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Production and Characterization of Cyanide Hydratase from *Micromonospora braunna*

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Abstract:

Cyanide hydratase [E.C. 4.2.1.66] is an intracellular enzyme detected in few fungi and bacteria. It catalyzes the reaction of hydration of cyanide, a potent toxin, to form formamide, a non-poisonous amide intermediate of cyanide degradation. When asked to name a poison, everybody nominates cyanide. This evil reputation of cyanide worth paying attention because of its increasing occurrence due to natural synthesis and also due to the large scale industrial production of inorganic cyanide as CN^- and HCN as well as organic cyanides as nitriles, RCN. As cyanide is highly toxic, it must be detoxified in the effluent before discharging into the sewers. Although several processes of cyanide detoxification are available, the biological treatment of cyanide waste is cost-effective and ecofriendly. Cyanide hydratase is becoming important green catalyst for this process, which converts cyanide into formamide and ammonia without employing extreme conditions of reaction temperature and hydrogen ion concentration. The present project was undertaken with the view of approaching to the bioremediation of cyanide effluent. Potential cyanide degrader actinomycetes, identified as *Micromonospora braunna* was isolated and acclimatized in the minimal medium containing 1000 ppm cyanide. The detection of formamide in the culture broth confirmed the cyanide hydratase activity. 76.66 enzyme units were detected in the production broth supplemented with dextrose as carbon source and potassium cyanide as sole nitrogen source. The enzyme cyanide hydratase was extracted and its reaction parameters were determined. The kinetics studied revealed the Km value as 33mM and Vmax 39 µmoles/ml/min.

Keywords : Cyanide hydratase, Formamide, *Micromonospora braunna*, Green catalyst, Bioremediation.

1.0 Introduction:

Cyanide hydratase is an intracellular, inducible enzyme, having cyanide and metallocyanides as inducers. Cyanide undergoes hydrolysis to formamide by cyanide hydratase (formamide hydrolyase E.C. 4.2.1.66) which in turn is hydrolyzed to ammonia and formic acid. 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 (Fry,1992). However, the extent of cyanide release in the environment due to rapid industrialization is the main factor that makes us to think for designing the process for degradation of cyanide and to avoid its accumulation in the nature, which otherwise may result in poisoning effects. Cyanide is a respiratory inhibitor. It affects the living cell by binding with the cytochrome–C-oxidase. It also affects other metalloproteins. (Solomonson, 1981). 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. Longer term effects include neuropathy, optical atrophy and pernicious anemia (Way, 1983). Cyanide poisoning presents one of the most difficult challenges in disaster medicine and forensic science, due to its high toxicity, fast action, a number of possible sources of exposure and some limitations of analytical methods for cyanide determination (Andriana, 2012).

There are many natural sources of cyanide, including plants, bacteria and fungi that synthesize and secrete it, however, the most significant sources of cyanide in environment are industrial waste (Ubalua, 2010). 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 and mining operations and coal manufacturing plants produce wastes and waste water with high cyanide content. Bulk of cyanide occurrence in environment is mainly due to metal finishing and mining industries. (Dash , 2009). Electroplating industrial wastes contain 0.5% to 20% cyanide. (Dwivwdi, 2011). The release of

cyanide from industries worldwide has been estimated to be more than 14 million kg/yr (Ebbs, 2011).. 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.

