyanide compounds are produced on the industrial scale to use in the metal extraction, electroplating, polymer, steel, carbonization, organic chemicals, pharmaceuticals and agricultural product industries. Cyanide is a respiratory inhibitor and it affects the living cell by binding with the enzyme cytochrome C oxidase. Cyanide released in the stream cause poisoning to animals and fishes in the water bodies. As cyanide is highly toxic, it must be detoxified before discharging into the sewers. Potential cyanide degrader Actinomycete was isolated and acclimatized in the minimal medium containing 1000 ppm cyanide. It was then identified as *Streptomyces phaeoviridae* by using International Streptomyces Project Standard Tests. Cyanide degradation by this organism was studied. The mechanism of cyanide degradation was found to be enzymatic and the detection of formamide in the culture broth confirmed the cyanide hydratase [E.C. 4.2.1.66] activity. Parameters for cyanide hydratase production using *Streptomyces pheoviridea* were optimized. The enzyme was extracted and optimum conditions for its activity with respect to pH, temperature and substrate concentration were determined. The kinetics studied revealed the Km value as 33 mM and Vmax 35 mM/ml/min.

Keywords: Cyanide hydratase, Formamide, *Streptomyces phaeoviridae*, cyanide.

INTRODUCTION

Cyanide is a respiratory inhibitor. It affects the living cell by binding with the cytochrome–C-oxidase. It also acts by binding with other metalloproteins[1]. The lethal dose of cyanide is just 0.5 to 3.50 mg/kg body weight. Acute cyanide poisoning in human can lead to convulsion, vomiting, coma and death.[2] Longer term effects include neuropathy, optical atrophy and pernicious anemia.[3] In nature, HCN is liberated in the rhizosphere soil by some plants on the hydrolysis of cynogenic glucosides or other nitriles. Cyanide production is a natural defense of plants.[4] Cyanogenesis has also been reported in few fungi of Ascomycete and Basidiomycete group and in certain bacteria as exemplified by *Pseudomonas* and *Achromobacter*. They produce HCN mainly by oxidative decarboxylation of glycine. The ability of certain plants and microorganisms to produce, to degrade or to assimilate cyanide states that the cyanide micro cycle operates in the nature.[5] Cyanide as HCN, KCN, NaCN is produced on the industrial scale for its use in the metal extraction, electroplating, polymer, steel, carbonization, organic chemicals, pharmaceutical and agricultural product industries. All these industries along with the industries of cyanide products generate waste water containing fairly large quantities of cyanide compounds. Metal finishing and mining industries produce large amount of cyanide containing waste.[6] The extent of cyanide release in the environment due to rapid industrialization is the main factor that adds to the environmental pollution. Such waste if not properly detoxified, can become a reason for human and animal poisoning as well as poisoning to the fishes and other aquacultures present in that water bodies. Hence we must think for designing the process for degradation of cyanide and to avoid its accumulation in the nature, which otherwise may result in poisoning effects.

Chlorination, ozonation, wet air oxidation and sulphur based technologies are some of the important methods used to treat cyanide containing waste. Most of these methods have their drawbacks as cost of operation and problem of disposal of the reaction products.[7,8,9] Scattered reports of microbial degradation of cyanide waste by mixed population in the acclimatized sludge are available.[10,11] The ability of certain phytopathogens to attack cyanide producing plants focuses on the evolved microbial systems for the detoxification or degradation of cyanide. Cyanide hydratase and rhodanase are main enzymes reported in cyanide degrading microorganisms. Cyanide hydratase is an intracellular enzyme that catalyzes hydrolysis of cyanide to formamide, a less toxic amide. The enzyme is reported in some fungi and aerobic bacteria.[12,13,14,15] Studies on pure cultures of cyanide degrading microorganisms have been concentrated on fungi and aerobic bacteria.[16] An Actinomycetes member, *Streptomyces lavendulae* is reported for the

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presence of rhodanase enzyme system, that transfer sulphur from thiosulphate to cyanide forming thiocyanate. Considering the fact that the Actinomycetes are the organisms with diverse degradative potential, efforts were made to find out potential cyanide degrading Actionomycete and to study cyanide hydrates production and characterization.

