Molecular Characterization of Cyanide Degrading Bacterium-Enterobacter sakazakii Isolated from Sago Factory Effluent

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ABSTRACT

Sago effluent was collected from Varalakshmi factory Mallur, Salem Dist. for the isolation of Enterobacter sakazakii. Rhizosphere soil collected for the isolation of Azotobacter sp. and root nodule for Rhizobium sp. These organisms tested for the cyanide tolerance. Enterobacter sakazakii showed MIC of 5,000 ppm KCN and Azotobacter and Rhizobium showed MIC of 50 ppm KCN each. Enterobacter sakazakii wild type strain showed 99% degradation after 96 hrs. and mutated strain showed 100% degradation with in 96 hrs. From the present study it is sure that Enterobacter sakazakii stands as a best cyanide degrading organism. By performing plasmid curing experiment it was confirmed that Enterobacter sakazakii bear plasmids for the degradation of cyanide and plasmid had a size of ~8kb. The restriction digestion of the plasmid with EcoRI produced five fragments indicating that it have five restriction sites and a ligated
fragment formed. In transformation studies, transformation increased the efficiency of the recipients. Transformants, *Azotobacter* sp. showed MIC of 700 ppm KCN and *Rhizobium* sp. showed MIC of 400 ppm KCN. From the present investigation, it is concluded that *Enterobacter sakazakii* have 100% efficiency to degrade cyanide making its possibility to use as an ecofriendly strain in the cyanide contaminated environment. Transformed strains of *Azotobacter* sp. and *Rhizobium* sp. can also tolerate and utilize high level of cyanide. To improve cyanide degrading ability of *Enterobacter sakazakii* genetic engineering techniques involving use of expression vector system has to be considered.

**Keywords:** Sago effluent, Cyanide tolerance, Cyanide degradation, *Enterobacter sakazakii*.

**Key word Abbreviations:** MIC-Minimum inhibitory concentration, KCN-Potassium Cyanide

**INTRODUCTION**

The general term “cyanide” refers to numerous compounds, natural and manmade having chemical group CN. Several plants, some soil bacteria and several species of invertebrate organisms produce natural cyanide and related compounds (Dixon 1999). Selected edible plants contain potentially toxic concentrations of various cyanide like compounds eg. Cassava, bamboo, lima beans, almonds and sorghum (Eister 1991). Cyanides are released into the environment by metal plating, aluminium electrolysis, and coal gasification, coal cooking and leaching. Some pharmaceutical and agricultural industries release low levels of cyanide (<1 mg 1⁻¹) into the environment. Chemical treatment of cyanide is costly, so biological treatment of cyanide is
preferred. Considerable amounts of cyanide in the form of hydrocyanic acid are released into the effluent during the process of root tubers of the plant cassava (*Manihot esculenta* Crantz). This plant has cyanide content in all parts including root tubers in order to protect it from disease causing agents. In India, Tamil Nadu, Kerala and Andra Pradesh play an important role in the manufacture of sago and starch. In Tamil Nadu, at Salem and Namakkal districts, there are around 800 sago factories discharging large quantities of cyanide containing effluent into the environment.

A number of microorganisms have been reported to possess the ability to produce, assimilate or detoxify hydrogen cyanide. (Castric 1981). Biological treatment of cyanide containing waste depends on the enzyme system Rhodanese or cyanide sulftransferase in the bacteria. Different enzymes are involved in the enzyme system are β cyanoalanine synthase, rhodanese cyanide hydratase, cyanase, nitrogenase and cyanide oxygenase. The enzymes present in the microorganisms can convert the cyanide to less toxic formamide and ammonia then to CO₂. Cyanide in the effluent can be treated by either chemical or biological methods. Bacterial detoxification is of interest both in order to understand how cyanide may be dealt with in the environment and to evaluate the economic viability of bacterial system for cyanide detoxification. Many number of bacteria have been reported to detoxify and convert cyanide to less toxic thiocyanate. Pellet & Ware (1954) first isolated a cyanide degrading bacterium.
MATERIAL AND METHODS

