A PRELIMINARY STUDY ON MOLECULAR CHARACTERIZATION OF THE EUBACTERIA IN A THERMOPHILIC, POULTRY WASTE FED ANAEROBIC DIGESTER

UN ESTUDIO PRELIMINAR DE LA CARACTERIZACIÓN MOLECULAR DE EUBACTERIAS EN UN DIGESTOR ANAEROBIO, TERMOFILICO ALIMENTADO CON DESECHOS DE UNA POLLERÍA

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Abstract

Biomethanation is a unique process which aids the recovery of carbon present in the wastes as methane, an energy rich product. The presence and activity of a variety of bacterial and archaeal microorganisms play a vital role in this process. An understanding of the microbial groups present in the anaerobic digester is important for augmenting the recovery of carbon and also will help in solving the digester related problems as the functioning of the digester depends on the microbial activity. Molecular characterization of the anaerobic bacteria present in a thermophilic (55ºC) anaerobic digester fed with poultry waste was analyzed by DNA extraction followed by PCR amplification, cloning and sequencing of the obtained clones. Results showed that more than 75 per cent of the clones represented uncultured bacteria and among the different genera recorded, *Clostridium* sp. was dominant. Results demonstrated the need for obtaining more number of clones and a combination of different methods for reliable molecular characterization of an anaerobic digester.

Keywords: anaerobic digester, poultry waste, fermentative anaerobes, molecular analysis.

Resumen

La biometanación es un proceso único que ayuda la recuperación del carbono presente en la basura como el metano, un producto rico en energía. La presencia y actividad de una variedad bacteriana y los microorganismos del archaea juegan un papel vital en este proceso. Una comprensión de los grupos microbianos presentes en el digestor anaerobio es importante para aumentar la recuperación de carbono y también ayudará resolver los problemas relacionados con el digestor ya que depende de la actividad microbiana. La caracterización molecular de grupos de bacterias anaerobias presente en un digestor termofilico (55ºC), anaerobio alimentado con los desechos de la pollería se analizó por extracto de ADN seguido por la amplificación de PCR, clonando y secuenciado de los clones obtenidos. Los resultados mostraron que más del 75 por ciento de los clones representaron las bacterias no cultivadas y entre diferente genero registrado, *Clostridium* sp. era dominante. Los resultados demostraron claramente la necesidad de obtener más número de clones y la combinación de los métodos diferentes para una caracterización molecular confiable de un digestor anaerobio.

Palabras clave: digestor anaerobio, desechos de pollería, anaerobias fermentativas, análisis molecular.

1. Introduction

Management of animal manures is of growing concern due to the environmental risk such as contamination of ground and surface water, pathogens and offensive odor. Approximately 1000 birds produce 10-15 tons of litter annually (Flora and Riahi-Nezhad, 2006) and about 9×10^6 t of livestock wastes are produced annually in USA (Espinosa-Solares *et al*., 2006). Anaerobic digestion of these organic wastes for biogas production is a desirable method of waste treatment, as it produces renewable energy in the form of methane (Ahring, 2003). In nature, anaerobic microbial degradation of organic matter to methane and carbon dioxide occurs in a variety of habitats such as intestinal tracts, soil, sediments and in wetlands. This natural process is exploited on a large scale as a simple and effective biotechnological process to reduce the pollution caused by organic wastes. This technology became more and more attractive in the recent past because new reactor designs significantly improved the

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reactor performance (Verstraete and Vandevivere, 1998). Trophical structure of anaerobic ecosystems has revealed that anaerobic metabolism proceeds in a stepwise manner where several metabolic groups of bacteria interact in the mineralization of organic matter to methane (McInerney, 1999). Consequently, the efficiency and robustness of wastewater treatment systems mainly depend on the composition and activity of its microbial community. In addition, the failure in reliability of many anaerobic digesters to perform at stable efficiency has underlined the need for more basic information on the biological aspects of the anaerobic digestion ecosystem. But, though biological wastewater treatment systems are being used for more than a century, studies on the microbiology of this process (black-box) has made little progress due to the methodological limitations to study different anaerobic groups. With the recent progresses that has been made in microbial molecular techniques (Schramm and Amann, 1999; Chouari *et al*., 2005) it is being possible to determine the composition and dynamics of microbial communities in these systems, to identify the key microbial players, to identify the problems in the anaerobic process and to find solutions to the problems associated with the process. With this background, the aim of this work was to characterize the anaerobic fermentative groups, the primary important group of bacteria that play a major role in the biomethanation of poultry wastes.