MATERIALS AND METHODS

Isolation and identification of cyanide degrading Actinomycete:

Garden soil samples were suspended in sterile saline and agitated properly. Loopful of supernatant from each sample were spreaded on starch casein agar and on glycerol asparagine agar plates. Plates were incubated at 28°C. for 4 to 5 days. Well grown colonies were subjected to staining to confirm the presence of Actinomycete. The isolates were maintained on glycerol asparagine agar slants. Then each isolate was spot inoculated on minimal medium containing potassium cyanide as sole nitrogen source and glucose as carbon source. The plates were observed after 5 days for growth.

The isolate growing most efficiently on the cyanide medium was selected and characterized with respect to its growth characteristics and carbohydrate utilization tests. Melanin production was studied on peptone iron agar and on tyrosine agar. Morphological characterization was done by scanning electron microscopy. The isolate was identified according to the International Streptomyces Project Standard Tests Identification Key. [17]

Acclimatization of the isolate:

Cultures were grown in glycerol asparagines broth for 5 days. Cell pellets were separated by centrifugation at 3000 rpm and washed repeatedly with alkaline saline to remove the traces of residual carbon and nitrogen from the growth medium. These cells were then acclimatized to KCN as the nitrogen source with its increasing concentration from 10 mg/liter to 1000 mg/liter. Acclimatization was confirmed by growing the cultures on solid medium containing potassium cyanide as the sole nitrogen source.

Studies on cyanide degradation by *Streptomyces phaeoviridae*:

The suspension of *Streptomyces phaeoviridae* was inoculated in the cyanide dextrose broth and incubated for 5 days. Cyanide degradation by the isolate *Streptomyces phaeoviridae* was studied by observing decrease in the initial level of cyanide in the broth and by the simultaneous detection of ammonia in the broth. Effect of various initial concentrations of cyanide from 10mg/lit to 100mg/lit on cyanide degradation was determined. Effect of different sugars as arabinose, ribose, dextrose, sorbitol, lactose, sucrose, glycerol and starch was studied. Finally, appearance of formamide was detected in the fermented cyanide dextrose broth with initial 10 mg/lit KCN concentration by a modified method for amide estimation. Cyanide estimation was done by pyridine-barbituric acid method while Nessler's method was adopted for ammonia estimation[17].

Production of Cyanide hydratase using *Streptomyces phaeoviridae*:

For getting cyanide hydratase, 72 hour old broth culture was used as inoculum. The enzyme induction was done by adding KCN at the final concentration of 10 mg/lit. The broth was further incubated for next 24 hours. Finally, cells were harvested with 0.05M phosphate buffer and allowed to disrupt by ultrasonication (labsonic-U) at 350 Hz. The crude extract of cyanide hydratase was collected by cold centrifugation at 25000 g for 60 minutes. About 5 ml crude enzyme was obtained from 500 mg cell mass.

Characterization of crude Cyanide hydratase:

The enzyme activity was conducted by mixing 0.5 ml crude enzyme to 0.1 ml KCN as substrate, final amount made 1 ml with double distilled water. The reaction mixture was incubated for 30 minutes at 30°C. The enzyme reaction in which cyanide nitrogen is converted to formamide was studied by detecting the appearance of formamide in the reaction mixture. The rate of enzyme reaction was determined as the amount of the product formed per minute in one ml. Effect of pH, temperature and initial substrate concentration on the rate of reaction was determined.

Formamide assay:

A modified amide estimation method was adopted for formamide assay. 2 ml of formamide was allowed to react with 4 ml 1:1 mixture of sodium hydroxide and hydroxylamine. The reacting mixture was kept at 60°C. for 30 minutes. To this, 2ml of 4N HCl and 2 ml $FeCl_3$ reagent is added and the absorbance was recorded at 530 nm.

Cyanide assay:

20 ml test solution was taken in the 50 ml volumetric flask. 4 ml of 3 N sodium phosphate buffer and 2 ml chloramine T was added to this. Immediately after the addition, 5 ml pyridine-barbituric acid reagent was added with gentle constant swirling. The reacting mixture was diluted to 50 ml with distilled water and the absorbance was recorded at 578 nm, after 8 minutes but within 15 minutes.

