Biotransformation of Cyanide by Native Bacteria from Sago Wastewater Under Anaerobic Conditions

Sujatha Kandasamy and Kumar Krishnamoorthy*

Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore - 641 003, India.

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The efficiency of anaerobic digestion is closely tied to the structure of its microbial community. Determination of microbial dynamics at different depths in biogas plants from sago industries showed the population of total heterotrophic bacteria \((58.4 \times 10^6 \text{ cfu/ml})\), fungi \((10.1 \times 10^3 \text{ cfu/ml})\), fermenting bacteria \((1.4 \times 10^9 \text{ MPN/ml})\), starch hydrolyzing- \((19.5 \times 10^5 \text{ cfu/ml})\) and cyanide degrading \((11.6 \times 10^3 \text{ cfu/ml})\) bacteria were dominant in the bottom zone, denitrifiers \((9.4 \times 10^6 \text{ MPN/ml})\) in the middle and methanogens were recorded higher \((4.8 \times 10^6 \text{ MPN/ml})\) at the top of biogas plants. This implied that the composition of wastewater could affect the evolution of quantitative community structure in an anaerobic process. With the study of native cyanide degrading bacteria, six facultative anaerobic bacteriaviz., \(P. \) putida, \(B. \) anthracis, \(B. \) cereus C1, \(B. \) cereus C2, \(S. \) maltophilia and \(B. \) weihenstephanensis were found to metabolize cyanide into ammonia and formate via a hydrolytic pathway. Higher growth in terms of optical density and a maximum cyanide removal was obtained with \(S. \) maltophilia. Ammonia production and glucose consumption were well correlated with disappearance of cyanide.

Key words: Sago wastewater, Biogas plants, Anaerobic conditions, cyanide

There is a growing interest in alternate energy sources as a result of increased demand for energy coupled with a rise in the cost of available fuels. Rapid industrialization has resulted in the generation of a large quantity of effluents with high organic contents, which if treated suitably, can result in a perpetual source of energy. In recent years, considerable attention has been paid towards the development of anaerobic reactors for treatment of wastes leading to the conversion of organic molecules into biogas. Great advances have been made over the last 20 years in anaerobic reactor design and in understanding the complex processes. Anaerobic technology for the treatment of wastes was known in India from the beginning of the 20th century. Presently in India 12 million biogas plants exists which generates \(\text{CH}_4\) during anaerobic organic digestion with the advantage of low sewage sludge production. It is a kind of flow through system with gas collector and suggested retention time of 15 days. It is known that potentially, all organic waste materials contain adequate quantities of the nutrients essential for the growth and metabolism of the anaerobic bacteria in biogas production. This biogas is a renewable high-quality energy source that should be explored, particularly in developing countries where energy is costly and is much needed for developmental activities.

Cassava processing is an important agroindustry with considerable importance in southern states of India, especially Tamil Nadu. By nature, cassava processing for starch extraction
produces large amounts of effluent high in organic content, as a result of the dissolution of organic compounds that take place during the crushing steps. The high organic content presents in the cassava processing wastewater makes it especially suitable for processes based on anaerobic technologies. At present, there are more than 100 units producing biogas using Tarpaulin cover over the conventional anaerobic lagoons and utilizing the biogas produced for roasting of sago and for generation of electrical energy. The sago factories in Salem districts release the wastewater in short periods, because of lacking biogas plant capacity. Hence, the present study was determined to study the microbial community structure in the biogas plants and anaerobic cyanide degradation by native bacterial isolates under laboratory conditions.

**MATERIALS AND METHODS**

**Sampling**

The sago wastewatersamples were obtained at different depths (top, middle and bottom) of the biogas plants from the cassava processing industries located in Salem districts of Tamil Nadu, India. The physico-chemical characteristics and microbial dynamics of the wastewater were determined according to APHA.2

**Enrichment and isolation**

Cyanide degrading microorganisms were enriched from wastewater collected from biogas plants. The enrichment and isolation medium (MSM) consisted of (g/L): K2HPO4 · 2H2O 1.0, MgSO4·7H2O 0.2, CaCl2·2H2O 0.01, NaCl 0.01, MnSO4·4H2O 0.02, CuSO4·5H2O 0.2 and ZnSO4·7H2O 0.2 with pH 7.0. Cyanide (1 mM NaCN) and glucose (2 g/l) were filter sterilized (0.2 µm; Sartorius, Germany) before adding to medium. About 1 ml wastewater was added to 100 ml MSM medium and incubated at 28°C, 120 rpm. The enrichment cultures were subcultured thrice in MSM before plating on MSM-agar plates. Distinct colonies were purified on nutrient agar and maintained at 4°C.

