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

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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 showedthe population of total heterotrophic bacteria (58.4 x 10^6 cfu/ml), fungi (10.1 x 10^3cfu/ml), fermenting bacteria (1.4 x 10^{10} MPN/ml), starch hydrolyzing-(19.5 x 10^5cfu/ml) and cyanide degrading (11.6 x 10^3cfu/ml) bacteria were dominant in the bottom zone, denitrifiers (9.4 x 10^6 MPN/ml) in the middle and methanogens were recorded higher (4.8 x 10^6 MPN/ml) at the top of biogas plants. This implied that the composition of wastewater could affect the evolution ofquantitative community structure in an anaerobic process. With the study of native cyanide degrading bacteria, sixfacultative anaerobic bacteriaviz., *P. putida, B. anthracis, B. cereus C1, B. cereus C2, S. maltophila* and *B. weihenste phenensis* were found to metabolize cyanideinto ammonia and formatevia a hydrolytic pathway. Higher growth interms of optical density and a maximum cyanide removal was obtained with *S. maltophila*. 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[1]. 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 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[2].

Cassava processing is an important agroindustry withconsiderable importance in southern states of India, especially TamilNadu. By nature, cassava processing for starch extraction

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produceslarge amounts of effluent high in organic content, as aresult of the dissolution of organic compounds that takesplace during the crushing steps. The high organic contentpresent in the cassava processing wastewater makes itespecially suitable for processes based on anaerobic technologies[3]. 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 or for generation of electrical energy. The sago factories in Salem districts release the wastewater in short periods, because of lacking biogas plant capacity[4]. 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 dynamicsof the wastewater were determined according to APHA[5].

Enrichment and isolation

Cyanide degrading microorganisms were enriched from wastewater collected from biogas plants. The enrichment and isolation medium (MSM) consisted of (g/L): $K_2HPO_4.2H_2O$ 1.0, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.01, NaCl 0.01, $MnSO_4.4H_2O$ 0.2, $CuSO_4.5H_2O$ 0.2 and $ZnSO_4.7H_2O$ 0.2 with pH 7.0. Cyanide (1 mMNaCN) 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, MSMcomposed of (g/L): NaCl 1.0, $MgSO_4 \cdot 7H_2O$ 0.486, $CaCl_2 \cdot 2H_2O$ 0.15, KCl 0.5, KH_2PO_4 0.2 and NH_4Cl 0.25 respectively,1 ml of each trace element solution[Na_2.EDTA 5200, $FeSO_4.7H_2O$ 2100, $CoCl_2.6H_2O$ 190, $ZnSO_4.7H_2O$ 144, $MnCl_2.4H_2O$ 100, $Na_2MoO_4.2H_2O$ 36, H_3BO_3 30, $NiCl_2.6H_2O$ 24, $CuCl_2.2H_2O$ 2 and $CuSO_4.5H_2O$ 29 (mg/L)], Vitamin mixture[Pyridoxine-dihydrochloride 15, Nicotinic acid 10, Calcium-D (+)pantothenate5, 4-aminobenzoic acid4, D(+) Biotine1 (mg/100 ml)] Thiamine, Riboflavin, Vitamin-B_{12}, glucose (1mM) and cyanide (1 mM) were added under a constant stream of N_2/CO_2. 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 N_2-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 N_2/CO_2 (80:20 [vol:vol] and bottles were tightly sealed with rubber stoppers. Individual bacterial isolates grown in nutrient media (A_{660}=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

Cellgrowth 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 OD_{660} nm.

Cyanide

The cyanide content in MSM was determinedas 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 Na_2CO_3 andplaced 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[6].

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_2SO_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'[7] 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 micro-organisms are activating independently viz., fermentative, proton reducing acetogenic and methanogenic bacteria[8]

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_2S), and water[9].It was almost that 70 per cent of methane was formed from acetate and the rest was formed from carbon dioxide and hydrogen[10].

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 (58.4×10^6 cfu/ml), fungi (10.1×10^3cfu/ml), fermenting bacteria (1.4×10^{10} MPN/ml), starch hydrolyzing- (19.5×10^5cfu/ml) and cyanide degrading (11.6×10^3 cfu/ml) bacteria were found to be dominant in the bottom zone of the biogas plants. Nimaradee[11]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[12].

The denitrifiers which can grow in the presence of high nitrate concentrations were observed to be maximum (9.4×10^6 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[10]. The methanogens were recorded higher (4.8×10^6 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[13].