Several processes are discussed and used to reduce the toxicity of cyanide waste. Chlorination, ozonation, wet air oxidation and sulphur based technologies are some of the important methods among them. Most of these methods have their drawbacks as cost of operation and problem of disposal of the reaction products. (Palmer, 1988; Mudder, 1983). On the other hand, the ability of certain phytopathogens to attack cyanide producing plants focuses on the evolved microbial systems for the detoxification or degradation of cyanide. Bhalla, (2012) has reported a large number of microorganisms to degrade nitriles and cyanide. Studies on pure cultures of cyanide degrading microorganisms have been concentrated on fungi such as *Fusarium solani* and aerobic bacteria such as *Alcaligens sp, Klebsiella oxytoca.* etc. (Barclay, 2002, 1998, Kao et al, 2003, Richardson, 1987; Ingverason, 1991). An Actinomycetes member, Streptomyces *lavendulae* is reported for the presence of rhodanase enzyme system, that transfer sulphur from thiosulphate to cyanide forming thiocynate, a less toxic compound Scattered reports of microbial degradation of cyanide waste by mixed population in the acclimatized sludge are available. (Richards, 1989; Fallon, 1992). Manolov, (2005) had designed a degradation process in a packed bed reactor. In this view, cyanide hydratase can become significant 'green catalyst'. The enzyme was reported in some fungi and aerobic bacteria (Dumestre, 1998, Barclay, 1998, 2002, Wang, 2012).

Actinomycetes are the organisms with diverse metabolic potential. Our aim for the present project was to isolate and acclimatize potential cyanide degrading actinomycetes for its possible biotechnological use in cyanide waste treatment. Efforts were made to optimize parameters for cyanide hydratase production and to understand its activity as well as to find out optimum pH and temperature for its activity.

2.0 Materials and Methods:

2.1 *Isolation and acclimatization of cyanide degrading Actinomycetes*

Actinomycetes were isolated from garden soil on starch casein agar and on glycerol asparagine agar. The isolates were maintained on glycerol asparagine agar slants. Cultures were grown in glycerol asparagine 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 10mg/liter to 1000mg/liter. The medium contained dextrose, potassium phosphate dibasic and trace salt solution of $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, and $ZnSO_4.7H_2O$. Acclimatization was confirmed by growing the cultures on solid medium containing respective amount of potassium cyanide.

2.2 *Identification of the isolate*

The isolate growing most efficiently in the minimal medium containing potassium cyanide as nitrogen source was selected and identified according to the Bergey's manual of systematic bacteriology Ed. 1, Vol. 4., by characterization 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.

2.3 *Studies on cyanide degradation by Micromonospora braunna*

Cyanide degradation by the isolate *Micromonospora braunna* was studied by observing decrease in the initial level of cyanide and by the simultaneous detection of formamide and ammonia in the cyanide dextrose broth culture of the organism. Effect of various initial concentrations of cyanide from 10mg/lit to 1000mg/lit was determined. Effect of different sugars as arabinose, ribose, dextrose, sorbitol, lactose, sucrose, glycerol and starch was also studied.

2.4 *Ammonia estimation*

Nessler's method was adopted for ammonia estimation.

2.5 *Formamide assay*

Formamide was detected spectrophotometrically, using a modified method for amide estimation. 2 ml fermented broth was taken in a test tube. To this 4 ml 1:1 v/v mixture of 3.5 M NaOH and 2.3 M hydroxylamine hydrochloride was added. The reaction mixture was kept at 60^o C. for 30 minutes. Then 2ml of 4 N HCl and 2 ml 1.23 M $FeCl_3$ was added. Absorbance was recorded at 530 nm. Similar method was adapted to estimate formamide in the enzyme reacted mixture to determine the enzyme activity. A standard graph

was prepared using formamide at the concentration 0.5mM/ml to 5 mM/ml.

2.6 *Cyanide assay*

Cyanide estimation was done by pyridine-barbituric acid method. (Shiger, 1984). For this 4ml fermented broth was taken. 2 ml Chloramine-T was added to this. Immediately after this 5 ml pyridine-barbituric acid reagent was added with constant gentle swirling and the mixture was then diluted to 50 ml with double 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 minutes but within 15 minutes. A standard graph was prepared using potassium cyanide concentrations within range of 0.2µg/ml to 0.3µg/ml.