**Biodegradation of cyanide**

For anaerobic studies, MSM composed of (g/L): NaCl 1.0, MgSO4·7H2O 0.486, CaCl2·2H2O 0.15, KCl 0.5, KH2PO4 0.2 and NH4Cl 0.25 respectively, 1 ml of each trace element solution [Na2·EDTA 5200, FeSO4·7H2O 2100, CoCl2·6H2O 190, ZnSO4·7H2O 144, MnCl2·4H2O 100, Na2MoO4·2H2O 36, H3BO3 30, NiCl2·6H2O 24, CuSO4·5H2O 29 (mg/L)], Vitamin mixture [Pyridoxine-dihydrochloride 15, Nicotinic acid 10, Calcium-D (+) pantothenate 5, 4-aminobenzoic acid 4, D(+)-Biotin 1 (mg/100 ml)], Thiamine, Riboflavin, Vitamin-B12, glucose (1 mM) and cysteine (1 mM) were added under a constant stream of N2/CO2. The head space was then changed to N2/CO2 (80:20 [vol:vol]) and 30 ml of sodium bicarbonate was added and pH was adjusted to 7. Finally, sodium sulfide (5 ml) and cysteine (1 ml) was added by means of N2-flushed sterile syringe. The medium (40 ml) was dispensed, via glass dispenser into 100 ml sterile serum bottles. In each case, the head space was gassed with N2/CO2 (80:20 [vol:vol]) and bottles were tightly sealed with rubber stoppers. Individual bacterial isolates grown in nutrient media (A660=1) were inoculated into serum bottles and incubated at 37°C. Determination of optical density, cyanide, ammonia, glucose and formate concentration was estimated after 15 days after incubation.

**Analytical methods**

**Growth**

Cell growth was monitored by determining the optical density (O.D) of 1 ml culture at 660 nm by Spectrophotometry (GENESYS 10 UV-Vis Scanning, Thermo Scientific) and expressed as OD660 nm.

**Cyanide**

The cyanide content in MSM was determined as follows: aliquots (0.05 ml) of cyanide solutions (centrifuged at 15,000 g, 10 min, 4°C) were added to 0.1 ml solution containing 0.5% (w/ v) picric acid and 0.25 M Na2CO3 and placed in a boiling water-bath (5 min), diluted to 1 ml and cooled for 30 min. The absorbance was read at 520 nm against a blank of distilled water and picric acid reagent.

**Ammonia determination**

Aliquot (2.5 ml) of sample (after centrifugation at 15,000 rpm for 10 min at 4°C) was added to 1.25 ml of Na-nitroprusside solution and vortexed briefly for a few seconds. To this, 500 µl of dichloroisocyanurate was added and samples were incubated for 30 min in the dark. The absorbance was measured at 625 nm using ammonium chloride as standard.
**HPLC analysis**

Glucose and formate were assayed by HPLC (Sykam, Gilching, Germany), equipped with an HPX-87 P ion-exchange column Aminex (Bio-Rad Lab, USA). The mobile phase was 5 mM H$_2$SO$_4$ with the flow rate 0.8 ml/min. The column was maintained at room temperature and the absorbance was measured at 210 nm. All samples were filter-sterilized (0.2 µm pore size) to remove cells and other particles before analysis.

**Identification of the bacterial isolates**

Identification of the strains was carried out by using 16S rRNA gene sequencing method. The 16S rRNA gene was amplified and sequenced using universal primers forward MF: 5’-GAG TTT GAT CMT GGC TCA G-3’ and reverse MR: 5’-ACG GYT ACC TTG TTA CGA CTT-3’ and the PCR products were purified using spin columns according to the manufacturer’s instructions (Qiagen, Germany). Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer.

**RESULTS AND DISCUSSION**

**Dynamics of microbial community in biogas plants**

The efficiency of anaerobic digestion is closely tied to the structure of its microbial community. In the anaerobic process, a complex mixture of interacting microorganisms, mainly bacteria, carries out the complete degradation of organic materials to biogas. The decomposition of the complex organic compounds occurs in a 3 stage process, where 3 main groups of microorganisms are activating independently viz., fermentative, proton reducing acetogenic and methanogenic bacteria.