Ammonia estimation:

Ammonia developing during the reaction was estimated by Nessler's method.

RESULTS

Out of 15 isolates, 10 isolates were adapted to 120 mg/lit KCN in the dextrose broth. Out of these only two cultures showed ability to grow luxuriously. These cultures were further acclimatized to 1000 ppm cyanide in the broth. One of them was identified as *Streptomyces phaeoviridae*. The organism belongs to the section reticulum apertum (RA), showing flexous ends and open loop sporophores, with smooth surfaced oval spores having 1.8-2.5 µm length and 1.4 µm breadth (Figure 1). Every spore showed pronounced concavity on one side to give a flattened view and typical spore arrangement (Figure 2). The isolate was growing with gray coloured aerial mycelium and black reverse pigment on oat agar. Yellow diffusible pigment and melanin production was observed. (Table 1)

Table.1: Major Characteristics of *Streptomyces phaeoviridae*, on incubation at 30° C. for 4 days.

Sr. No.	Characteristic	Observation
01	Growth of substrate mycelium on SCA	Luxuriant
02	Colour of aerial mycelium	Gray
03	Reverse pigment	Black
04	Diffusible pigment	Yellow
05	Average number of spores per sporophore	20
06	Spore shape	Oval
07	Spore length	1.8-2.5 µm
08	Spore breadth	1.4 µm
09	Melanin production on TA	+ ve
10	H_2S production	+ ve
11	D- Glucose	A
12	Fructose	A
13	Arabinose	A
14	D-Xylose	A
15	D-Ribose	- ve
16	Mannitol	A
17	Inositol	- ve
18	Sucrose	- ve

