

# Cyanide Degradation by Consortium of Bacterial Species Isolated from Sago Industry Effluent

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## Abstract

The present study emphasizes degradation of cyanide under aerobic condition using microbial consortia isolated from wastewater of sago industries. The consortium contains species of *Bacillus*, *Klebsiella* and *Pseudomonas*. More than 98% of cyanide was removed as ammonia within 96 hours at pH range of 7.0-8.0 by the chosen microbial consortia. However, the individual species displayed only 50-75% cyanide degradation. The higher percentage of cyanide removal observed with the consortium than individual organism suggested the existence of synergism in the consortium.

**Keywords:** Bacterial consortium, Cyanide, Cassava, Degradation, Sago industrial effluent

## 1 Introduction

Cassava (*Manihot esculenta*) is an important root crop used as an energy rich food supplement by millions of people in India, Africa and South America. The tuber consists of 2-25% starch, and limited quantities of proteins, fats, vitamins and minerals. Additionally, the roots also contain large quantities of cyanide as an anti-nutrient factor. Environmental pollution from medium scale cassava processors is more difficult to deal with cyanide that enters the environment from both natural processes and industrial activities. Large quantities of cyanide originate as wastes in metal plating, steel tempering and mining industries [1,2,3]. Nevertheless, plants are the main source of cyanide in the biosphere [4]. Cyanide occurs naturally in cassava roots, potato like tubers grown in tropical countries in the form of cyanogenic glucosides (the precursors of HCN) in various concentrations depending on the variety and growth condition. The bound cyanide is converted into free cyanide during milling operations such as peeling, slicing, squeezing and crushing. About 70% of total cyanide is present in the wastewater generated and 30% is in the fibrous residues.

At high concentrations, cyanide becomes toxic to soil microorganisms and can pass through soil into underground water streams. Apart from toxic nature, cyanide is also known for its metabolic inhibitory effects. Hence, cyanide-containing effluents cannot be discharged without any proper treatment. The permissible level of cyanide is 0.1mg/l [5].

With regard to the removal of cyanide, microorganisms such as bacteria, fungi and actinomycetes are capable of degrading and utilizing the cyanide for their growth as carbon or nitrogen source. As cyanide is a one-carbon compound, and the utilization of other C<sub>1</sub> compound by microorganisms has been extensively studied because of their scientific and industrial importance [6]. Microbial degradation of cyanide, cyanate and thiocyanate under laboratory scale and found utilization of cyanide improved by addition of dextrose to the influent [7]. Up to 60 mg of cyanide per liter of effluent was completely degraded, with recovery of the cyanide nitrogen as ammonia and nitrate plus nitrite. Degradation of cyanide in the presence of significant concentration of heavy metals [8].

Report available on a mixed culture comprising *Fusarium solani* and *Trichoderma polysporum* obtained by enrichment with tetracyanonickelate [KfI (CN)] at pH 4 and another mixed culture consisting of *Fusarium oxysporum*, *Scytalidium thermophilum*, and *Penicillium miczynski* obtained by enrichment with hexacyanoferrate [K Fe (CN)] at pH 4 were able to grow on K Fe (CN) as the sole source of nitrogen under acidic conditions [9]. Further, the authors suggest growth was associated with progressive removal of cyanide from the culture supernatant and after the termination of growth; at least 50% of the total cyanide had been degraded.

The literatures suggested, biological removal of cyanide from wastewater using selective microbes or microbial cultures. Hence, in the present study, an attempt was made on to have microbial cultures of sago industrial wastewater origin and evaluate the efficacy of the isolated species individually as well as in mixed form on removal of cyanide from wastewater generated from sago industries. Further, the study has been extended to assess the influence of various physical and nutrient factors on removal of cyanide.

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## 2 Materials and Methods

### 2.1. Collection of sample

The industrial effluent sample was collected from a sago industry located at Salem, Tamilnadu, India. The samples were transported to the laboratory within 2 to 3 hours of collection in an ice box for microbiological analysis.