In the fermentative stage, organic materials (protein, cellulose, lipid, and starch) were broken down by fermentative microorganisms to lower molecular weight molecules. The second stage was acid-forming stage, in which products from the first stage were converted by acetogenic bacteria (acetate and H$_2$-producing bacteria) into acetate, hydrogen gas, carbon dioxide and few other volatile fatty acids such as propionic- and butyric acid. The third stage was methanogenic stage, where volatile fatty acids are known to be important intermediates in the degradation of organic matter. The methanogens produce methane, carbon dioxide, trace gases (e.g., H$_2$S), and water. It was almost that 70 per cent of methane was formed from acetate and the rest was formed from carbon dioxide and hydrogen.

Biogas production processes from cassava starch effluent was started with hydrolysis of the complex organic materials by fermentative bacteria to simple organic material. Accordingly, in the present study, the population of total heterotrophic bacteria (58.4 x 10$^6$ cfu/ml), fungi (10.1 x 10$^3$ cfu/ml), fermenting bacteria (1.4 x 10$^{10}$ MPN/ml), starch hydrolyzing (19.5 x 10$^5$ cfu/ml) and cyanide degrading (11.6 x 10$^3$ cfu/ml) bacteria were found to be dominant in the bottom zone of the biogas plants. Nimaradee explained that accumulation of suspended solid mainly occurs near the inlet zone of ponds, where complex organic matter was fed into the reactor, was an area of intense enzyme catalytic activity for the fast growing hydrolytic- and fermentative non-methanogens. Most of the complex organic matter was broken down into soluble monomers to support the growth of the non-methanogens, and then the soluble monomers must be converted into volatile fatty acids. The initial high bacterial load of the slurries may be due to the fact that large populations of aerobic- and facultative anaerobic organisms are usually involved in the hydrolytic- and acidogenic stages whereas only strict or obligate anaerobes are involved in the methanogenesis stage.

The denitrifiers which can grow in the presence of high nitrate concentrations were observed to be maximum (9.4 x 10$^6$ MPN/ml) in the middle of biogas plants. Finally methanogenic bacteria became established in biomethanation tank and use the end product from the acid forming bacteria to produce methane. The methanogens were recorded higher (4.8 x 10$^6$ MPN/ml) at the top of the biogas plants. A possible explanation is that high pH might have favoured methanogenic activity. The dominance of different species in different environments are considered to be a reflection of their different survival strategies.

**Biodegradation of cyanide**

Adaptation to low cyanide concentrations can result in successful treatment of anaerobic systems, but there has been only little evidence...
Table 1. Microbial population at three different depths of biomethanation plants from a medium scale sago factory in Salem district

<table>
<thead>
<tr>
<th>Microbial population</th>
<th>Medium BB</th>
<th>Medium AB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Top</td>
<td>Middle</td>
</tr>
<tr>
<td>cfu/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total aerobic bacteria (x 10^n)</td>
<td>9.6 (±0.55)</td>
<td>15.1 (±0.87)</td>
</tr>
<tr>
<td>Starch degrading bacteria (x 10^5)</td>
<td>11.4 (±0.66)</td>
<td>2.6 (±0.15)</td>
</tr>
<tr>
<td>Cyanide tolerating bacteria (x 10^3)</td>
<td>4.9 (±0.28)</td>
<td>1.12 (±0.01)</td>
</tr>
<tr>
<td>Fungi (x 10^3)</td>
<td>15.5 (±0.89)</td>
<td>3.4 (±0.20)</td>
</tr>
<tr>
<td>MPN/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fermenting bacteria</td>
<td>1.1 (±0.05) x 10^7</td>
<td>1.1 (±0.08) x 10^10</td>
</tr>
<tr>
<td>Denitrifying bacteria</td>
<td>6.9 (±0.36) x 10^2</td>
<td>3 (±0.17) x 10^5</td>
</tr>
<tr>
<td>Methanogenicarchaea</td>
<td>2.5 (±0.16) x 10^2</td>
<td>4.8 (±0.23) x 10^6</td>
</tr>
</tbody>
</table>

Values are mean ± SE of three replicates. BB – Before Biomethanation; AB - After Biomethanation.

