

STUDIES ON HYDROLYTIC BACTERIA FROM VEGETABLE WASTE, CATTLE DUNG SLURRY AND BIOGAS DIGESTER EFFLUENT

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ABSTRACT

Biomethanation is the anaerobic digestion of biodegradable organic matter in an enclosed space under controlled operational conditions to produce biogas and manure. Biomethanation is considered as eco-friendly microbiological treatment method for management of different kinds of agro-industrial wastes. The types of microorganisms involved in biogas production process are mainly bacteria, yeast, molds and actinomycetes. Hydrolytic microorganisms play an important role in initial step of biomethanation. The present paper deals with the study of bacteria from vegetable waste slurry, cattle dung slurry and biogas digester effluent. The biomethanation experiment was carried out in 5 litre capacity digester under ambient temperature conditions. Standard plate count of bacteria from vegetable waste slurry, cattle dung slurry and digester effluents were found to be 3.27×10^7 , 2.44×10^9 and 2.25×10^{11} CFU/mL respectively. In present study sixteen, twelve and seventeen bacteria were isolated and identified from vegetable waste slurry, cattle dung slurry and digester effluent respectively using standard methods. Further all the bacterial isolates were tested for their potential to produce hydrolytic enzymes. The present study has provides useful information about the bacteria associated with substrates and digester effluent that have important role in biogas generation. Further, the potent hydrolytic bacterial isolates can be used to develop consortia to increase the efficiency of vegetable waste and other agro-industrial organic wastes based biomethanation process and also for its composting.

Key Words : Vegetable waste, Environmental pollution, Biomethanation, Bacteria, Hydrolytic enzymes

INTRODUCTION

India being one of the largest vegetables producer, the large amount of vegetable waste is also generated in vegetable markets, in the municipal corporations, municipalities and villages in India. The considerable amount of vegetable waste is also generated in kitchens, hotels and restaurants. Out of 320 million tones of agricultural wastes generated in India annually, the bulk is constituted by vegetable waste¹. The vegetable waste contains high moisture and is putrescible. The collection, transportation and disposal of vegetable waste is a very serious problem today. The present

unscientific treatment methods results in environmental pollution². The biomass of vegetation can be used through chemo-thermal processes (combustion, gasification and pyrolysis) or biochemical methods. The one of the best biochemical process in the vegetable waste management is anaerobic digestion which produces biogas production and leachate of high fertilizer value that have potential to replace conventional energy source and chemical fertilizers, respectively³. The biomethanation being ecofriendly, play significant role in maintaining the balance in the environment^{2,4}.

Biomethanation is accomplished by a consortium of microorganisms working synergistically. Aerobic, facultative anaerobic and obligate

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anaerobic microflora are responsible for transformation of organic matter into biogas. Generally four main reactions occur during the entire process of the biomethanation: hydrolysis, acidogenesis, acetogenesis and methanogenesis⁵. The hydrolysis step degrades both insoluble complex organic matter and high molecular weight compounds into soluble monomers⁵. This reaction is catalyzed by extracellular enzymes (amylase, proteases, lipases, cellulases and ligninases) produced by hydrolytic and fermentative microorganisms. There are several reports on isolation of hydrolytic fungi from biogas digester run on different agro-industrial organic residues⁶⁻⁹. Bacteria also have been isolated from biogas digester run on different agro-industrial organic wastes^{7,9,10-12}.

OBJECTIVES

The objectives of the present study were to isolate and identify bacteria associated with vegetable waste slurry, cattle dung slurry and effluent of biogas digester and to determine their potential to produce hydrolytic enzymes.

MATERIAL AND METHODS

Collection and preparation of samples

Vegetable waste collected from local vegetable market was segregated into individual vegetable waste types. The equal proportions of dominating individual wastes were used to prepare a paste and stored at 4°C. The physico-chemical analysis of vegetable waste was determined according to standard methods¹³.

Biomethanation of vegetable waste at 5 litre (L) level

Biomethanation study was carried out in a floating dome design type of 5 L capacity locally fabricated digesters. Inoculum was obtained from an active mesophilic digester of cattle dung based biomethanation plant. The 5 L digesters were operated at organic loading rate (OLR) 0.320 g VS/l.d and pH 7.0 of substrate under ambient temperature conditions with two cycles of 20 days hydraulic retention time (HRT). The volumes of biogas were recorded daily. Combustibility testing and % methane were also determined.