SCA – Starch Casein Agar, TA – Tyrosine Agar, A - Acid

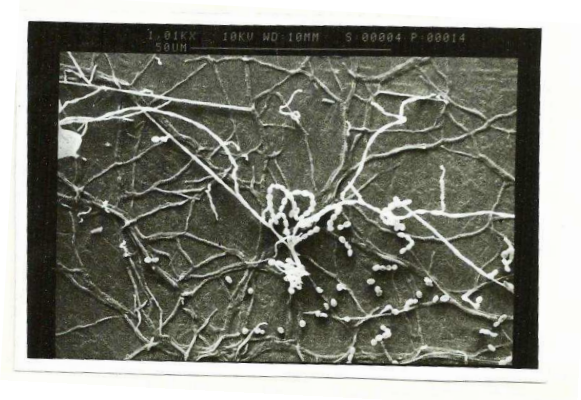

Figure.1: SEM of *Streptomyces phaeoviridae* colony on asparagine agar medium

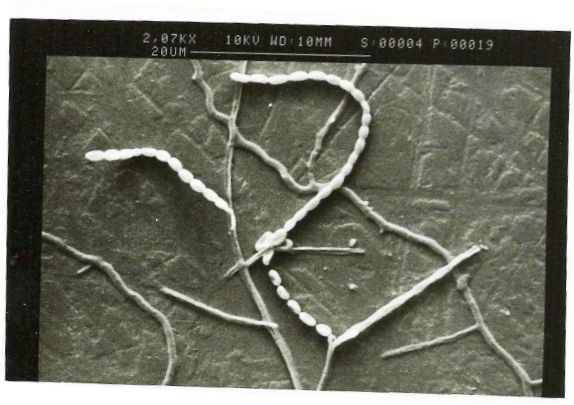

Figure.2: SEM of *Streptomyces phaeoviridea* showing spore chain

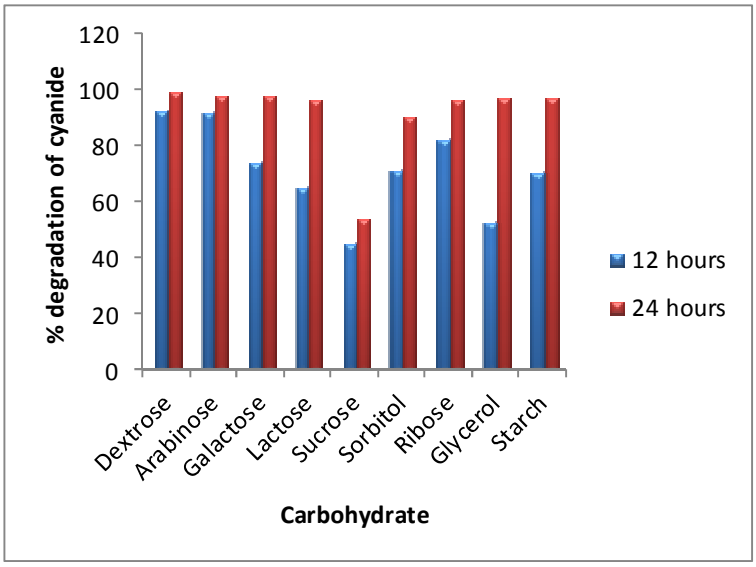

Figure.3: Effect of different carbon sources on cyanide degradation by *Streptomyces phaeoviridae*

The maximum 99.81% Cyanide degradation was observed in the presence of 1% dextrose with 80mg/lit initial KCN concentration. There was little fluctuation in the percentage of degradation due to the presence of various sugars in the medium. Sucrose was found least efficient sugar for cyanide degradation. Cyanide undergoes hydrolysis to form formamide by inducible cyanide hydratase enzyme. Some amount of ammonia was detected in all cases. The appearance of ammonia may be because of further hydrolysis of formamide to formic acid and ammonia. In the control sets where abiotic process of cyanide depletion was studied the generation of ammonia was observed very high, whereas in the microbial degradation the extent of ammonia production was reduced by 90.80% (Table 2). During the reaction study, formamide was noted as the major end product confirming the presence of cyanide hydratase [Formamide hydrolase, E.C. 4.2.1.66].

Table.2: Cyanide degradation by *Streptomyces phaeoviridae*, in 20 mg/lit KCN broth, on incubation at 30° C. for 18 hours

Sr. No.	Cyanide broth	% degradation of cyanide	Ammonia produced µg/ml	Formamide produced mM/ml
01	*Streptomyces phaeoviridae*	98.87	490	1.35
02	Control	93.65	5000	00

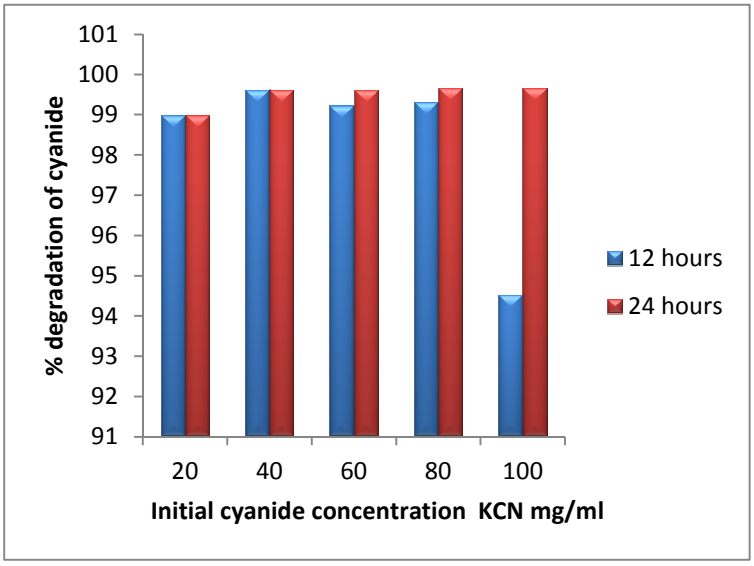

Figure.4: Effect of different initial cyanide concentrations on cyanide degradation by *Streptomyces phaeoviridae*