2. Materials and methods.

2.1. DNA extraction

Slurry samples for DNA extraction were collected from a pilot scale thermophilic anaerobic digester (55ºC) fed with poultry waste. DNA was extracted using PowerSoil™ DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA) and by following the protocols given in the kit. The quality and quantity of the extracted DNA was determined by using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and analyzed in 0.8% agarose-TAE gel electrophoresis.

2.2. Amplification of 16S rDNA, cloning and screening

The 16S rDNA genes were amplified in a 96-well GeneAmp® PCR System (Model 9700, Applied Biosystems, Foster city, CA, USA) reaction using 341F (5'-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTRAGTTT-3') primers, which target for eubacteria. PCR conditions involved one cycle of initial denaturation at 94ºC for 5 min, 30 cycles of annealing (66ºC for 1 min); elongation (72ºC for 30 s); and denaturation (94ºC for 1 min) and one cycle of extension (72ºC for 4 min). The quality of PCR amplicon was determined using 0.8% agarose gel using TAE buffer. The PCR amplicon (566 bp) was cloned in TOPO® vector using TOPO TA cloning® kit (Invitrogen Corporation, Carlsbad, CA, USA) and transformed into One Shot® Mach1™-T1® chemically competent *E. coli* (Invitrogen Corporation, Carlsbad, CA, USA). The ampicillin resistant clones were screened and selected using LB agar added with ampicillin (100 $\mu g.ml^{-1}$). A clone library was constructed and the clones were grown on LB broth with ampicillin for overnight and the plasmids were isolated using QIAprep Spin columns (QIAGEN Inc., Valencia, CA, USA) and by the protocols given by the manufacturer.

2.3. Sequencing and phylogenetic analysis

The plasmid DNA of the clones were prepared by using ABI BigDye® Terminator v3.1 cycle sequencing kits by following the protocols of the supplier, amplified using 341F primer (45 cycles of 94ºC, 10 s; 50ºC, 5 s and 60ºC, 4 min) and the DNA sequencing was carried out in a 48 capillary DNA Analyzer (Model 3730; Applied Biosystems, Foster city, CA, USA). The sequences were analyzed by comparison with the sequences available in RDP database (Cole *et al*., 2005). DNA sequence comparisons between the clones were performed with Lasergene software (DNAStar Inc., Madison, WI, USA) and the phylogenetic tree was constructed by employing clustalW analysis using MegAlign algorithms of Lasergene software. Nucleotide substitutions and similarity index of the clones were also estimated using the same program.

3. Results and Discussion.

Anaerobic digestion is gaining importance for treatment of organic wastes as it produces energy in the form of methane apart from various benefits such as reduced space requirements, low sludge production, etc. Studies on the different microbial communities in relation to their type, numbers, spatial distribution (structural parameters) and as well as their activities (functional parameter) are important in order to monitor and manipulate the efficiency of the process. As cultivation-dependent methods have limitations in elucidating diversity of the complex microbial ecosystems since many of the component species remain to be identified (Suau *et al*., 1999), nucleic acid based methods aid in unraveling the microbial biodiversity in many different ecosystems (Schramm and Amann, 1999). The results on the molecular characterization of the fermentative anaerobic bacterial diversity in a thermophilic, poultry waste fed anaerobic digester are presented.

Mean concentration of DNA extracted from the slurry samples from the digester was 45.3 $\mu g.\mu l^{-1}$,

which indicated an efficient extraction of DNA from the digester slurry. O'Donnel and Görres (1999) reported that the yield of DNA generally varied between 2 and 35 mg g^{-1} dry soil. The $A_{260/280}$ was1.876, which indicated the high quality of extracted DNA (Drábková *et al*., 2002).

The use of molecular biological techniques, especially those that take advantage of the small-subunit (SSU) rRNA molecule, has eliminated the dependence on isolation of pure cultures as a means of studying the diversity and structure of microbial communities (Schramm and Amann, 1999). The microbial consortia in soil, sediments, anaerobic digesters and different other systems have been analyzed using this approach (Godon *et al*., 1997a; 1997b; O'Donnel and Görres, 1999; Mladenovska *et al*., 2003). Most of the ecosystems are open-field systems, and only one sample was analyzed. Even with a representative sample, it is difficult to distinguish between endogenous and transient microorganisms. Others such as anaerobic reactors are closed systems, but the microorganism's analyzed span either a small number of clones or only one taxon. As little is known about this ecosystem, a molecular inventory is the first step to describe these dynamic microbial communities without cultivation.