Standard plate count (SPC) and Isolation of bacteria from vegetable waste, cattle dung slurry and digester effluent

A one mL well mixed portions of the vegetable waste slurry, cattle dung slurry and digester effluent were subjected to serial dilutions and

SPC for bacteria was determined in triplicates using standard plate count agar. Plates were incubated at room temperature (R.T.) for 24-48 hrs and then SPC was calculated. The representative bacterial isolates were preserved on respective media at 4°C.

Demonstration of enzymatic capabilities of bacterial isolates

Starch agar, Skim milk agar, Gelatin agar, Tributyrin agar, Carboxy Methyl Cellulose (CMC) agar and Mineral salt medium (MSM) agar containing lignin with methylene blue as indicator dye were used for determining amyolytic, caseinolytic, gelatinolytic, lipolytic, cellulolytic and ligninolytic potential of bacterial isolates respectively. The individual cultures were spot inoculated onto respective media plates and incubated at R.T. for 48-72 hours. Amyolytic activity was detected by exposing the starch agar plates to iodine crystals for 5 minutes. Skim milk agar plates and tributyrin agar plates were observed for clear zones around growth. Gelatin agar plates were flooded with Fraziers reagent to observe clear zones. Cellulolytic activity was detected by flooding the plates with 1% congo red for 20 minutes at R.T. followed by washing with 1M sodium chloride solution. A clear zone around the growth against the dark red background was taken as indication of cellulase activity¹⁴. The MSM-lignin agar plates were observed for decolorization around the bacterial growth¹⁵.

Identification of bacterial isolates

The bacteria from vegetable waste slurry, cattle dung slurry and digester effluents were identified to the species level according to characteristics described in Bergey's Manual of Systematic Bacteriology¹⁶⁻²⁰.

RESULTS AND DISCUSSION

Potato (*Solanum tuberosum* L.), Onion (*Allium cepa* L.), Cabbage (*Brassica oleracea* L. var. capitata), Cauliflower (*Brassica oleraceae* L. var. botrytis), Tomato (*Lycopersicon esculentum* Mill.) and Brinjal (*Solanum melongena* L.) wastes dominated the composition of vegetable waste. The paste prepared from these wastes was used for biomethanation study. The moisture content of the waste was found to be 89.50%. The chemical oxygen demand and biochemical oxygen demand were 174000 mg/kg and 97150 mg/kg respectively. Starch, cellulose, hemicelluloses, lignin, fat and proteins were present in 9900 mg/kg, 8700 mg/kg, 2400

mg/kg, 2200 mg/kg, 4000 mg/kg and 9081 mg/kg respectively. The high moisture and carbohydrate content indicated its suitability for biomethanation.

The range of biogas produced was 510-1340mL/d and average amount of biogas was 0.633L/g VS added/d. The biogas burning test showed blue flame indicating rich methane content in the biogas. Gas chromatographic analysis of biogas revealed 59 % methane.

The bacterial count of the digester effluent was significantly higher than those of vegetable waste slurry and cattle dung slurry. The average bacterial counts from vegetable waste, cattle dung slurry and digester effluent were 3.27×10^7 , 2.44×10^9 and 2.25×10^{11} CFU/mL respectively. The bacteria isolated from the slurry, could be the normal inhabitants on the surface of the vegetable waste, cattle dung slurry and some coming as contaminants from environment.

Sixteen bacteria were isolated and identified from vegetable waste slurry. Out of 16 bacterial isolates, eight isolates were Gram positive (50%) and remaining 8 were Gram negative (50%). Amongst the Gram positive isolates *Bacillus species* was in abundance, which was 7 in number and included *B. amyloliquefaciens*, *B. cereus*, *B. circulans*, *B. megaterium*, *B. coagulans*, *B. mycoides* and *B. sphaericus*. The remaining Gram positive isolate was *L. plantarum Subsp plantarum*. Gram negative isolates were found to be *P. aeruginosa*, *P. alcaligenes*, *Proteus vulgaris*, *Brevundimonas diminuta*, *Enterobacter aerogenes*, *Serratia marcescens*, *Flavobacterium aquatile* and *Flavobacterium indicum*. Most of these bacteria were hydrolytic in nature. They also showed fermentation of glucose, trehalose, xylose, mannitol and sucrose. Many of them showed hydrolysis of starch, cellulose, lecithin, gelatin and arginine, while others showed production of catalase, oxidase and nitrate reductase enzymes.

