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Studies on Molds from Vegetable Waste, Cattle Dung Slurry and Biogas Digester Effluent

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A B S T R A C T

Biomethanation is the anaerobic digestion of biodegradable organic matter in an enclosed space under controlled conditions of temperature, moisture, pH, etc. The different types of microorganisms mainly bacteria, yeast, molds and actinomycetes are involved in biogas production process. Hydrolytic microorganisms play an important role in the hydrolysis step of biomethanation. The present paper deals with the study of molds 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 molds from vegetable waste slurry, cattle dung slurry and digester effluents were found to be 4.1 X 10^2, 2.78 X 10^3 and 7.73 X 10^3 colonies/mL respectively. In present study six molds were isolated and identified from each vegetable waste slurry and digester effluent whereas four molds were isolated and identified from cattle dung slurry using standard methods. Further all the mold isolates were tested for their potential to produce hydrolytic enzymes. This study has provided useful information about the molds associated with substrates and digester effluent that have important role in biogas generation.

Introduction

Vegetable waste is produced in large quantities during harvesting, poor and inadequate transportation, storage facilities, marketing practices and processing of vegetables. The collection, transportation and disposal of vegetable waste is a very serious problem today. The present unscientific treatment methods results in environmental pollution (Kumar *et al*., 2009).

Biomethanation is an attractive treatment option for vegetable wastes and play a triple role. First, it is a method of converting the energy contained in biomass into a biogas (Naik *et al*., 2010). Second, it is a method of recycling of organic wastes into stable soil additives, that is, valuable liquid fertilizer (Chami and Vivanco, 2007). Third, it is a method of wastes treatment aimed at a reduction of their hazardous effects on the environment (Kumar *et al*., 2009).

Biomethanation is accomplished by a consortium of microorganisms working synergistically. Aerobic, facultative anaerobic and obligate anaerobic (methanogenic and non methanogenic) 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 methanogenesis (Nijaguna, 2012; Charles *et al*., 2009; Khanal, 2008; Davidsson, 2007). The hydrolysis step degrades both insoluble complex organic matter and high molecular weight compounds into soluble monomers in order to allow their transport through microbial cell membrane (Nijaguna, 2012). This reaction is catalyzed by extracellular enzymes (amylase, proteases, lipases, cellulases and ligninases) produced by hydrolytic and fermentative microorganisms. The aim of the present work was to identify mold isolates obtained from vegetable waste slurry, cattle dung slurry and effluent of biogas digester and to determine their potential to produce hydrolytic enzymes.

Materials and Methods

Collection and preparation of samples

Vegetable wastes for the present study were collected from the local vegetable market. The collected wastes were further segregated, shredded separately and ground in a kitchen blender to make paste. The physico-chemical analysis of vegetable waste was determined according to standard methods (APHA, 1998).

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 located at Degaon village, M.I.D.C., Satara (M.S.), India. 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 molds 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 molds were carried out in triplicates using Martin Rose Bengal Agar (MRBA) medium. Plates were incubated at room temperature for 72 hours to get practicable colonies and then counts were recorded. The representative mold isolates were preserved in triplicates on MRBA slants at refrigeration temperature for further studies.

Identification of mold isolates

The preliminary identification of the mold isolates upto genus level was done on the basis of colonial, morphological and microscopic observation of the wet mounts with reference to Barnett and Hunter (1972), Domsch *et al* (1980), Aneja (2001), Gilman (2001) and Nagamani *et al* (2006). The confirmation of these mold isolates at species level was done at National Fungal Culture Collection of India, Mycology and Plant Pathology Group, Agharkar Research Institute, Pune, M.S., India.

Demonstration of enzymatic capabilities of mold isolates

Starch agar, Skim milk agar, Gelatin agar, Tributyrin agar, Czapek Dox Agar medium containing carboxy-methyl cellulose and Mineral salt medium (MSM) agar containing lignin with methylene blue as indicator dye were used for determining amylolytic, caseinolytic, gelatinolytic, lipolytic, cellulolytic and ligninolytic potential of mold isolates respectively. The individual cultures were spot inoculated onto respective media plates and incubated at room temperature for 48-72 hours. Amylolytic activity was detected by exposing the starch agar plates to iodine crystals for 5 minutes to observe the starch degradation zone. Skim milk agar plates and tributyrin agar plates were observed for clear zones around growth which indicated caseinolytic and lipolytic activity respectively. Gelatin agar plates were flooded with Fraziers reagent to detect gelatinolytic activity of mold isolates. Cellulolytic activity was detected by flooding the plates with 1% congo red for 20 minutes at room temperature followed by washing with 1M sodium chloride solution. A clear zone around the growth against the dark red background was taken as indication of cellulose activity (Lu *et al*., 2004). The MSM-lignin agar plates were monitored for fungal growth and decolorization of methylene blue dye (Bandounas *et al*., 2011).

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 9900mg/kg, 8700 mg/kg, 2400mg/kg, 2200 mg/kg, 4000mg/kg and 9081mg/kg respectively. The high moisture and carbohydrate content indicated its suitability for biomethanation.