In this study, different PCR conditions for amplification were tested and PCR amplicon (566 bp) obtained under optimum conditions was inserted in TOPO vector, transformed into chemically competent *Escherichia coli* and the clones were selected by resistance for ampicillin. From the clone library, ten clones were picked and multiplied in LB broth at 37ºC for overnight. Simultaneously the clones were also dot streaked in LB agar with ampicillin using sterile tooth picks to preserve the clones for further studies. The plasmid DNA (3.9 kb) of the clones grown in LB broth was isolated and checked for their purity (Fig. 1). The isolated Plasmid DNAs were amplified using 341F primer (45 cycles) and sequenced with ABI BigDye® Terminator v3.1 cycle sequencing kits. Sequences of the clones were compared with database in RDP and a phylogenetic tree was constructed by ClustalW analysis (Fig. 2) and the distance of the clones based on similarity percent is presented in Fig. 3. In the present study sequence analysis showed that the majority of the clones pertained to uncultured clostridia.

Buzzini *et al*. (2006) observed that structure of the microbial community in an anaerobic digester is complex and most of the population belonging to the domain *Bacteria* remained stable through the process, but was sensitive to operational changes. Earlier, Godon *et al*. (1997a) reported that the presence of more than 146 different organisms belonging to the three domains *Bacteria*, *Eucarya*, and *Archaea* in an anaerobic digester. Among the 139 OTUs (Operational Taxonomic Unit) recorded, 133 were from the *Bacteria* domain. These observations indicated that there is rich diversity of bacteria in an anaerobic digester, many of which are not yet identified. However, in this study the eubacterial species diversity was less and further they pertained to uncultured bacterial groups. In accordance, Tauber *et al*. (2007) reported that the species diversity of bacteria was less in thermophilic anaerobic digester. Apajalahti *et al*. (2004) reported that 90% of the bacteria in the chicken gastrointestinal tract represented previously unknown species and this could be related to the higher presence of unidentified bacteria in this study, as the digester is fed with poultry litter. Earlier, Godon *et al*. (1997b) also observed that the most frequent bacterial OTU in an anaerobic digester were less than 5% of the characterized bacterial population and the majority of them were not closely related to other hitherto-determined sequences.

The results of this study also showed that the clostridia were predominant among the clones. This could be related to the nature of the poultry waste, which contained high cellulose (20% on dry basis) and hemicellulose (11% on dry basis) content (Espinosa-Solares *et al*., 2006) and it is widely known that cellulose hydrolysis is the rate-limiting step in the anaerobic digestion of organic solid wastes (O'Sullivan *et al*., 2005).

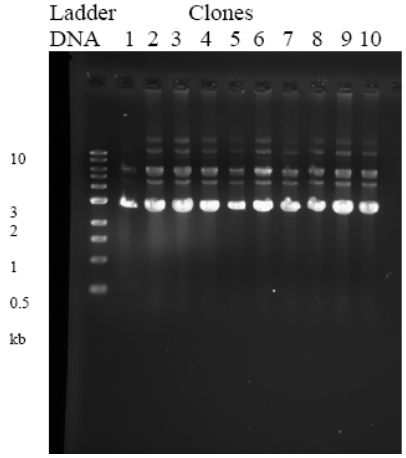

Fig. 1. Plasmid profile of the clones.

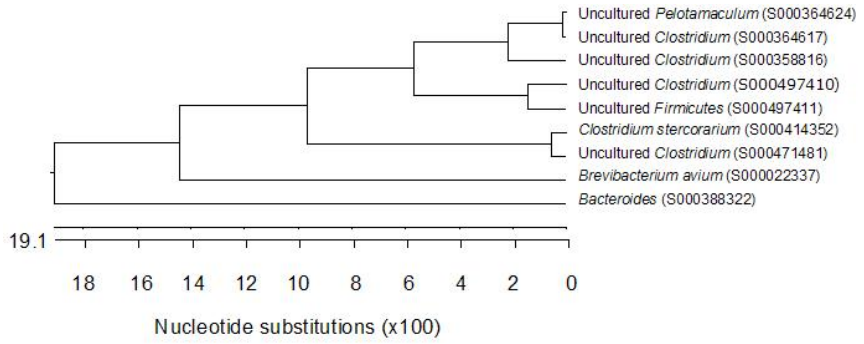

Fig. 2. Phylogenetic tree based on the sequence analysis of the clones.