**Sample collection:** Effluent samples (250 ml) with cyanide were collected from Varalakshmi factory Mallur, Salem Dist. in sterile containers at 4°C. Then they were transferred to the laboratory and processed immediately. 10ml of effluent sample was inoculated with 90ml of mineral salt medium and incubated for 24-48 hrs at 370 C to enrich the culture growth. A loopful of culture streaked on the nutrient agar medium and was incubated at 370C for overnight for the isolation of bacteria. 10g of rhizosphere soil sample collected, serially diluted and streaked on the Ashby’s medium and incubated at room temperature for 2-3 days for the isolation of *Azotobacter*. Root nodules were collected, sterilized with HgCl₂ (0.1%) crushed and streaked on Yeast extract mannitol agar medium with congo red dye and incubated at room temperature for 2-3 days for the isolation of *(Aneja 2002).* Different tests were done for the identification of *Enterobacter sakazakii, Azotobacter sp.* and *Rhizobium sp.* Characterization of isolates done according to the methods given in “Bergey’s Manual of Determinative Bacteriology” 9th edition (1985).

**TOLERANCE OF CYANIDE –MINIMUM INHIBITORY CONCENTRATION (MIC):**

The minimum inhibitory concentration (MIC) of cyanide was determined by using mineral salt medium. Different concentrations of filter sterilized potassium cyanide solution (100 ppm, 500 ppm, 1000ppm, 2000ppm, 3000ppm, 4000 ppm, 5000ppm, 6000ppm) was added to the medium before inoculation of the isolates (2% inoculum). Potassium cyanide served as the principle nitrogen source. Experiment was conducted in sterile test tubes with 1ml of mineral
salt medium and the tubes were incubated for 48 hours before being, scored for growth by observing turbidity. As described above, the growth of *Enterobacter sakazakii* was tested using mineral salt medium with potassium thiocyanate (100-5000 ppm) as the carbon and nitrogen source. Growth of *Azotobacter* and *Rhizobium* tested by using the same medium with potassium thiocyanate (100-500ppm) as the carbon and nitrogen source.

**CYANIDE DEGRADATION:** Mineral salt medium, which was introduced by Shivaraman and Parhad (1985). Composition shown above. MSM was used for the degradation process. The medium was sterilized and 200 ppm of filter sterilized KCN was added into the medium. Degradation was studied at pH 7.0 and at room temperature by shake flask culture method. During degradation of cyanide, three parameters were checked, which were determination of growth, determination of biomass and estimation of cyanide. At 12 hour intervals samples (1 ml) were collected and OD values taken at 610 nm for determination of growth. 10 ml culture broth was centrifuged and the pellet washed twice with double distilled water. It is filtered with Millipore filter (0.45μm pore size). Filter was dried to a constant weight at 103°C for 2 hrs and dry weight of the filter was determined for the determination of biomass. For cyanide estimation, a standard graph was prepared using known standard cyanide solution. Distilled water was used as blank. The samples were removed from the medium at 8 hour intervals and centrifuged and the supernatant was used for the cyanide estimation. To 5 ml broth, 5ml of 0.5M Na₂CO₃ was added followed by the addition of 5 ml of 1 % picric acid solution making the volume 15 ml. The tubes were immersed in boiling water bath for 5
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minutes. The tubes were taken and cooled to room temperature. OD values were taken at 540nm using UV-VIS spectrophotometer. After studying degradation with wild type strain, mutants also tested for the degradation. In order to produce mutants, which can tolerate high cyanide concentration. *Enterobacter sakazakii* was irradiated using UV and EMS (Ethyl methyl sulfonate). The cyanide tolerant capacity of mutants tested by adding the KCN (100-10,000ppm) into the mineral salt medium and incubating at 37°C for 48 hours. Cyanide degradation by mutants studied by shake flask culture method as described above. Growth pattern and biomass determined and residual cyanide level estimated. Plasmid curing done to confirm the presence of plasmid, The plasmid bearing degradation capacity isolated and restriction digestion with Eco RI done to find out the restriction sites & ligation also done.