Biomethanation of vegetable waste at 5 L level was carried out at HRT 20 days, OLR 0.320 g VS/l.d, pH 7.0 of influent and ambient temperature conditions (30-40 ^{o}C). Range of biogas produced was 510-1340mL/d, total amount of biogas produced in 40 days experiment was found to be 40515mL and average amount of biogas was 0.633L/g VS added/d. The biogas burning test produced blue flame indicating rich methane content in the biogas. Gas chromatographic analysis of biogas revealed 59 % methane.

SPC of molds from vegetable waste slurry, cattle dung slurry and digester effluents were determined using MRBA plates and incubating at room temperature for 72 hours (Table 1).

Six molds were isolated and identified from vegetable waste slurry, which included *Aspergillus niger*, *Aspergillus* sp., *Cunninghamella echinulata*, *Alternaria raphani*, *Penicillium* aff. *granulatum* and *Penicillium* sp., while four mold were isolated and identified from cattle dung slurry which included *Aspergillus niger*, *Aspergillus* sp., *Trichoderma pseudokoningii* and *Penicillium* sp. Six molds were isolated and identified from digester effluent included *Aspergillus niger,*

Aspergillus sp., *Trichoderma longibrachiatum, Trichoderma pseudokoningii, Penicillium* aff. *granulatum* and *Penicillium* sp. (Table 2-4). Figure 1 represents colonial morphology of mold isolates obtained from vegetable waste slurry, cattle dung slurry and digester effluent.

It was observed that some of the mold isolates were common to vegetable waste slurry and digester effluent, cattle dung slurry and digester effluent (Table 5). The results showed that large number of molds was introduced by these two inoculums in the anaerobic digester, and many of the molds from this inoculums got established in the anaerobic digester and caused hydrolytic and methanogenic activities leading to generation of biogas from organic matter.

Table 6 represents the enzymatic capabilities of selected mold isolates obtained from vegetable waste slurry, cattle dung slurry and digester effluent. All the mold isolates obtained from vegetable waste slurry, cattle dung slurry and digester effluent showed strong amylolytic, gelatinolytic, caseinolytic, lipolytic, cellulolytic and ligninolytic activity. Thus, it is evident that these hydrolytic molds along with other hydrolytic microorganisms from the digester play an important role in transformation of organic matter into intermediates that are further utilized by other microorganisms to produce biogas.

Table.1 SPC of molds from different sources

Source	SPC of molds (colonies/mL)
Vegetable waste slurry	4.10×10^2
Cattle dung slurry	2.78×10^3
Digester effluent	7.73×10^3

Table.2 Identification of molds from vegetable waste slurry

Isolate code No.	Morphological identification	Family
VWM-1	*Aspergillus niger* gr.	Trichocomaceae
VWM-2	*Aspergillus* sp.	Trichocomaceae
VWM-3	*Cunninghamella echinulata* (Thaxt.)Thaxt.	Cunninghamellaceae
VWM-4	*Alternaria raphani* J.W.Groves and Stolko	Pleosporaceae
VWM-5	*Penicillium* aff. *granulatum* Bainier	Trichocomaceae
VWM-6	*Penicillium* sp.	Trichocomaceae

Table.3 Identification of molds from cattle dung slurry

Isolate code No.	Morphological identification	Family
DSM-1	*Aspergillus niger* gr.	Trichocomaceae
DSM-2	*Aspergillus* sp.	Trichocomaceae
DSM-3	*Trichoderma pseudokoningii* Rifai	Hypocreaceae
DSM-4	*Penicillium* sp.	Trichocomaceae

Table.4 Identification of molds from digester effluent

Isolate code No.	Morphological identification	Family
EM-1	*Aspergillus niger* gr.	Trichocomaceae
EM-2	*Aspergillus* sp.	Trichocomaceae
EM-3	*Trichoderma longibrachiatum* Rifai	Hypocreaceae
EM-4	*Trichoderma pseudokoningii* Rifai	Hypocreaceae
EM-5	*Penicillium* aff. *granulatum* Bainier	Trichocomaceae
EM-6	*Penicillium* sp.	Trichocomaceae

Table.5 Molds found common to anaerobic digester effluent and to that of vegetable waste slurry and cattle dung slurry

