

Isolation and Characterization of Potential Cyanide Degrading *Bacillus nealsonii* from Different Industrial Effluents

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Abstract: Cyanide causes severe environmental problems when formed in high amounts by anthropogenic activities. Cyanide resistant *Bacillus* species were isolated from the waste waters of sago, steel and tannery industries located at Salem and Namakkal, Tamil Nadu, India. Out of eight *Bacillus* isolates (S₁- S₈) only two species (S₁ and S₂) had the cyanide resistance up to 6000 ppm. Growth rate of *Bacillus* isolate (S₂) was higher than *Bacillus* isolate (S₁) in basal mineral medium. After 96 hrs, cyanide degradation level was measured as 170 µg/mL and 100 µg/mL for *Bacillus spp* (S₁) and *Bacillus spp* (S₂) respectively. *Bacillus spp* (S₂) was identified as *Bacillus nealsonii* by 16S rRNA analysis.

Keywords: cyanide, *Bacillus* sp, degradation, effluent.

Introduction

Cyanide is a carbon nitrogen radical, which may be found in a wide variety of life forms and their large scale presence in the environment is attributed to the manufactured sources which are used extensively in industries¹. Cyanide is highly toxic to living organisms^{2,3}, particularly in inactivating the respiration system by tightly binding to terminal oxidase⁴. Cyanide was toxic to humans and mammals because it binds to key iron containing enzyme Cytochrome oxidase required for cells to use oxygen⁵. The occurrence of cyanide compounds in industrial wastes presents a major environmental and ecological hazard as most of these compounds are highly toxic and some are mutagenic as well as carcinogenic⁶. However, large amounts of cyanide are used in industries involved in the metal-plating, pharmaceuticals, synthetic fibers, plastics, coal gasification; coal coking, ore leaching, gold mining, and electroplating^{7,8,3}. Exposure to high level of cyanide, a powerful and rapid-acting poison might harm brain and heart leading to coma and death⁹.

Consequently, organisms growing in the presence of cyanide must have a cyanide-insensitive metabolism, such as the alternative oxidase described for the plants¹⁰ or the cytochrome bd (or cyanide-insensitive oxidase) in bacteria^{11,12}. From a chemical point of view, the biological treatment of effluents contaminated with cyanide requires an alkaline pH in order to avoid the formation of volatile HCN. In addition, since cyanide is known to react chemically with some keto groups, the use of glucose or similar carbon sources should be avoided. Microbial degradation of cyanide at neutral or acidic conditions has been reported^{13,14}.

Even though, there are different chemical and physical degradation methods used to reduce the cyanide related compounds, huge amount of toxic byproducts are released in the aquatic environment¹⁵. Based on this fact, the strategy to eliminate cyanide containing waters has turned to biodegradation process using micro

organisms¹⁶. Strains adapted to survive in the presence of cyanide at alkaline pH but unable to degrade it has also been described¹⁷. By contrast, references describing cyanide biodegradation at alkaline pH are scarce one of them refers to the fungus which uses cyanide as the sole nitrogen source¹⁷.

The cyanide remains as an important form of nitrogen source for some microorganisms including fungi and plants. Although some organisms synthesize cyanide, a greater number are capable of degrading cyanide using some pathways. The existence of these pathways has allowed the development of biotechnologies to degrade cyanide compounds in industrial waste streams. Disposal of cyanide wastes was studied by Pettet and Ware in 1954¹⁸ and cyanide utilizing bacterium was first isolated by Ware and Painter in 1955¹⁹. Numbers of microorganisms that are able to tolerate or degrade cyanide and its metal complexes have been described to date⁹.

Recently, biological processes have been studied because they are inexpensive and no effect to the environment^{20,21}. For these reasons, the objective in this study was to isolate and identify the *Bacillus spp* which may have role in detoxification of cyanide present in different industrial effluents and also to know their tolerance and degradation level.

