Growth and Cyanide Degradation of *Azotobacter vinelandii* in Cyanide-Containing Wastewater System

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*Azotobacter vinelandii*, a strict aerobic nitrogen-fixing bacterium, has been extensively studied with regard to the ability of N₂-fixation due to its high expression of nitrogenase and fast growth. Because nitrogenase can also reduce cyanide to ammonia and methane, cyanide degradation by *A. vinelandii* has been studied for the application in the bioremediation of cyanide-contaminated wastewater. Cyanide degradation by *A. vinelandii* in NFS (nitrogen-free sucrose) medium was examined in terms of cell growth and cyanide reduction, and the results were applied for cyanide-contaminated cassava mill wastewater. From the NFS medium study in the 300 ml flask, it was found that *A. vinelandii* in the early stationary growth phase could reduce cyanide more rapidly than the cells in the exponential growth phase, and 84.4% of cyanide was degraded in 66 h incubation upon addition of 3.0 mM of NaCN. The resting cells of *A. vinelandii* could also reduce cyanide concentration by 90.4% with 3.0 mM of NaCN in the large-scale (3 L) fermentation with the same incubation time. Finally, the optimized conditions were applied to the cassava mill wastewater bioremediation, and *A. vinelandii* was able to reduce the cyanide concentration by 69.7% after 66 h in the cassava mill wastewater containing 4.0 mM of NaCN in the 3 L fermenter. Related to cyanide degradation in the cassava mill wastewater, nitrogenase was the responsible enzyme, which was confirmed by methane production. These findings would be helpful to design a practical bioremediation system for the treatment of cyanide-contaminated wastewater.

Key words: *Azotobacter vinelandii*, bioremediation, cyanide, nitrogenase, wastewater

Cyanide is highly toxic to living organisms and particularly inactivates the respiration system by tight binding to cytochrome c oxidase. Cyanide is used in many industries such as chemical synthesis, pharmaceuticals, coal gasification, electroplating, plastics processing, gold and silver extraction, ore leaching, tanning, metallurgy, and agricultural chemistry [18]. Food processing industries handling crops like cassava and bitter almond also generate considerable quantities of cyanide waste from the cyanogenic glucosides in the plant material [1]. Because acute exposure to cyanide can cause death, and chronic exposure to even a low concentration can damage the thyroid and neuron system, the Environmental Protection Agency regulates its maximum tolerance level below 0.2 mg/l, and the Korean National Institute of Environmental Research requires a cyanide detection limit of 0.01 mg/l in drinking water.

Cyanide ion (CN⁻) and hydrogen cyanide (HCN) are often referred to as free cyanide. The relative amounts of both forms of free cyanide are mainly controlled by pH. The acid dissociation constant of HCN is 9.2 and most free cyanide in natural waters, which have a pH ranging between 6.0 and 8.5, is present as HCN. The percentage of HCN continues to increase as the pH drops, and at pH 7.0 about 99.5% of the cyanide exists as HCN, which is also highly soluble in water.

Currently, cyanide-containing wastewater is generally treated by chemical oxidation methods [17]. However, these methods are expensive, and hazardous chemicals of chlorine gas, permanganate, and ozone are used as the...
reagents. Alternatively, bioremediation using microorganisms would be an environmentally preferred method for cyanide removal, compared with other techniques currently in use [3]. *Azotobacter vinelandii* is a widespread Gram-negative, strict-aerobic, and free-living bacterium that fixes nitrogen through the action of nitrogenase [14]. This organism has evolved a number of physiological mechanisms to allow it to fix nitrogen aerobically, despite the inherent oxygen sensitivity of nitrogenase enzyme. The biochemical study of nitrogen fixation by *A. vinelandii* is presented in many literatures [4, 9], because *A. vinelandii* grows very fast aerobically and produces a large quantity of nitrogenase under diazotrophic conditions. Nitrogenase is a robust enzyme that can also reduce many small molecules, including H\(^+\), acetylene, HCN, SCN\(^-\), and N\(_2\) [4]. *Azotobacter* species have been used for biological treatments of olive-oil wastewater [6] and pulp-paper industry wastewater [8]. However, there are few studies of *A. vinelandii* for the degradation of cyanide from contaminated wastewater [10].

While we were working on the nitrogenase mechanism [12, 15], we noticed that no basic study on cyanide degradation by *A. vinelandii* has been done in the aspect of microbial biotechnology. Here we present cyanide degradation by *A. vinelandii* in NFS (nitrogen-free sucrose) medium in terms of cell growth and cyanide reduction. We also present a possible application in the bioremediation of cyanide-contaminated wastewater by means of the cassava-mill-wastewater model system.