2.7 Production and e*xtraction of cyanide hydratase from Micromonospora braunna*

72 hour old dextrose broth culture was used as inoculum to inoculate the production medium containing dextrose and trace metal solution. The enzyme induction was done by adding KCN at the final concentration of 10 mg/lit. The medium was incubated for 24 hours. Then well grown cells were harvested by centrifugation and suspended in 0.05M phosphate buffer. The cells were disrupted 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.

2.8 *Estimation of Cyanide hydratase activity*

The enzyme activity was conducted by mixing 0.5 ml crude enzyme to 0.1 ml of 500mM KCN as substrate. The final amount was 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 formamide formed per minute in one ml.

2.9 *Parameter optimization for cyanide hydratase activity*

Effect of pH, temperature and initial substrate concentration on the rate of reaction was determined. For determining the optimum pH, the enzyme substrate reaction was carried out at various pH values from 2 to 10 and resultant formamide was estimated. Optimum temperature for the enzyme activity was determined by allowing the reaction to occur at different temperatures from 20°C. to 60°C. and then estimating the amount of formamide formed during the reaction.

2.10 *Determination of Km and Vmax values*

Michelis Menten constant and maximum velocity of the cyanide hydratase reaction was determined by studying effect of various concentrations of the substrate potassium cyanide on the rate of enzyme reaction and then plotting MM graph.

3.0 Results and Discussion:

Out of 10 isolates those were adapted to 120 mg/lit KCN in the dextrose broth, only two showed growth at 1000 mg/lit cyanide concentration. One of the two, showing fast and consistent growth was identified as *Micromonospora braunna*. The isolate was growing with golden yellow coloured colony on starch casein agar with no diffusible pigment. The substrate mycelium with singly located smooth surfaced, cylindrical spores was characteristically observed. (Fig. 1). The spores were having 1.2 µm length and 0.6-0.8 µm width. Melanin was not produced (Table 1).

Table 1: Characteristics of *Micromonospora braunna*, on incubation at 30°C.for 4 days.

Sr. No.	Characteristics	Observation
01	Growth of substrate mycelium on SCA	Yellow brown
02	Colour of aerial mycelium	Absent
03	Reverse pigment	Yellow
04	Diffusible pigment	Absent
05	Spore arrangement	Single
06	Spore shape	Cylindrical
07	Spore length	1.2 µm
08	Spore breadth	0.6-0.8µm
09	Melanin production on TA	-ve
10	H_2S production	-ve
11	D- Glucose	A
12	Fructose	A
13	Arabinose	A
14	D-Xylose	-ve
15	D-Ribose	- ve
16	Mannitol	A
17	Inositol	- ve
18	Sucrose	+ ve

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

Cyanide degradation by *Micromonospora braunna* was observed maximum 98.79% in the presence of 1% dextrose and with 100 mg/lit initial KCN concentration. It was highest as 99.84% with 80 mg/lit initial cyanide concentrations. Little fluctuation in the percent degradation due to the presence of various sugars in the medium was noticed. However, presence of dextrose speeded up the cyanide degradation (Fig. 2) Maximum cyanide degradation observed with dextrose and with lactose was 68%, while minimum i.e. 95% cyanide degradation was observed with starch as carbon source. Ammonia was detected in all cases. The appearance of ammonia was possibly because of further hydrolysis of formamide by amidase enzyme to form formic acid and ammonia. Further, dehydrogenation reaction converted formic acid to carbon dioxide.

However, in control sets where abiotic process of cyanide depletion was observed the generation of ammonia was very high. The extent of ammonia production was reduced by about 85%, in microbial degradation of cyanide as compared with the abiotic process. (Table2).

During the study of the reaction of cyanide degradation by *Micromonospora braunna*, formamide was noted as the major end product confirming the presence of cyanide hydratase [Formamide hydrolase, E.C. 4.2.1.66] in the fermented broth.