### 2.2. Enrichment, screening and isolation

One mL of the sample was taken and inoculated into 100 mL of isolation medium and kept in a shaker (150 rpm) at 30 °C for 24 h. Then the above enriched sample was subjected to serial dilution and plating methods. The isolation media contains (g/L), Glucose - 1.0 g, Tryptone - 1.0 g, Potassium cyanide - 1 mM (65.12 mg), Dipotassium hydrogen phosphate -1.0 g, Magnesium sulphate - 0.2 g, Calcium chloride - 0.01 g, Manganese Sulphate - 0.2 mg, Copper sulphate - 0.2 mg, Zinc Sulphate - 0.2 mg, Agar - 20 g, Distilled water - 1000 mL. The pH of the medium was adjusted to 7.6 ±0.2.

Followed by isolation, cyanide utilizing bacterial species was screened further using Nitrogen free glucose medium containing Glucose - 0.8 %, Disodium hydrogen phosphate - 50 mM, Potassium dihydrogen phosphate - 100 mM, Magnesium sulphate -1 mM, Calcium chloride - 0.1 mM, Potassium cyanide - 1 mM, Distilled water - 100 mL and the pH of the medium was adjusted to 7.2.

### 2.3. Degradation of cyanide

The isolated microbial species were exposed to potassium cyanide at varied concentration ranging from 10 to 100 mM in pre-sterilized nitrogen free glucose medium under aseptic condition and incubated for 24-48 h under shaking condition (150 rpm) at 30 C. Followed by incubation, the biomass free supernatant was subjected to estimation of cyanide according to the standard procedure [11]. In brief, the cell free supernatant (100 µL) was mixed with 200 µL of the picric acid assay solution (0.5% of Picric acid in 0.25 M sodium carbonate) and kept at 100 °C in a heating block for 6 min and then diluted with 700 µL of distilled water and measured the absorbance at 520 nm and the concentration of cyanide was calculated from the standard curve.

The total ammonia content of the sample was estimated according to the standard procedure [11]. In brief, one mL of supernatant obtained from the above step was diluted to 80 ml. To this solution added 2 mL of Nessler's reagent and then made up to 100 mL and measured the absorbance at 570 nm. The concentration of ammonia was calculated from the standard graph.

### 2.4. Effect of pH and Temperature on cyanide removal

The impact of pH on cyanide removal by the selected isolates was studied at different pHs, viz., 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0. The percentage of cyanide removal and ammonia release was determined as summarized in the above said paragraphs. Similarly, the growth medium with chosen isolates were incubated at 20 to 45 °C with 5 °C increment and measured the percentage of cyanide and ammonia release accordingly to assess the influence of temperature on cyanide removal.

### 2.5. Effect of salt concentration, carbon sources and metal salts on cyanide removal

Further, the effect of sodium chloride on removal of cyanide from sago industries wastewater was studied at 1, 1.5, 2, 2.5, 3, 3.5, 4 and 5 % concentration of sodium chloride. In brief, the NFG medium was supplemented with the said concentration of sodium chloride and inoculated with the chosen isolates. The percentage reduction in cyanide concentration was calculated accordingly. Similarly, the influence of carbon source on the percentage removal of cyanide was also studied using glucose, fructose and sucrose as carbon sources at 0.05, 0.25, 0.5, 0.75 and 1% concentrations. Salts of Mg, Mn, Ca and Cu were supplemented to the growth medium at 0.5% concentration and then the growth and cyanide removal was studied accordingly.

### 2.6. Statistical analysis

The effect of various factors such as time, pH, temperature, concentration of glucose, sucrose and fructose on cyanide degradation were tested for their significance by performing One way ANOVA. The post hoc tests for the significant factors were done by Tukey's HSD multiple comparison tests. All the analysis was carried out by using the statistical software package SPSS – student version 11.

## 3 Results

### 3.1 Isolation studies

The isolation studies followed in the present study yielded only few isolates shown growth in the presence of KCN (1 mM). Further screening with NFG medium was displayed different growth pattern. Good growth was observed with less number of bacterial isolates and some isolates were shown moderate and slight growth. The isolates shown good growth were identified at genus level as *Bacillus* spp. CY1, *Klebsiella* spp. CY2, *Pseudomonas* spp. CY3 and *Pseudomonas* spp. CY4.