Experimental

Sample collection

Effluent samples (200 mL) were collected from sago, steel and tannery industries in Salem and Namakkal regions, Tamil Nadu, India. The samples were collected in sterile glass containers and stored at 4 °C for analysis of cyanide resistant bacteria.

Enumeration of Heterotrophic bacteria

To the 100 mL of the nutrient broth with 100 ppm of filter sterilized potassium cyanide (KCN), 1.0 mL of the effluent was added and kept on rotary shaker (100rpm) for 24 hrs. 1.0 mL of effluent was serially diluted and 0.1 mL of diluents (10^{-3} to 10^{-7}) was spread plated on to nutrient agar medium and plates were incubated at 37°C for 24 hrs.

Identification of cyanide resistant bacteria

The different bacteria appeared on the nutrient agar medium were identified by using the key characters given in Cowan and Steel's manual for the identification of bacteria²². All the bacterial isolates with different colony morphology were kept stored on agar slants and were subjected for Gram's staining, spore staining and biochemical tests.

Growth of isolates in minimal media

The bacterial isolates were grown in minimal medium (without ammonium chloride), Glucose (1%) was used as a carbon source and pH was adjusted to 7.0. The appropriate nitrogen source (filter sterilized KCN) was added from sterilized stocks at different concentrations (1,000ppm to 10,000ppm) and it was incubated at room temperature for 24 to 48 hrs in water bath set at 30°C. At 24 hrs intervals, 2 mL of sample was collected and OD values were taken at 600 nm using UV spectrophotometer.

Plasmid Curing

Curing was done using agents ($\mu\text{g/mL}$) acridine orange. Tubes containing 10 mL peptone water was supplemented with using different concentrations of agents (100 - 400 μg). Inoculated with 0.1 mL of the overnight culture and incubated at 37 °C for 3-4 hrs. After incubation, the highest growth was taken. From that 0.1 mL of culture was spread plate on the nutrient agar plates to get the single colonies after 24 hrs of incubation at 37 °C. Resulting colonies were tested for the loss of plasmid and the presence of chromosomal DNA on the nutrient agar plates incorporated with the appropriate cyanide.

Estimation of cyanide

Alkaline sodium Picric acid based method²³ used for estimation of cyanide. The isolates were inoculated in the appropriate medium (minimal medium) and incubated at 30 °C for overnight. After incubation, the cells were pelleted and 100 μL of the supernatant was added to 200 μL of the picric acid assay solution. This was

immediately placed in a 100 °C heating block for 6 mins. The solution was diluted with 700 µl of distilled water and the absorbance at 520 nm was measured spectrophotometrically.

Estimation of ammonia

The ammonia was estimated in the culture filtrate by following Nessler's reagent method²⁴. 1 mL of culture filtrate was pipette out into 100 µl of standard flask and diluted to about 80mL. Then 2mL of Nessler's reagent was added and made upto the volume with distilled water. This solution was allowed to stand for 10 minutes, the colour of the unknown solution was compared to the standard solution and the solution was measured spectrophotometrically at 570 nm.

Molecular identification of *Bacillus spp* (S₂)

Genomic DNA was extracted from the isolate by the standard phenol- chloroform extraction method²⁵. The 16S rDNA genes were amplified using PCR with Taq polymerase (Sunbiotech) and the universal primer pair of 20F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500R (5'- GGTTACCTTGTTACGACTT-3'). The amplified fragment was sequenced by SciGemom, Kerala. The BLASTN search tool²⁶ was used to determine sequence homology and related sequences were obtained from the GenBank database (National Center for Biotechnology Information, NCBI). A phylogenetic tree constructed with BioEdit (Version 7.0.9.0) software.

Results

Cyanide-degrading bacteria were isolated from the effluent samples collected from sago, steel and tannery industries after serial dilution and plating on nutrient agar medium. 21 bacterial isolates from different genera were obtained. Out of 21 isolates, 8 were belonging to the genus *Bacillus* was confirmed by external morphology and biochemical tests (Table 1).