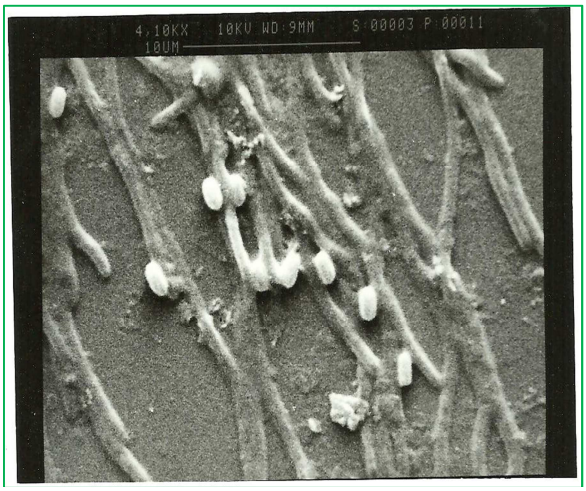

Figure 1: SEM showing sessile spores of *Micromonospora braunna*

During optimization of reaction condition crude enzyme extract showed pH 9 as optimum at 30^{o} C. where 40µM formamide was produced per ml per minute. Increase in the rate of the enzyme reaction was observed from pH 6. At neutrality the reaction rate was 70% (Fig. 3). The optimum temperature range observed was 30^{o}C.-40^{0}C. after which the rate of reaction decreased sharply. (Fig. 4)

The rate of reaction increased with the increase in KCN concentration in the reaction mixture from 25 mg/lit to 100mg/lit. [Figure e].Vmax and Km values were decided by plotting Michaelis Menten graph. Vmax was calculated as 39 µM/ml/min and Km as 33 mM. Jandhyala, (2005) reported that the fungal cyanide hydratase has notably high Km and Vmax values.

Table 2: Cyanide degradation by *Micromonospora braunna* in 100 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	*Micromonospora braunna*	98.79	740	0.95
02	Control	93.65	5000	00