### 3.2 Cyanide tolerance by isolates

Figure 1 depicts the growth of the four isolates, namely *Bacillus* spp. CY1, *Klebsiella* spp. CY2, *Pseudomonas* spp. CY3 and *Pseudomonas* spp. CY4 at 25, 50, 75 and 100 mM concentration of cyanide. It has been observed that among the four isolates *Pseudomonas* CY3 and *Pseudomonas* CY4 sps shown tolerance up to 75 mM of cyanide compared to *Bacillus* CY1 and *Klebsiella* CY2 species, however, they have shown tolerance to cyanide only up to 25 mM.

### 3.3 Removal of cyanide by chosen isolates

Figure 2 a-d illustrates the residual cyanide concentration and the corresponding concentration of ammonia release observed for the four different isolates at different period of incubation. It has been observed that irrespective of the isolates there was about 50% reduction in cyanide concentration after 48 hours of incubation. However, the isolates CY3 and CY4 showed more than 80% reduction in cyanide concentration after 72 hours. An increase in the concentration of ammonia release was observed correspondingly for all the isolates and the maximum ammonia release was around 120 h of incubation.

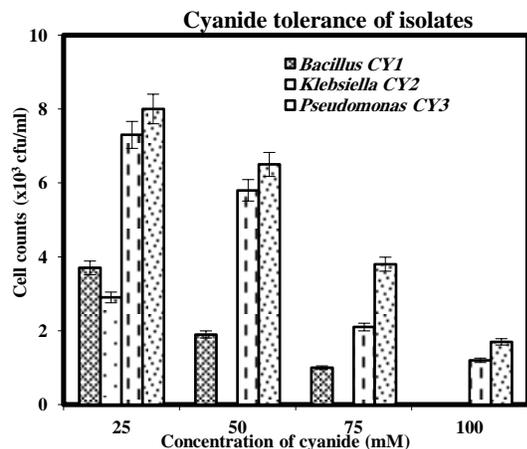


Fig 1 Tolerance of isolates with respect to increasing concentration of cyanide

### 3.4. Removal of cyanide by bacterial consortia

Followed by the observations with individual isolates, experiments were further extended to mixed consortium. Figure 3 depicts the cyanide removal and ammonia release pattern at different incubation period with mixed consortium. About 98 % reduction in cyanide concentration within 96 h of incubation was observed with the corresponding increase in ammonia concentration.

### 3.5 Effect of temperature and pH on cyanide removal

With regard to the influence of physical factors, carbon sources and metal salts on removal of cyanide, at pH 7.0-9.0, more than 80% reduction was observed in all the four isolates and meager reduction was at pH above 9.0 (Fig. 4a). Amongst the various temperatures studied, only at 35 °C the maximum percentage reduction of more than 85% was observed irrespective of the isolates (Fig. 4b). However, the isolate CY4 showed more than 50% reduction even at 40 °C.

### 3.6. Concentration of carbon source on cyanide removal

Studies on effect of different carbon sources and at different concentrations, it has been observed that, additional supplementation of carbon source in the form of glucose, fructose and sucrose suppress the cyanide removal when the concentration increased above 0.05%.

### 3.7. Effect of metal ion on cyanide degradation

With reference to influence of metal ion concentrations supplied in the form of salts of Cu, Mn, Ca and Mg at 0.5 g/L, Cu and Ca suppress the growth, whereas, Mg and Mn salts delay the growth, however, Mg salts alone have meager influence on the cyanide degradation.

### 3.8 Salt tolerance on cyanide isolates

Further, the salt tolerance of the isolates CY1, CY2, CY3 and CY4 in the presence of NaCl concentration of 1.0-5.0% showed, all the isolates displayed growth up to 3% salt concentration and no growth was at 4-5% concentration.

### 3.9. Statistical analysis

One way ANOVA tests for all the influencing factors for cyanide degradation by the four isolates namely, CY1, CY2, CY3, and CY4 were conducted. The result did not show any significant variation among the isolates for all the test factors. But however the variations in cyanide degrading capacity were highly significant between each of the factors. The Tukey's HSD multiple comparison analysis showed significant variations with time, temperature, pH, Glucose concentration, fructose concentration and sucrose concentration resulted in the homogenous subsets for all the factors which were listed in Table 1.