Biodegradation of cyanide

Adaptation to low cyanide concentrations can result in successful treatment of anaerobic systems, but there has been only little evidence

for anaerobic biological breakdown of cyanide. Applications of anaerobic digestion for cassava wastewater treatment show that among the anaerobic processes, methanogenesis is most sensitive to cyanide toxicity[14]. Gijzen[15] demonstrated that the effect of CN-inhibition on methanogenic activity was more pronounced for aceticlastic- than hydrogenotrophic methanogens. A possible site of inhibition is formed by the metabolically important metalloproteins in these organisms. The observation of cyanide sensitivity of methanogens has resulted in minimal attention for anaerobic treatment of wastewater containing this compound. Fallon[16] observed a strong correlation between microbial activity and cyanide removal in anaerobic digestion and suggested that the removal mechanism depends on microbial activity.

In this study, enhancement of cyanide degradation by the acclimation of native bacterial

Table 1. Microbial population at three different depths of biomethanation plants from a medium scale sago factory in Salem district

Microbial population	Medium BB	Medium AB		
		Top	Middle	Bottom
cfu/ml				
Total aerobic bacteria (x 10^6)	9.6 (±0.55)	15.1 (±0.87)	21.5 (±1.24)	58.4 (±0.42)
Starch degrading bacteria (x 10^5)	11.4 (±0.66)	2.6 (±0.15)	4.3 (±0.25)	19.5 (±0.55)
Cyanide tolerating bacteria (x 10^3)	4.9 (±0.28)	1.12 (±0.01)	2.07 (±0.06)	11.6 (±0.21)
Fungi (x 10^3)	15.5 (±0.89)	3.4 (±0.20)	3.2 (±0.18)	10.1 (±0.58)
MPN/ml				
Fermenting bacteria	1.1 (±0.05) x 10^7	1.1 (±0.08) x 10^{10}	1.1 (±0.06) x 10^{10}	1.4 (±0.09) x 10^{10}
Denitrifying bacteria	6.9 (±0.36) x 10^2	3 (±0.17) x 10^5	9.4 (±0.52) x 10^6	6.9 (±0.31) x 10^2
Methanogenicarchaea	2.5 (±0.16) x 10^2	4.8 (±0.23) x 10^6	6.9 (±0.40) x 10^5	2.5 (±0.16) x 10^2

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

Isolates	Growth (O.D. at 660 nm)	Cyanide (mM)	Ammonia (mM)	Formate (mM)	Residual Glucose (mM)
CD 1	0.005^{f}	-	-	-	-
CD2	0.002^{f}	-	-	-	-
CD3	0.072^{c}	0.051^{d}	0.885 (±0.05)^{a}	2.012 (±0.12)^{a}	0.342 (±0.02)^{b,c}
CD4	0.043^{e}	0.091 (±0.01)^{f}	0.736 (±0.04)^{b}	0.280 (±0.02)^{g}	0.400 (±0.02)^{d}
CD5	0.087 (±0.01)^{d}	0.039 (±0.01)^{c}	0.892 (±0.05)^{a}	0.478 (±0.03)^{f}	0.279 (±0.02)^{a,b}
CD6	0.008^{c}	-	-	-	-
CD7	0.049^{b}	0.064^{e}	0.861 (±0.05)^{a}	1.288 (±0.07)^{c}	0.358 (±0.02)^{c,d}
CD8	0.145 (±0.01)^{a}	0.018 (±0.01)^{a}	0.916 (±0.05)^{a}	1.507 (±0.09)^{b}	0.258 (±0.01)^{a}
CD9	0.097 (±0.01)^{a,b}	0.027 (±0.01)^{b,c}	0.897 (±0.05)^{a}	0.461 (±0.03)^{f}	0.261 (±0.02)^{a}
CD10	0.004^{f}	-	-	-	-

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.

isolatesfrom 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, CD8and CD9 recorded positive growth in MSM with 1 mMcyanide 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*[17]and cyanide metabolism of *A. xylosoxidans*subsp. *dentrificans* DF3[18] could be induced by cyanide during growth. The cyanide oxygenase and cyanide nitrilase/hydratase 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[16] 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[19] 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 producedby*S. maltophila*, while minimum (0.736 mM) with *B. anthracis*. The isolate *P. putida* was found to produce a maximum formateof 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[20].

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 *Stenotrophomonasmaltophila* (98 per cent) and *Bacillus weihenstephenensis* (99 per cent). respectively.

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