The bacterial isolates obtained from cattle dung slurry were 12 in number including 8 Gram positive (67%) and 4 Gram negative (33%) members. Among the eight Gram positive isolates, 3 were species of genus *Bacillus* indicating its abundance in cattle dung slurry. In the remaining 5 Gram positive isolates were two

Lactobacillus species and one from each *Micrococcus*, one *Kocuria species* and one *Arthrobacter species*. Gram positive isolates included *Bacillus amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, *L. brevis*, *L. helveticus*, *Micrococcus luteus*, *Kocuria rosea* and *Arthrobacter globiformis*. The Gram negative bacteria included *P. fluorescens*, *P. putida*, *Brevundimonas diminuta* and *Klebsiella pneumoniae*. Many of these bacteria were hydrolytic in nature, which showed hydrolysis of starch, cellulose, lignin, casein, gelatin and lipids. Thus, bacterial isolates of all these inoculum materials showed a variety of hydrolytic members, which were important in biomethanogenesis.

The bacterial isolated from digester effluent were seventeen of which 10 were Gram positive (59%) and seven were Gram negative isolates (41%). The results showed abundance of Gram positive bacteria. Among the 10 Gram positive isolates, genus *Bacillus* indicated predominance and included *Bacillus amyloliquefaciens*, *B. subtilis*, *B. megaterium*, *B. licheniformis*, *B. firmus* and *B. mycoides*. In the remaining 04 Gram positive isolates were *Lactobacillus helveticus*, *Micrococcus flavus*, *Arthrobacter aureus* and *Arthrobacter citreus*. The Gram negative bacteria included *Flavobacterium indicum*, *Flavobacterium aquatile*, *Brevundimonas diminuta*, *P. aeruginosa*, *Serratia marcescens*, *Citrobacter freundii* and *E. coli*. Many of these bacteria were hydrolytic in nature.

Bacterial isolates showed production of several hydrolytic enzymes (**Table 1**). Many bacterial isolates from digester effluent were common to vegetable waste slurry and cattle dung slurry (**Table 2**). The microorganisms which were common to vegetable waste, cattle dung slurry and digester effluent indicated that, when these are subjected to anaerobic digestion for biomethanation of vegetable waste, probably sustained, grown and contributed to the hydrolytic activities serving the substrates for methanogenesis. This indicates that these hydrolytic microorganisms probably have come from vegetable waste and cattle dung slurry and acclimatized in the anaerobic digester.

Table 1 : Enzymatic capabilities of bacterial isolates common to substrates and digester effluent

S/N	Mold isolate	Source	Enzyme production					
			Amylase	Caseinase	Gelatinase	Lipase	Cellulase	Ligninase
1	<i>Bacillus megaterium</i>	Vegetable waste and Effluent	+	+	+	-	+	+
2	<i>Bacillus mycoides</i>	Vegetable waste and Effluent	+	+	+	+	+	+
3	<i>Bacillus amyloliquefaciens</i>	Vegetable waste, Cattle dung slurry and Effluent	+	+	+	+	+	+
4	<i>Bacillus licheniformis</i>	Vegetable waste, Cattle dung slurry and Effluent	+	+	+	-	+	+
5	<i>Brevundimonas diminuta</i>	Vegetable waste, Cattle dung slurry and Effluent	-	+	+	-	+	+
6	<i>Pseudomonas aeruginosa</i>	Vegetable waste and Effluent	+	+	+	+	+	+
7	<i>Serratia marsescens</i>	Vegetable waste and Effluent	+	+	+	+	+	+
8	<i>Flavobacterium aquatile</i>	Vegetable waste and Effluent	+	-	+	-	-	-
9	<i>Flavobacterium indicum</i>	Vegetable waste and Effluent	+	+	+	-	-	-

(+) positive, (-) negative

Table 2 : Hydrolytic bacteria common to anaerobic digester effluent and to that of vegetable waste slurry and cattle dung slurry

S/N	Materials	Common bacterial isolates
1	Vegetable waste slurry and digester effluent	<i>Bacillus megaterium</i>
		<i>Bacillus mycoides</i>
		<i>Bacillus amyloliquefaciens</i>
		<i>Brevundimonas diminuta</i>
		<i>Pseudomonas aeruginosa</i>
		<i>Serratia marsescens</i>
		<i>Flavobacterium aquatile</i>
		<i>Flavobacterium indicum</i>
2	Cattle dung slurry and digester effluent	<i>Bacillus amyloliquefaciens</i> ,
		<i>Bacillus licheniformis</i>
		<i>Brevundimonas diminuta</i> .