**Materials and Methods**

**Microorganism**

*A. vinelandii* was supplied by Microbiological Resources Centre (MIRCECN) of Thailand Institute of Scientific and Technological Research (TISTR) (Bangkok, Thailand). The microorganism from stock culture was streaked to activate on slants of NFS agar medium, and incubated aerobically at 30°C for 3 days before use.

**Growth Medium and Inoculum Preparation**

An NFS medium consisting of 2.0% sucrose, 0.2 g/l MgSO\(_4\)-7H\(_2\)O, 0.073 g/l CaCl\(_2\)-2H\(_2\)O, 10 mM Na\(_2\)MoO\(_4\)-2H\(_2\)O, and 20 \(\mu\)M FeCl\(_3\)-6H\(_2\)O in pH 7.4 phosphate buffer (2.0 mM) was used in the experiments [16]. When it was necessary, 20 mM of urea was added as well. *A. vinelandii* grown on the slant was transferred in a cotton-wool-plugged 500 ml Erlenmeyer flask containing 300 ml of NFS medium. The culture was grown in a shaking incubator (New Brunswick Scientific, CT, USA) with 200 rpm at 30°C for 30 h, and the culture was used as a starter for cyanide biodegradation experiments.

**Cell Growth Determination**

Cell growth was determined by two different methods. The total cell count (direct microscopic cell counts) was determined using a hemacytometer (HBG Henneberg-Sander GmbH, Germany). Samples were centrifuged at 3,000 \(\times\) g for 5 min and then washed twice with 2 mM phosphate buffer (pH 7.4). Appropriated dilutions were used for the total cell count. Alternatively, the viable cell count was adopted by using the plate count technique. An appropriate dilution (0.1 ml of samples) was spread on NFS agar plates. The plates were incubated at 30°C for 48 h, and colony counts were enumerated for viable organisms. All the measurements were duplicated and the average values were shown in the data.

**Cyanide Degradation by Different Growth Phases of A. vinelandii**

After obtaining the growth curve, cell culture was harvested by centrifuging at 7,800 \(\times\) g and 4°C for 5 min, when the cells were grown to the mid-exponential (24 h) or the early-stationary phase (30 h). The supernatants were discarded and cell pellets were resuspended with NFS medium by adjusting the absorbance to 1.0 at 600 nm. NaCN was then added to the cell suspension to give the desired final concentrations. The cell suspensions were then incubated on a shaking incubator with 200 rpm at 30°C. NFS medium with the same concentrations of NaCN without *A. vinelandii* was also measured for the cyanide reduction and used as a control.

**Cyanide Analysis**

Cyanide was assayed by a modified ninhydrin method described by Drocchioi [5]. The sample was centrifuged at 3,000 \(\times\) g for 5 min, and 2.0 ml of diluted supernatant was mixed with 2.0 ml of ninhydrin solution. After 10 min incubation at room temperature, the absorbance was measured at 485 nm, using NaCN in the range of 0–150 ng/ml as a standard solution. All the measurements were duplicated and the average values were shown in the data.

**Fermenter Experiments**

The experimental set-up basically consisted of a 5 L fermenter (B. Braun Biotech International, Germany) with a 3 L working volume of NFS medium. The fermenter was sterilized by autoclaving at 15 psi for 15 min. Fermentation was maintained at an agitation rate of 200 rpm at 30°C. Aeration was performed with a sparger at the flow rate of 2 L/min. The gas scrubber was filled with 0.1 N NaOH placed at the exit of the air stream to trap the evaporation of HCN. When the cells were grown to the stationary phase (30 h), the aeration was stopped and NaCN was added to give final concentrations of 3.0, 3.5, and 4.0 mM, respectively, in the separated experiments. The cell growth and cyanide degradation were measured with the time intervals.

**Cassava Mill Wastewater Preparation**

The cassava mill wastewater was collected directly from a cassava mill processing site near Khon Kaen, Thailand. Samples were centrifuged at 5,000 \(\times\) g, for 10 min at 4°C. The supernatant from the centrifuged cassava mill wastewater was analyzed and used in this experiment. The major components in the wastewater such as cyanide concentration, ammonia, COD, BOD, total nitrogen, and total carbohydrate were analyzed using the standard method [2]. For the controlled experiments, cyanide in the cassava mill wastewater was eliminated by autoclaving at 15 psi for 15 min. Fermentation was maintained at an agitation rate of 200 rpm at 30°C. Aeration was performed with a sparger at the flow rate of 2 L/min. The gas scrubber was filled with 0.1 N NaOH placed at the exit of the air stream to trap the evaporation of HCN. When the cells were grown to the stationary phase (30 h), the aeration was stopped and NaCN was added to give a final concentration of 4.0 mM. The cell growth and cyanide degradation were measured with the time intervals.