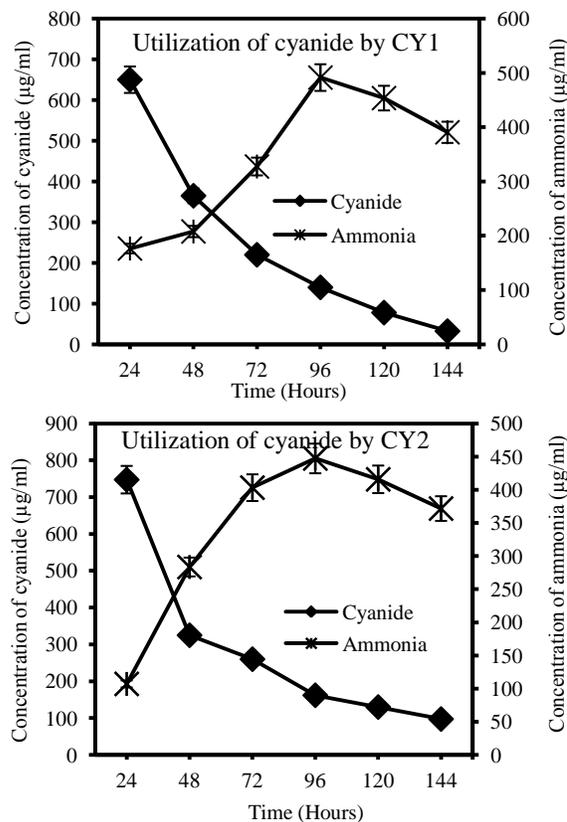


Fig. 2 a-d. Profile of cyanide removal and ammonia release by the chosen four isolates

## 4 Discussion

According to the earlier work [12], microbes readily degrade free cyanide, CN and HCN. Free Cyanide degradation by microorganisms was described by Howe in 1965 and reviewed by Knowles in 1976 and further it was considered in 1990s as a "green" technology [13,14]. However, when cyanide is present in the form of thiocyanate, metal-cyanide complexes such as Zn (CN)<sub>2</sub>, Ni (CN)<sub>2</sub>, Na(CN), KCN, etc., and also the spent cyanides in the wastes generated from various processing industries, microbial degradation is a challenging process [15-18].