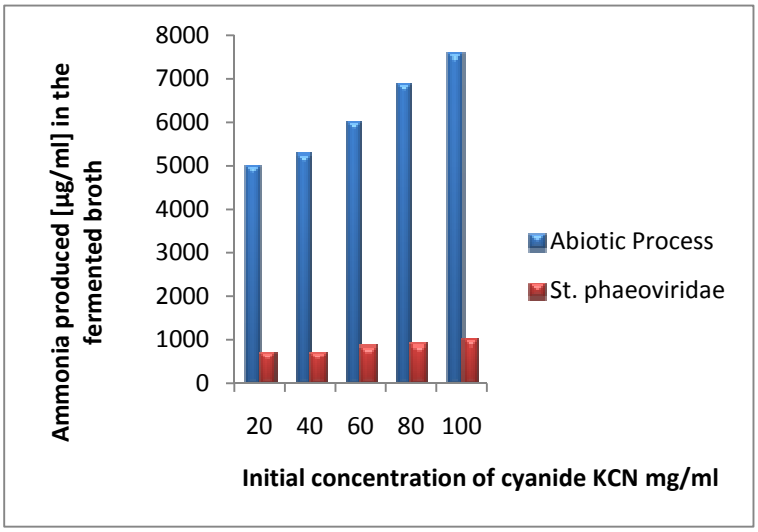

Figure.5: Ammonia production during cyanide degradation by *Streptomyces phaeoviridae*