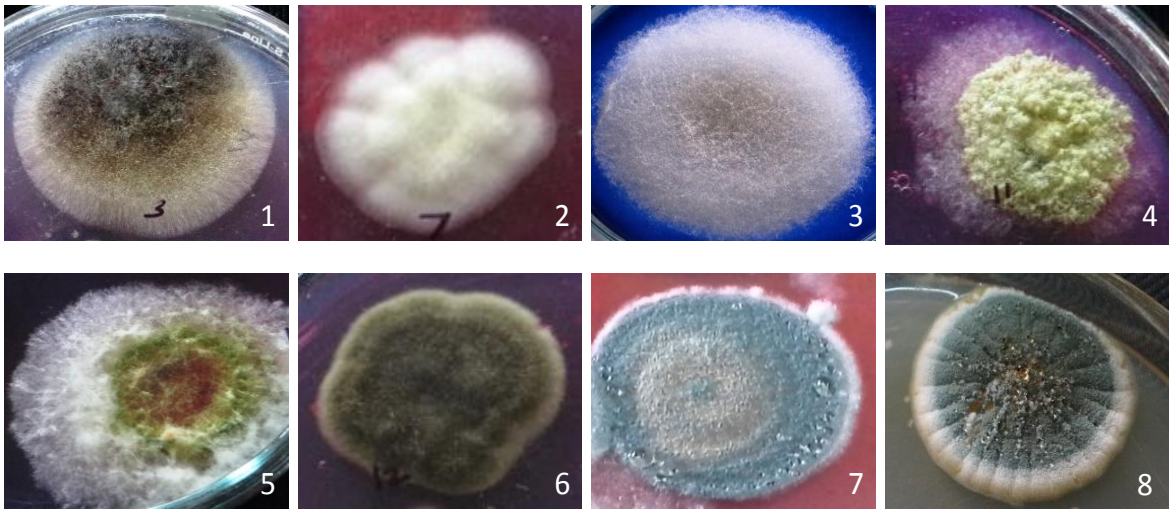
Sr.No.	Materials	Common mold isolates
1	Vegetable waste slurry and digester effluent	1) *Aspergillus niger* gr.
		2) *Aspergillus* sp.
		3) *Penicillium* aff. *granulatum* Bainier
		4) *Penicillium* sp.
2	Cattle dung slurry and digester effluent	1) *Aspergillus niger* gr.
		2) *Aspergillus* sp.
		3) *Trichoderma pseudokoningii* Rifai
		4) *Penicillium* sp.

Table.6 Enzymatic capabilities of selected mold isolates

Sr. No.	Mold isolate	Source	Enzyme production					
			Amylase	Caseinase	Gelatinase	Lipase	Cellulase	Ligninase
1.	*Aspergillus niger gr.*	Vegetable waste,Cattle dung slurry and Effluent	+	+	+	+	+	+
2.	*Aspergillus* sp.	Vegetable waste,Cattle dung slurry and Effluent	+	+	+	+	+	+
3.	*Cunninghamella echinulata*	Vegetable waste	+	+	+	+	+	+
4.	*Trichoderma longibrachiatum*	Effluent	+	+	+	+	+	+
5.	*Trichoderma pseudokoningii*	Cattle dung slurry and Effluent	+	+	+	+	+	+
6.	*Alternaria raphani*	Vegetable waste	+	+	+	+	+	+
7.	*Penicillium aff. granulatum*	Vegetable waste and Effluent	+	-	+	+	+	+
8.	*Penicillium* sp.	Vegetable waste, Cattle dung slurry and Effluent	+	+	+	+	+	+

(+) positive, (-) negative

Fig.1 Colonial morphology of mold isolates obtained from vegetable waste, cattle dung slurry and digester effluent



1=*Aspergillus niger*, 2=*Aspergillus* sp., 3=*Cunninghamella echinulata*, 4=*Trichoderma longibrachiatum*, 5=*Trichoderma pseudokoningii*, 6=*Alternaria raphani*, 7=*Penicillium affinity granulatum*, 8=*Penicillium* sp.

There are reports on isolation and identification of molds from different substrates and digester effluent. Deshmukh (2004) identified *Aspergillus* sp., *Fusarium* sp., *Neurospora* sp., *Penicillum* sp., *Tricoderma* sp. from digester effluent run on mixture of distillery waste and *Ipomoea* weed. Kale (1986) isolated *Aspergillus, Cladosporium, Fusarium, Mucor, Penicillium, Rhozopus* and *Candida* from anaerobic lagoons of distillery waste treatment. The hydrolytic capabilities of these molds isolated from different soil samples has been reported by several researchers (Gupta, 2015; Manoorkar and Gachande, 2015; Kaur and Joshi, 2015; Reddy *et al*., 2014; Saleem *et al*., 2013; Chukwudi and Bamidele, 2013; Devi and Kumar, 2012; Khokhar *et al*., 2012).

In conclusion, SPC of molds from vegetable waste slurry, cattle dung slurry and digester effluents were found to be 4.1 X 10^2, 2.78 X 10^3 and 7.73 X 10^3 colonies/mL respectively. The mold isolates found were the species of *Aspergillus*, *Cunninghamella, Trichoderma, Alternaria* and *Penicillium.* The aerobic and facultative anaerobic, hydrolytic molds isolated from digester effluent included *Aspergillus niger*, *Aspergillus* sp., *Trichoderma longibrachiatum, Trichoderma pseudokoningii, Penicillium* aff. *granulatum* and *Penicillium* sp. The hydrolytic molds common to this digester effluent and to that vegetable waste slurry and cattle dung slurry included *Aspergillus niger*, *Aspergillus* sp., *Trichoderma pseudokoningii*, *Penicillium* aff. *granulatum* and *Penicillium* sp. This indicated that these hydrolytic molds probably have come from primary inoculum materials and acclimatized in the digester and contributed to the biogas production along with the help of other microorganisms.

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