**PREPARATION OF CELL FREE EXTRACTS AND ENZYME ASSAY:** Cells were treated with 0.5M Tris HCl buffer (pH 8.5-8.6) overnight at 5°C then centrifuged and resuspended in 0.01M Tris HCl containing 3M Na$_2$S$_2$O$_2$ at pH 7.8 forming 20% cell suspension. Thiosulphate was added to stabilize the enzyme. The cells were disrupted by lysozyme and incubated at 37°C for 30 minutes. Whole cells and debris were removed by centrifugation at 10,000 rpm for 30 minutes. The supernatant containing crude extract was then centrifuged at 12,000 rpm at 4°C in a high speed cold centrifuge. The clear yellow supernatant was removed and the pellet resuspended in the buffer and centrifuged at 10,000 rpm for 45 minutes. About 98% of the enzymatic activity of the crude extract was found in the supernatant fraction. Enzyme was purified by dialysis technique. For enzyme assay, the cell free extract was diluted in the presence of 0.125M thiosulphate and 0.025% bovine
serum albumin. 0.5 ml of enzyme was added to a mixture of 1.0 ml of 0.0125M Na₂S₂O₃. 0.5 ml of 0.20M KH₂PO₄ and 0.5 ml of 0.25 M KCN. The optimum pH is 8.5 for the bacterial enzyme. The test reagents were added in a 50 ml Erlenmeyer flask kept at a temperature of 20°C and the enzyme was added. After 30 minutes the reaction was stopped by the addition of 0.5 ml of 38% formaldehyde and ferric nitrate reagent was added. When ferric ions were added to a solution containing thiocyanate in an excess of thiosulfate, a blue colour appears due to the formation of a complex of ferric ions and thiosulphate but rapidly fades. Then a red colour of the iron thiocyanate complex become visible.

**Transformation**: The competent cells of *Rhizobium* and *Azotobacter* was prepared by suspending cells in CaCl₂ solution. Transformants were selected and tested for the efficiency in tolerating high level of cyanide.

**RESULTS**

Sago effluent was collected from Varalakshmi sago factory at Mallur, Salem Dist. for the isolation of cyanide degrading bacterium. Based on bergy’s manual of microbiology, the isolate identified as *Enterobacter sakazakii*. Rhizosphere soil sample and groundnut root nodules were collected for the isolation of *Azotobacter* sp. and *Rhizobium* sp. respectively. The isolated *Enterobacter sakazakii* from sago effluent was used for the cyanide degradation. At first the tolerance of potassium cyanide was tested (50-8000 ppm KCN) *Enterobacter sakazakii* wild type showed minimum inhibitory concentration (MIC) of 5,000 ppm KCN. *Azotobacter* sp. and *Rhizobium* sp. showed minimum inhibitory concentration of 50 ppm KCN each. Using chemical mutagen EMS (Ethyl methyl sulfonate) and UV irradiation, mutants were developed and were
tested for the tolerance of potassium cyanide (KCN). Mutated strain of *Enterobacter sakazakii* showed the minimum inhibitory concentration (MIC) of 7,000 ppm. In presence of potassium thiocyanate (KSCN), *Enterobacter sakazakii* showed minimum inhibitory concentration of 5,000 ppm. *Azotobacter* sp. showed MIC of 200 ppm and *Rhizobium* sp. showed MIC of 300 ppm potassium thiocyanate. Potassium cyanide (KCN) and its common primary metabolite (KSCN) were used as nutrients for the growth of *Enterobacter sakazakii* as carbon as well as nitrogen source. During the study of cyanide degradation, the growth of the organisms, both wild type and mutated type of strains were checked by determining the growth, cell dry weight and estimation of residual cyanide level. The study of degradation of cyanide by wild strain of *Enterobacter sakazakii* showed increase in growth up to 60 hrs but there was a steady decline after 60 hrs (Fig1) and the degradation by mutated strain of *Enterobacter sakazakii* showed growth up to 48 hrs and there is a decline in growth (Fig 2).
Fig:1 Showing the growth pattern of Enterobacter sakazakii (wild strain) in mineral salt medium with 100ppm KCN during degradation of Cyanide.

Fig:2 Showing the growth pattern of Enterobacter sakazakii (mutated strain) in mineral salt medium with 100ppm KCN during degradation of Cyanide.