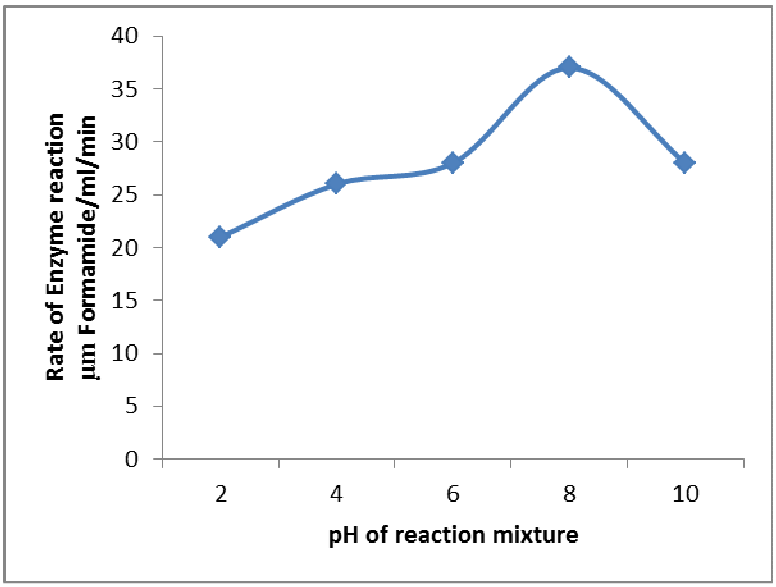

Figure.6: Effect of pH on the rate of cyanide Hydratase reaction

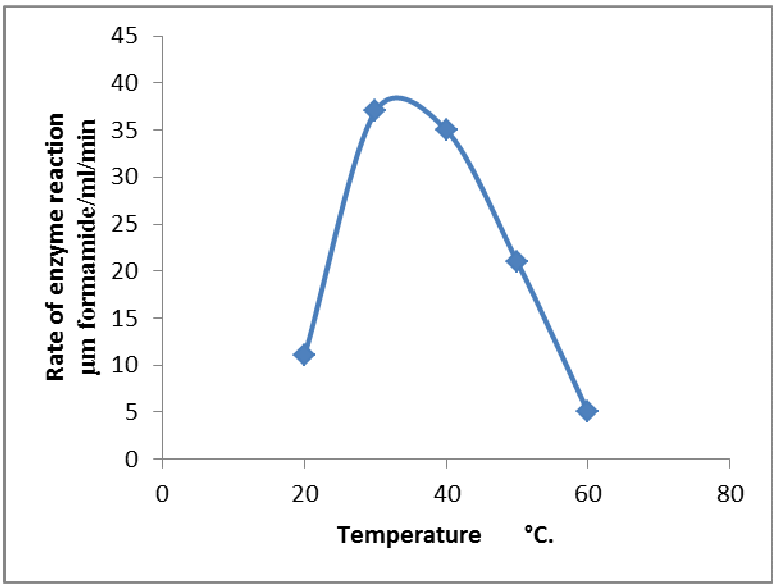

Figure. 7: Effect of temperature on the rate of cyanide Hydratase reaction

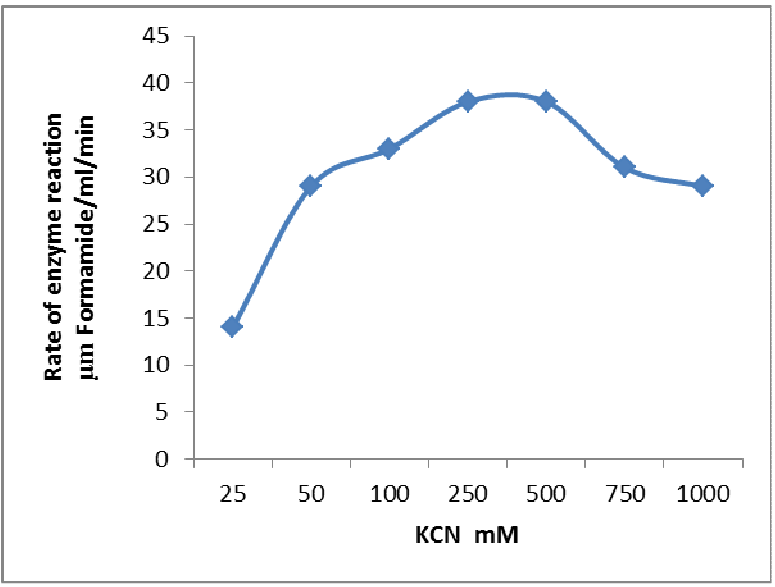

Figure.8: Effect of substrate concentration the rate of cyanide Hydratase reaction

Reaction condition optimization with the crude enzyme preparation revealed pH 8 as optimum pH where 37µm formamide was produced per ml within one minute. The rate of reaction was dropped by 10% at neutrality and by 24% at acidic pH as well as at pH 10. The optimum temperature range was found as 30ºC.-37ºC. The substrate concentration (KCN) needed for efficient enzyme production was detected as 500 mM/ml in the dextrose cyanide broth. Michaelis Menten constant was calculated as 33 mM/ml and Vmax was 35µm/ml/min. As against this record the fungal cyanide hydratase has notably high Km and Vmax values.[19]

DISCUSSION AND CONCLUSION

A variety of enzymatic pathways for cyanide degradation have been described. These include conversion of cyanide into thiocyanate,[20] formation of bicarbonate and formate,[11] formation of bicarbonate and ammonia,[21] formation of carbon dioxide and ammonia and formation of methane and ammonia.[22] Cyanide hydrolysis to formate and ammonia by cyanidase from *Alcaligen xylosoxidans sp. denitrificans*[23] and cyanide hydration by cyanide hydratase from *Stemphylium loti* and *Fusarium lateritium* are also reported. *Micromonospora braunna* was reported as a good source of cyanide hydratase in our previous work.[24] Out of scanty reports available for microbial treatment of cyanide waste, *Streptomyces lavendulae* reported by Oi, in 1977[25]; has rhodanase enzyme system. This converts cyanide into thiocyanate.

Using Microorganism is an ecosociable approach for removal of cyanide industrial wastes. It can be less expensive and much faster than chemical and physical methods.[26] Biological treatment of cyanide waste may lead to generate less toxic amide and less amounts of acidic products, carbon-dioxide and mainly ammonia. Present work reports *Streptomyces phaeoviridae* as the useful Actinomycetes with its possible application in the cyanide waste treatment operated under aerobic conditions. Less amount of ammonia generation with the production of formamide, a non-toxic compound is a favorable feature of cyanide degradation by this organism. The extracted enzyme system is promising as it removed cyanide rapidly from the experimental system at unobjectionable pH and maintainable temperature. All these observations studied during the project explain the superiority of using *Streptomyces phaeoviridae* for cyanide waste treatment than going for pure abiotic process.

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