Eight *Bacillus* isolates were named as S₁ - S₈ and their minimum inhibitory concentration value for cyanide was tested by growing them in minimal medium with different concentrations of cyanide (1000 – 10,000 ppm). Isolates S₁ and S₂ showed high minimum inhibitory concentration values for cyanide 7000 ppm and 6000 ppm respectively (Table 2).

Bacillus spp. S₁ & S₂ appeared as white mucous colonies on the nutrient agar medium (Figure 1). The growth pattern for *Bacillus spp.* (S₁ & S₂) was studied using minimal medium with 1000 ppm KCN at 37 °C up to 96 hrs. Growth curve showed maximum absorbance as 0.55 for *Bacillus spp.* S₂ and 0.45 for *Bacillus spp.* S₁.

Table: 1 Biochemical tests results for *Bacillus spp.* Isolates from industrial effluents

Tests	Number of <i>Bacillus spp.</i> isolates							
	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆	S ₇	S ₈
Gram's staining	+	+	+	+	+	+	+	+
Spore staining	C	T	T	C	C	C	T	T
Motility	M	M	M	M	M	M	M	M
Growth on MacConkey agar	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+
Indole Production	-	-	-	-	-	-	-	-
Methyl Red	+	+	+	+	+	+	+	+
Voges-proskauer	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+	+
Glucose utilization	+	+	+	+	+	+	+	+
Sucrose utilization	+	+	+	+	+	+	+	+

+ = positive; - = negative. C- Central, T- Terminal

Isolates 1- 4 from sago factory effluents

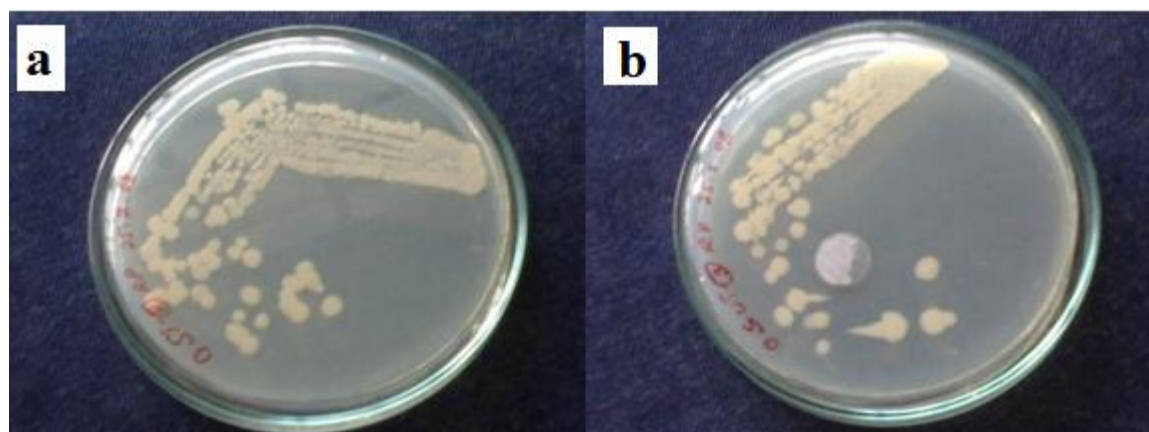
Isolates 5 & 7 from steel plant effluents

Isolates 6 & 8 from tannery factory effluents

Table: 2 Growth of *Bacillus spp.* isolates in different concentrations (1000 ppm to 10000 ppm) of KCN in minimal medium.