Table 2. Growth, cyanide and glucose content, ammonium and formate production by the bacterial isolates in mineral salts broth containing cyanide and glucose at 1 mM concentration each under anaerobic conditions

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Growth (O.D. at 660 nm)</th>
<th>Cyanide (mM)</th>
<th>Ammonia (mM)</th>
<th>Formate (mM)</th>
<th>Residual Glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 1</td>
<td>0.005f</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD 2</td>
<td>0.002f</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD 3</td>
<td>0.072c</td>
<td>0.051f</td>
<td>0.885</td>
<td>2.012</td>
<td>0.342</td>
</tr>
<tr>
<td>CD 4</td>
<td>0.043c</td>
<td>0.091</td>
<td>0.736</td>
<td>0.280</td>
<td>0.400</td>
</tr>
<tr>
<td>CD 5</td>
<td>0.087</td>
<td>(±0.01)f</td>
<td>(±0.04)b</td>
<td>(±0.02)f</td>
<td>(±0.02)f</td>
</tr>
<tr>
<td>CD 6</td>
<td>0.008c</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD 7</td>
<td>0.049b</td>
<td>0.064c</td>
<td>0.861</td>
<td>1.288</td>
<td>0.358</td>
</tr>
<tr>
<td>CD 8</td>
<td>0.145</td>
<td>0.018</td>
<td>0.916 (±0.05)c</td>
<td>1.507</td>
<td>0.258</td>
</tr>
<tr>
<td>CD 9</td>
<td>0.097</td>
<td>(±0.01)c</td>
<td>(±0.05)c</td>
<td>(±0.09)b</td>
<td>(±0.01)c</td>
</tr>
<tr>
<td>CD 10</td>
<td>0.004f</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Minus (-) sign indicates not estimated. Values are mean ± SE of three replicates.

In column, means having the same superscript letters are on par at $p \leq 0.05$ level by Duncan’s multiple range test.
isolates from sago wastewater was evaluated under anaerobic conditions. Out of ten cyanide degrading bacteria isolates from enrichment samples, six isolates (facultative anaerobes) viz., CD3, CD4, CD5, CD7, CD8 and CD9 recorded positive growth in MSM with 1 mM cyanide under anaerobic conditions, with highest growth (0.145) as evinced by OD value in S. maltophila. This suggests that the cyanide-degrading ability of bacterial isolates can be induced by cyanide acclimation process. For example, the cyanide degrading activity of P. fluorescens and cyanide metabolism of A. xylosoxidans subsp. denticrificans DF3 could be induced by cyanide during growth. The cyanide oxygenase and cyanide nitrilase/hydrazine in P. fluorescens, was induced in the cyanide-containing medium. The bacterial isolates showing growth were subjected to the measurement of cyanide and glucose content, ammonia and formate production (Table 2).

A significant cyanide reduction in the media was observed with all the positive isolates. The cyanide content varied between a minimum of 0.018 mM with S. maltophila and to a maximum of 0.091 mM with B. anthracis. Cyanide concentrations in medium without inoculation of bacterial cells remained consistent throughout the experimental process. This indicates that cyanide volatilization might not be significantly enough to cause the variation in cyanide concentration in this experiment. Thus, the reduction of cyanide concentration in sealed bottles containing bacterial cells was mainly due to biodegradation process.

Fallon demonstrated that under anaerobic conditions, cyanide is hydrolysed to form ammonia and formate, which is subsequently converted to bicarbonate. Accordingly, all these facultative anaerobes metabolize the cyanide and convert into ammonia and formate indicating a hydrolytic pathway without an intermediate, formamidewhich was not found in culture supernatant. The quantity of ammoniaproduced varied between 0.736 to 0.916 mM. Kunz proposed that the produced ammonia might be rapidly metabolized during the growth of bacteria, resulting in the immediate disappearance of ammonia. Among the isolates, maximum ammonia (0.916 mM) was produced by S. maltophila, while minimum (0.736 mM) with B. anthracis. The isolate P. putida was found to produce a maximum formate of 2.012 mM and a minimum of 0.280 mM with B. anthracis. The glucose concentration exhibited variation from 0.258 mM to 0.400 mM. The maximum glucose utilization was observed with S. maltophila (0.258 mM) and minimum with B. anthracis (0.400 mM). Ammonia production and the glucose consumption were well correlated with the disappearance of cyanide. The accumulation of formate in the medium resulted in the degradation of both NaCN and glucose.

Molecular analysis based on 16S rRNA gene sequencing revealed that isolate CD 3 and CD 4 was closely related to Pseudomonas putida (99 per cent) and Bacillus anthracis (98 per cent). The isolates CD 4, CD 5 and CD 7 showed maximum similarity with Bacillus cereus to about 98 per cent and Bacillus anthracis (98 per cent) each respectively. The isolates CD 8 and CD 9 showed the highest similarity to Stenotrophomonas maltophilia (98 per cent) and Bacillus weihenstephenensis (99 per cent) respectively.

REFERENCES