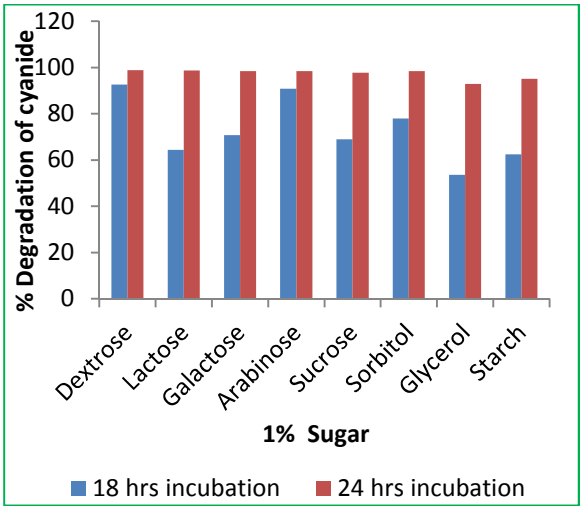

Figure 2: Effect of different sugars on cyanide degradation

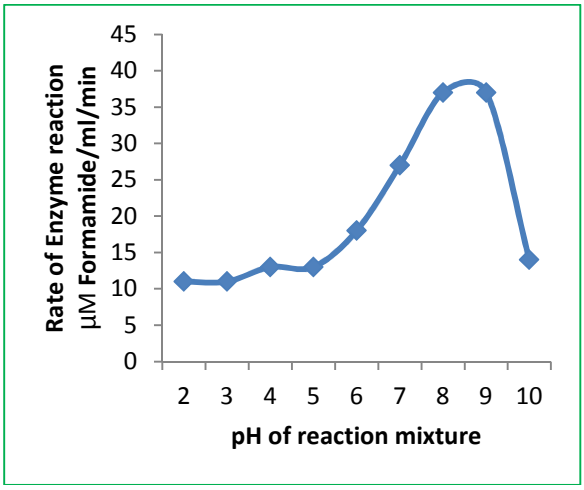

Figure 3: Effect of pH on rate of enzyme reaction

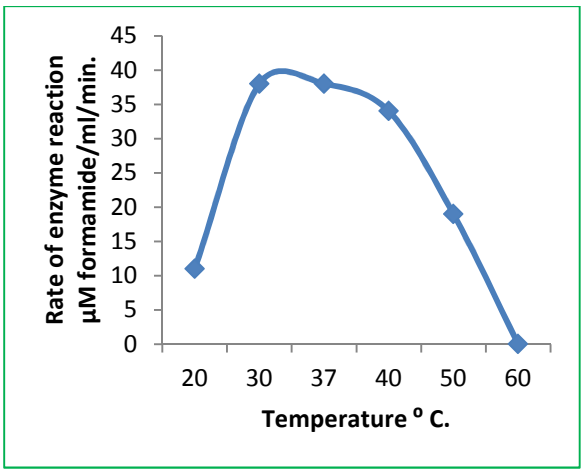

Figure 4: Effect of Temperature on rate of enzyme reaction

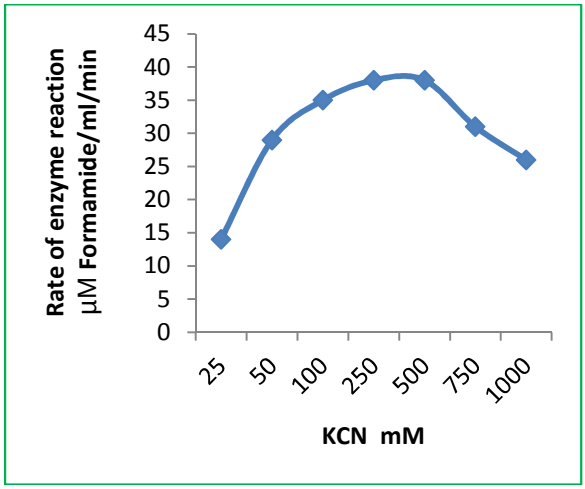

Figure 5: Effect of substrate concentration on rate of enzyme reaction

A variety of enzymatic pathways for cyanide degradation have been described. These include conversion of cyanide into thiocyanate, formation of bicarbonate and formate (Fallon, 1992), formation of bicarbonate and ammonia (Harris, 1983), formation of carbon dioxide and ammonia and formation of methane and ammonia (Fallon,1991). Cyanide hydrolysis to formate and ammonia by cyanidase from *Alcaligen xylosoxidans sp. denitrificans* (Ingvorse, 1991) and cyanide hydration by cyanide hydratase from *Stemphylium loti* and *Fusarium lateritium* (Fry, 1972) are also reported. Six bacteria with ability to degrade 60-94% cyanide are isolated by Agarry and Owabor, (2012) with the possible use in the biotreatment of cyanide waste effluent. One of the hurdle in using microorganism or their enzyme system for bioremediation of cyanide containing waste is that most microorganisms capable of biodegrading cyanide are sensitive to cyanide concentration, with biodegradation and/or growth rate decreasing above specific thresholds for each organism. *Micromonospora braunna* under present study, on the other hand was found to be growing at 1000 ppm cyanide concentration after acclimatization. Possibly its ability of cyanide tolerance can be further increased.

Out of scanty reports available for microbial treatment of cyanide waste, *Streptomyces lavendulae* reported by Oi, in 1977; has rhodanase enzyme system. This converts cyanide into thiocyanate. Biological treatment processes has a much lower operating cost when compared to chemical treatment processes and it allows both the removal of cyanide and denitrification of the ammonia produced as a result of the cyanide

removal. This in turn results in a much more environmentally friendly effluent.

Biological treatment is an ecosociable approach for removal of cyanide from industrial waste as well as from polluted natural bodies. It can be less expensive and much faster than chemical and physical methods. [Dwivwdi, 2011].

4.0 Conclusion:

The present work concludes that *Micromonospora braunna* is 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 cyanide hydratase 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 suggest possible application of *Micromonospora braunna* for cyanide waste treatment as alternative to the abiotic processes.

5.0 Acknowledgement:

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