Percent Identity

	1	2	3	4	5	6	7	8	9	
1		88.1	85.8	77.6	78.4	67.5	88.4	99.6	70.9	**1**
2	10.4		91.5	64.8	69.8	64.6	88.9	85.0	45.4	**2**
3	13.0	4.1		66.8	71.9	68.3	89.0	88.3	47.5	**3**
4	22.1	16.8	18.4		99.8	71.8	84.7	84.5	57.7	**4**
5	21.5	15.8	17.3	0.2		72.8	85.4	85.1	54.8	**5**
6	40.6	41.5	39.8	33.8	31.7		63.8	64.4	41.8	**6**
7	10.0	0.2	4.6	16.8	15.8	36.8		92.1	50.8	**7**
8	0.4	8.1	9.0	17.5	16.5	41.2	8.2		46.9	**8**
9	35.7	27.1	26.8	28.5	27.8	38.4	27.5	32.1		**9**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	

1 Uncultured *Clostridium* (S000497410)
2 Uncultured *Pelotamaculum* (S000364624)
3 Uncultured *Clostridium* (S000358816)
4 *Clostridium stercorarium* (S000414352)
5 Uncultured *Clostridium* (S000471481)
6 *Bacteroides* (S000388322)
7 Uncultured *Clostridium* (S000364617)
8 Uncultured *Firmicutes* (S000497411)
9 *Brevibacterium avium* (S000022337)

Fig. 3. Sequence distance of the clones based on percent identity.

Among the different bacterial species, *Clostridium* sp. has been implicated as the main anaerobic bacterial species contributing to cellulose degradation in anaerobic digesters (Lynd *et al*., 2002; Westlake *et al*., 1995). Leung and Topp (2001) also reported that *Clostridium* species was dominant in a thermophilic digester treating pig slurry. Van Dyke and McCarthy (2002) observed that cellulose degraders in a landfill were *Clostridium*. Burrell *et al*. (2004) observed that 16S rRNA gene clone libraries prepared from the attached fraction (biomass associated with cellulose particles) and also from the mixed fraction (attached and in the planktonic phase) were dominated by *Firmicutes* phylum sequences (100% of the attached library and 90% of the mixed library). Later Syutsubo *et al.* (2005) reported that *Clostridium* sp. strain JC3 was the dominant cellulose degrading bacterium in thermophilic methanogenic sludge. *Clostridium stercorarium* is a thermophilic anaerobic bacterium, widely reported for its capacity for degradation of cellulose and hemicelluloses (Ali *et al*., 2001) and *Bacteroides* sp. has been reported as a common cellulolytic anaerobic bacteria present in rumen (Stewart *et al*., 1997).

In addition to *Clostridium*, it was observed in the present study that the majority of the OTU's belonged to Firmicutes. Previously, Tang *et al*. (2004) and Chouari *et al*. (2005) also reported the predominance of Firmicutes in a thermophilic anaerobic digester treating municipal solid waste and an anaerobic sludge digester fed with municipal wastewater respectively. Similar to the results of this study, numerous novel 16S rRNA gene sequences have been retrieved during previous studies from both municipal and industrial wastewater treatment plants and the vast majority of species found in these communities have not been cultivated yet (Godon *et al*., 1997a; 1997b; Daims *et al*., 2001). Although cloning of PCR products revealed a higher diversity of species than culturing, there are differences in the species identified by this technique (Smit *et al*., 2001). It is probably not correct to assume that the OTU distribution in the sample is the same as the species distribution in the reactor. Several parameters could affect our ability to detect the actual species distribution (differential cell lysis, *Taq* polymerase specificity, primer specificity, chimera formation, and copy number of the SSU rRNA genes). However, the confidentiality of the results can be increased by constructing a clone library with large number of clones for sequence analysis. Dahllöf (2002) reported that there is no single approach that can aid in studying the microbial diversity in anaerobic digesters or other ecosystems. Results of this study recorded that more than 75 per cent of the clones represented uncultured bacteria and among the different genera recorded, *Clostridium* sp. was dominant. The presence of clostridia evokes a question about the pathogenic character of the slurry from anaerobic digester. But this fear can be

discounted due to the fact that the predominant clostridia were cellulolytic in nature, as reported by O'Sullivan *et al*. (2005). They employed fluorescence *in situ* hybridization (FISH) to determine the structure of cellulose degrading microbial community in anaerobic digesters and the predominant bacterial group belonged to Clostridium. Similarly Shiratori *et al*. (2006) reported that clostridia with diverse phylogenetic positions specifically occurred during the period of high fermentation efficiency in a thermophilic methanogenic digester. However, construction of a library that includes a higher number of clones will increase the reliability of the data and a parallel analysis using cultivation dependent methods could aid in determining microbial biodiversity and their role in the performance of the anaerobic digesters.

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