Bacillus amyloliquefaciens, *Brevundimonas diminuta* and *Serratia marsescens* were the potent hydrolytic bacteria from digester effluent which were found to be common to vegetable waste slurry. These organic matter decomposing potent bacteria isolated from digester effluent are also reported as potent environmentally friendly antifungal biocontrol agents. *B. amyloliquefaciens* strains are reported to produce various compounds like biofilm, siderophores, antibiotics, indole-3-acetic acid, phosphatases, phytases, hydrogen cyanide, zinc solubilisation, volatile organic compound of lipopeptides, polyketides^{21,22}. *B. diminuta* was reported to produce several hydrolytic enzymes and bioactive compounds^{23,24}. *Serratia marcescens* is reported to produce several hydrolytic enzymes and plant growth promoting metabolites and hence finds application in agriculture and environmental processes²⁵⁻³⁰. This highlights *Bacillus amyloliquefaciens*, *Brevundimonas diminuta* and *Serratia marsescens* as plant growth promoting bacteria can serve as good candidates for the development of biofertilizers and biocontrol agents against plant fungal pathogens because of production of several types of hydrolytic enzymes and bioactive metabolites.³⁴⁻³⁷

Gore identified the species of *Arthrobacter*, *Listeria*, *Citrobacter*, *Pseudomonas*, *Escherichia*, *Bacillus*, *Flavobacterium*, *Micrococcus* and *Lactobacillus* from effluent of digester run on cattle dung slurry³¹. Kale reported isolates of *Staphylococcus aureus*, several species of

Bacillus and *Proteus* genera, *Streptococcus faecalis*, *E. coli*, *Enterobacter cloacae*, *Citrobacter freundii*, *Alkaligenes faecalis*, *Flavobacterium rigense*, *Arthrobacter simplex*, *L. fermentatum*, *Micrococcus sp.* and *Pseudomonas sp.* from anaerobic lagoons of distillery waste³². Pathade identified species of *Citrobacter*, *Bacillus* and *Lactobacillus* genera from digester run on spent wash³³. Deshmukh reported various species from *Bacillus* genus, *Arthrobacter sp.*, *Cellulomonas flavigera*, *Citrobacter freundii*, *E. coli*, *Flavobacterium sp.*, *L. helveticus*, *Micrococcus varians*, *P. vulgaris*, *Pseudomonas aeruginosa*, and *Strep-tomyces sp.* from biogas digester run on admixture of Ipomoea biomass and distillery waste⁷.

CONCLUSION

The bacterial count of the digester effluent was significantly higher than those of vegetable waste slurry and cattle dung slurry. The sixteen, twelve and seventeen bacteria were isolated, identified and tested for their hydrolytic potential from vegetable waste, cattle dung and digester effluent slurries respectively. The hydrolytic bacteria common to this digester effluent and to that vegetable waste slurry and cattle dung slurry indicated that these hydrolytic bacteria probably have come from substrate and cattle dung slurry and acclimatized in the digester and contributed to the biogas production along with the help of other microorganisms. This study has provided useful information about the bacteria associated with substrates and cattle dung

slurry that have important role in biogas production. Further, the most potent hydrolytic bacterial isolates obtained from the digester effluent could be useful to increase the efficiency of the process for biomethanation of vegetable waste and other agro-industrial organic wastes. These potent hydrolytic bacteria could be useful in composting of variety of agro-industrial organic wastes. Some of the these potent hydrolytic bacterial isolates have been reported to have ability to produce variety of hydrolytic enzymes and bioactive compounds and hence with further studies can be used to develop biofertilizers and biocontrol agents for sustainable development of agriculture.

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