The biomass of wild type of strain increased significantly for the organism up to 96hrs (Fig 3). Biomass increased up to 72 hrs for the mutated strain. After 72 hours there is a decrease in cell dry weight. (Fig 4). Reduction in the cyanide level during degradation and the percentage of the cyanide degraded by the wild strain is given in (Fig 5). Wild type strain degraded cyanide within 60hrs and for the mutated strain the residual cyanide level and the percentage of cyanide degraded given in Fig 6. The mutated type degraded within 48 hrs. The degrading plasmid isolated from Enterobacter sakazakii by alkali lysis method was of ~ 8 kb in size.
restriction digestion of plasmid with EcoRI gave fire fragments with approximate sizes; ~10, ~7, ~4.3, ~2.3, ~2. Ligation with T₄ DNA ligase resulted in the formation of a single ligated fragment. Transformation of the microorganisms, *Azotobacter* sp. and *Rhizobium* sp. with plasmid DNA of *Enterobacter sakazakii* with better cyanide resistance character was done and the transformation frequency of these recipient organisms were calculated. The tolerance of potassium cyanide by the transformed cells were tested (100 – 1000 ppm). *Azotobacter* sp. showed the minimum inhibitory concentration (MIC) of 700 ppm KCN and *Rhizobium* sp. showed (MIC) of 400 ppm KCN.

![Fig 3: Changes in biomass of *Enterobacter sakazakii* (wild strain) during the growth in mineral salt medium with 100ppm KCN.](image)
Fig 4: Changes in biomass of *Enterobacter sakazakii* (mutated strain) during the growth in mineral salt medium with 100ppm KCN.

Fig 5: Showing the residual cyanide level in the mineral salt medium with 100ppm KCN during the cyanide degradation by wild strain of *Enterobacter sakazakii*. 
DISCUSSION

Reduction of cyanide toxicity in the sago effluent leads to safety of that ecosystem. Strains which act eco friendly in the environment should be produced for this purpose. Due to the high cyanide tolerance, *Enterobacter sakazakii* was used in the study. The efficiency of *Azotobacter* sp. and *Rhizobium* sp. to tolerate cyanide was tested. When they were tested for the MIC of cyanide, *Enterobacter sakazakii* showed maximum tolerance of 5,000 ppm KCN. Many cyanide tolerating bacteria *Pseudomonas fluorescens*, *Bacillus* sp and fungi, *Fusarium*, *Stemphylium* etc have been reported. (Kunz *et al.* 1994). The organisms ability to utilize the potassium thiocyanate also were tested. In this *Enterobacter sakazakii* grows well on KCN as well as KSCN and utilizes them as carbon an nitrogen sources. Harris and Knowles (1983).Shake flask culture method was used for the degradation study. Both the wild type and the mutated strains were used in cyanide degradation studies. Degradation was studied by using mineral salt medium (Shivaraman and parhad 1985). *Enterobacter sakazakii* mutants formed due to the action of EMS sondeveloped better resistance to KCN ie up to 7000 ppm in contrast of *Pseudomonas aeruginosa*. isolated and characterized by Cunningham and Williams 1995. Wild strain of *Enterobacter sakazakii* utilized 99% of cyanide after 96 hrs and mutated strain utilized 100% of cyanide with in 96 hrs. Biomass for wild strain was going on increasingly but mutated strains, cell dry weight decreased after 72 hrs of inoculation. Formation of formate and biocarbonate as the end products of cyanide degradation have been reported (Fallon 1992).
Enzymes active in the cyanide degradation were detected and assayed. Red colour formation indicated the presence of enzyme. Enzyme was intracellular in nature. Cyanidases are responsible for cyanide degradation. Cyanase is an inducible enzyme in *E.coli*. The peroxidation of thiocyanate which can be catalyzed by enzymes named rhodanese have been investigated (Sorbo and Westly 1975) Alexander and Volini ( 1987) have reported rhodanese with molecular weight of 20kb in size in *E.coli*. The cyanide degrading activity of *Enterobacter sakazakii* is borne on plasmid approximately 8 kb in size. Large plasmids are involved in the degradation of aromatic compounds in *Pseudomonas* (Duggleby et al. 1977) .The formation of number of fragments depends upon its number of restriction sites for the enzyme used. The used EcoRI produced five fragments indicating five sites study of restriction digestion is important in elucidating restriction mapping. The DNA from *Pseudomonas* having the molecular weight of 7.8 X 10⁷ has been isolated and restriction endonuclease cleavage sites have been mapped (Downing and Broda 1979).In transformation studies, *Azotobacter* sp. transformants showed tolerance of 700 ppm and the *Rhizobium* sp. showed tolerance of 400 ppm. Dubnau et al. (1971) reported the fate of transforming DNA following uptake by the competent *Bacillus subtilis*.

REFERENCES