Concentration of KCN (in ppm)	<i>Bacillus spp</i> isolates							
	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆	S ₇	S ₈
1000	+	+	+	+	+	+	+	+
2000	+	+	+	+	+	+	+	+
3000	+	+	+	+	+	+	+	+
4000	+	+	+	+	-	+	-	+
5000	+	+	-	+	-	+	-	-
6000	+	+	-	-	-	-	-	-
7000	-	+	-	-	-	-	-	-
8000	-	-	-	-	-	-	-	-
9000	-	-	-	-	-	-	-	-
10000	-	-	-	-	-	-	-	-

+ Growth Positive, - Growth Negative

**Figure 1: Morphology of *Bacillus spp.* S1(a) and S2(b) on nutrient agar medium after 24 hrs of incubation**

Detection of cyanide level reduction by alkaline sodium picrate method during the growth of *Bacillus spp* S₁ and S₂ in minimal medium at 37 °C for 96 hrs and the residual cyanide level for *Bacillus* isolate S₂ was found as higher (100 µg/mL- 921.66%) than S₁ (170 µg/mL-85.83%) (Figure 2).

Ammonia which was formed as byproduct during the cyanide (1200 µg) degradation by *Bacillus* S₁ and S₂ in minimal medium at 37°C for 96 hrs was detected by Nessler's reagent method. The amount of ammonia liberation was measured at 570nm by UV spectrophotometer as for *Bacillus spp.* S₁ & S₂ respectively (Figure 3).

16S rRNA gene for the *Bacillus spp* S₂ amplified through PCR and amplified product was observed approximately 1000 bp in size by using marker DNA ranging from 1000 bp – 6000 bp. Amplified product was sequenced and Phylogenetic tree was constructed by obtained similar sequences from BLASTN search tool. *Bacillus spp* S₂ identified as *Bacillus nealsonii* with 99% homology.

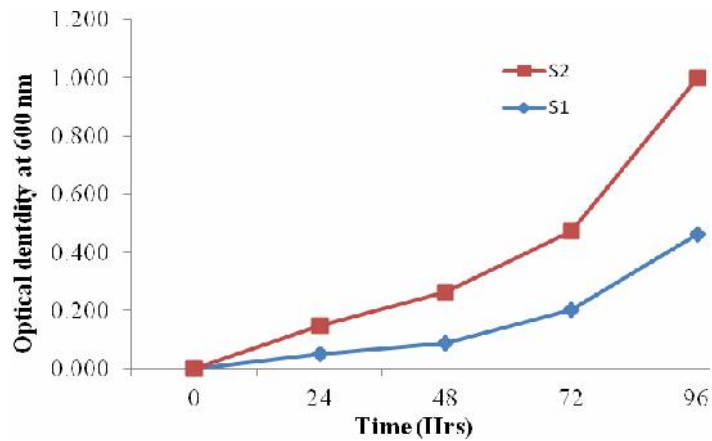


Figure 2: Growth pattern of *Bacillus* isolates S₁ and S₂ in minimal medium.

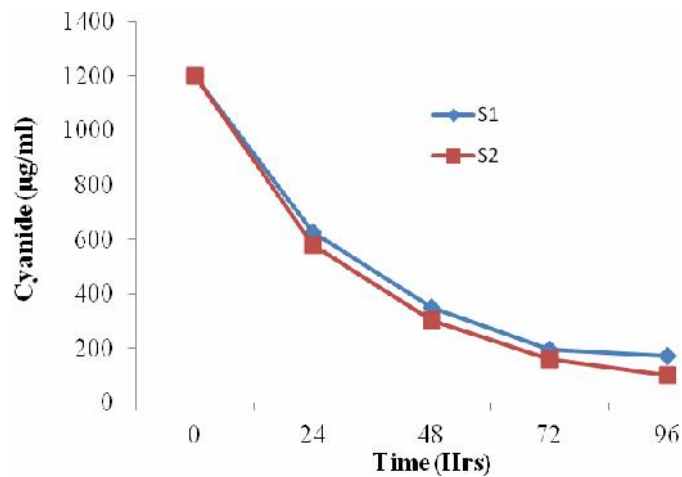


Figure 3: Detection of cyanide level reduction by alkaline sodium picrate method during the growth of *Bacillus* S₁ and S₂ in minimal medium at 37°C for 96 hrs.