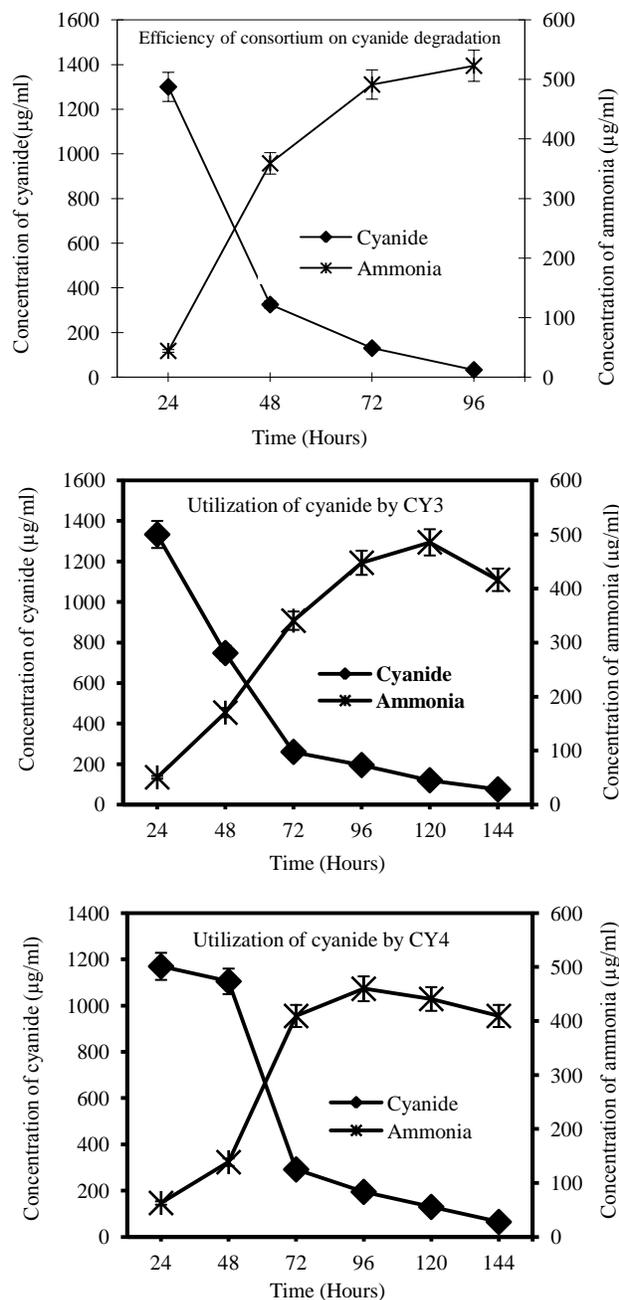


Fig. 3 Cyanide removal and ammonia release by the mixed consortium

In the present study, the isolation of bacterial species from cassava effluent samples yielded four potential species of different genera, *Bacillus sp.* (CY1), *Klebsiella sp.* (CY2) and *Pseudomonas sp.* (CY3 and CY4). Literature reports also suggested that *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Paracoccus*, *Pseudomonas*, *Thiobacillus* were the major bacterial population of wastewater generated from sago industries [19-22]. The recent studies on cyanide degradation by pure and mixed culture of a bacterial strains isolated from the effluent was reported [23]. The potential cyanide degradation

demonstrated by two bacterial consortia consisting of *Pseudomonas spp.* and *Bacillus spp.* [15] and of *Pseudomonas* and *Acinetobacter spp.* [19]. Both of these consortia were able to grow on cyanide containing mineral media at neutral pH values and metabolize cyanide at higher concentration and degrade 98.21% of cyanide in 96 h than the individual culture, which were able to degrade the same percentage at 144 h of incubation.

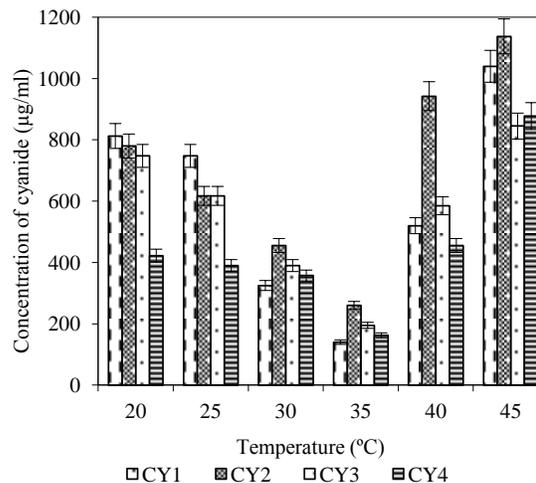


Fig. 4a: Effect of pH on removal of cyanide by the four isolates

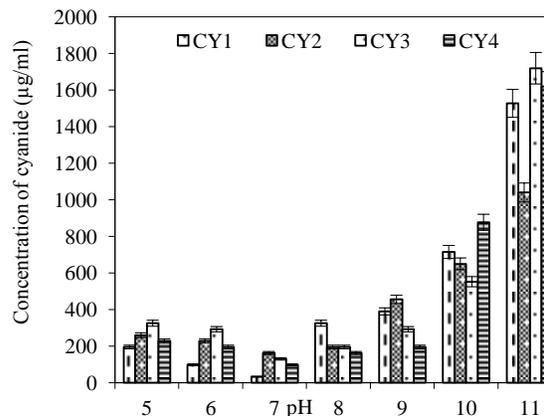


Fig. 4b Effect of temperature on removal of cyanide by the four isolates

A report [9] on the cyanide (as CN<sup>-</sup>) tolerance (but not growth) of fungi upto 60 mM concentration under neutral conditions including several *Fusarium sp.*, which, were grow on the iron cyanide complexes as the sole source of nitrogen. In the present study, the isolated bacterial strains of *Pseudomonas spp.* (CY3 and CY4) were able to tolerate cyanide up to 100 mM, whereas, the strain of *Bacillus spp.* (CY1) was able to tolerate up to 75 mM and the strain *Klebsiella spp.* (CY2) was able to tolerate only up to 25 mM concentration. The release of ammonia during cyanide degradation observed in the present study correlates well with the earlier reports [24,25]. According to them, cyanide is principally converted to formamide, formate and ammonia. The percentage release of ammonia was comparatively higher with mixed culture than with individual cultures.

**Table 1** Multiple comparison results for the test factors

S. No	Factor	Homogenous subsets by Tukey's HSD multiple comparison tests
1	Time (hrs) for residual cyanide concentration	144 120 96 72 48 24 0
2	Time (hrs) for ammonia concentration	24 48 72 144 190 120
3	pH	7 6 8 5 9 10 11
4	Temperature (°C)	35 30 25 40 20 45
5	Glucose concentration (µg/ml)	0.05 0.25 0.5 0.75 1.00
6	Fructose concentration (µg/ml)	0.05 0.25 0.5 0.75 1.00
7	Sucrose concentration (µg/ml)	0.05 0.25 0.5 0.75 1.00

All the factors were significant at P<0.05 level

It could be reasoned to the cometabolism, commensalism, synergistic and mutualistic effects among the bacterial species. Some bacterial strains were able to degrade cyanide and generate ammonia along with other kinetics such as carbon di oxide, formate, formamide etc [26]. The direct correlation between cyanide reduction and ammonia release are consistent with a number of the hydrolytic pathways known from aerobic organisms [27]. According to a report [28], the microbial consortium capable of utilizing metal cyanide as a source with the formation of ammonia and carbon di oxide as degradation products.

A research report [26] suggested that under anaerobic condition potassium cyanide complexes with reducing sugars present in the medium (Kiliani cyanohydrin's reaction) and the production of ammonia as a result of cyanide transformation by the cells in Nitrogen free glucose (NFG) medium suggests, cyanide could be used as an alternative nitrogen source for the growth of cells [29]. However, in the present study, aerobic conditions were provided and found growth of the isolates in 1 mM concentration of cyanide and reduction of cyanide and release of ammonia.

More than 50% of cyanide reduction irrespective of the species chosen with the individual species and more than 90% reduction with mixed consortium observed in the present study correlates well with the earlier report [8], where, the researchers showed 95% of cyanide reduction .

The influence of various parameters on the biodegradation of cyanide by the strain *Burkholderia cepacia* was reported previously [30]. The effect of pH on the hydrolysis of cyanide by resting cells and the intact cells exhibited maximum activity at pH 7.5 to 8.2 and only 35% activity at pH 9 [31]. In the present study, the maximum activity of above 90% degradation was observed at pH 7 – 8 and 75% at pH 9 irrespective of all the isolates. With regard to the influence of temperature, the optimum temperature for cyanide degradation was in the range between 35 to 40°C [16]. Similarly, in the present study, maximum degradation was observed at 35°C for the isolates chosen for the present study.

Studies on the influence of carbon sources on cyanide removal using selected carbon sources, glucose. Fructose and sucrose, demonstrate only at concentration 0.05% influences the degradation. Addition increase suppresses the degradation. The bacterium *Burkholderia cepacia* is able to consume cyanide optimally at pH 10 but it needs glucose as a carbon source and is relatively sensitive to metal ions, such as iron and copper [32]. With respect to metal ions, both Cu and Ca salts affect the growth as well as reduction of cyanide, whereas, Mg and Mn favours the growth and not influencing the cyanide reduction. The salt tolerance profile of the chosen isolates displayed all the isolates able to tolerate sodium chloride up to 3.0%.

## 5 Conclusions

The present study emphasizes degradation of cyanide in cassava industrial effluent by bacterial consortia. The degradation of cyanide quantity vary between the individual isolate and consortia and the bacterial consortia efficiently degraded the cyanide. The various parameters (temperature, pH, glucose, sucrose, fructose, sodium chloride) also influence the degradation of cyanide.

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