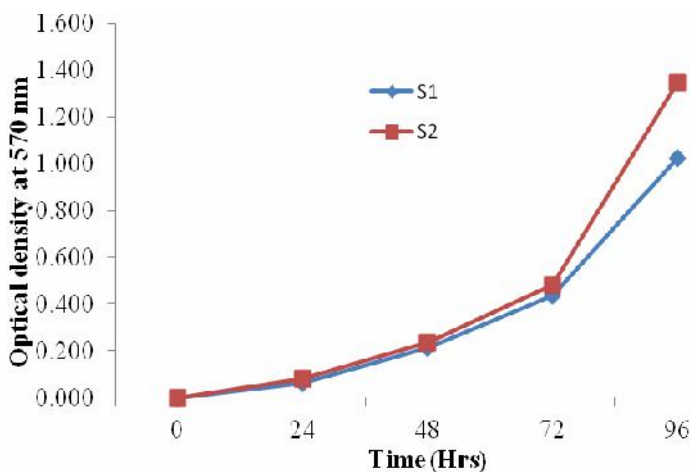


Figure 4: Detection of ammonia level by Nessler's reagent method during the cyanide (1200µg) degradation by *Bacillus* S₁ and S₂ in minimal medium at 37°C for 96 hrs.

Discussion

Several *Pseudomonas* species has been studied for cyanide degradation²⁷⁻³¹. Furthermore, *Klebsiella pneumoniae*, *Moraxella*, *Serratia*, and *Alcaligenes* species were isolated and identified as cyanide-degrading bacteria^{32,33} which utilized cyanide as a source of carbon and nitrogen. Chapatwala *et al.*³⁴ reported that the immobilized cell can degrade the higher concentration of cyanide more than the non-immobilized cell of *Pseudomonas putida*²¹. Sartry³⁵, reported some cyanide metabolizing bacteria were *Bacillus megaterium*, *B. pumilus*, *B. cereus*, *B. stearothermophilus* and the present study also support that *Bacillus* species had effective cyanide tolerance capacity. *Bacillus cerus* has reported to grow in trypticase soy broth containing 1mM cyanide³⁶. In the present study, isolate (S₁) was grow in minimal medium up to 6000 ppm and isolate (S₂) was grow to 7000 ppm of KCN.

The optimal cyanide biodegradation was carried at 25 – 37 °C, and the pH range was 6.0-7.5³⁷. As reported by them, the current cyanide degradation was at 37° C, and pH 7.0. According to the studies of Timmis and Puller³⁸, in bacteria majority of the degrading genes for organic matter are primarily located in the plasmid rather than chromosomal DNA. The cyanide degrading activity of *Bacillus* spp. (S₁ and S₂) was brone to the chromosomal DNA. The ammonia liberated of 50% to 70% by *Pseudomonas* spp. as reported by Dhillon and Shivaraman²⁹. In this present study, the reduction of cyanide levels were measured for *Bacillus* S₂ (100 µg/mL-91.663%) and *Bacillus* S₁ (100 µg/mL-85.83%).

In this study, it is evident that *Bacillus nealsonii* strain was able to degrade cyanide to ammonia and nitrate. The ammonia revealed the increasing concentration when the cyanide removal efficiency increased. But the nitrate exhibited the increasing concentration when the ammonia concentration decreased or did not detect. It may be due to cyanide can be degraded to ammonia and converted finally to nitrate as a final by-product^{39-42,21}.

Conclusion

In this work, *Bacillus* spp was selected for experiments since the strain evidently possesses huge potential to be useful in cyanide bioremediation. The cyanide removal by *Bacillus* spp was parallel with the accumulation of ammonia, suggesting that the production of ammonia from cyanide conversion would be acted as the alternate nitrogen source for the growth of bacteria. Due to the cyanide tolerant activity of *Bacillus nealsonii* can be used in the bioremediation of cyanide present in the waste water at high level. Improved strains using rDNA technology principles should be constructed in future to increase the rate of activity of cyanide degradation.

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