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Subtilase Genes Diversity in the Biogas Digester Microbiota

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Abstract Biogas digesters contain microbial assemblages that process a mass of extracellular polymeric substances from animal manure and domestic wastewater; however, due to the limitation of available technology in cultivation of majority of the micro-organisms in biogas digesters, the enzymatic potential of these microbial communities remains largely unexplored. In this study, to evaluate subtilase gene diversity in a biogas digester, the partial sequences of the gene were directly amplified from the metagenomic DNA by using consensus-degenerate primers. The desired PCR products were cloned into pGEM-T Easy vector, and thirty positive clones were chose for Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis, from which thirteen distinguished patterns were obtained and then sequenced. Phylogenetic analysis showed that ten out of the thirteen sequences were related to the subtilase genes in GenBank and were grouped into three families of the subtilases superfamily. The nucleotide sequences analysis through BLAST search revealed that none of the partial genes the authors isolated showed significant similarity against the non-redundant Nucleotide database of NCBI. Meanwhile, the deduced amino acid sequences of ten partial subtilase genes showed moderate identities to the previously identified sequences in GenBank, with a range from 39 to 61%. Collectively, the data indicate that there is a great diversity of subtilase genes in the biogas digester; and may be a rich reservoir for novel subtilase genes.

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Introduction

Biogas fermentation, widely used in developing countries, is a great approach for efficient utilization of agricultural wastes. People can benefit from the products of this process: biogas can be used as a fuel for cooking; lighting or generating electricity and the residue of the biogas fermentation process can be used as fertilizer [27]. China, as a large country of biogas exploitation and application in the world, has already had success in biogas fermentation application, especially in the countryside [4]. However, the application of biogas fermentation still need some improvements, low efficiency of raw materials utilization and low yield of biogas are two of the main problems. Biogas production is a microbial anaerobic digestion process. In the process, a overwhelming myriad of microorganisms produce various hydrolases like protease, lipase, amylase, cellulase,

xylanase, and pectinase which hydrolyze the undissolved polymeric compounds to dissolved small molecules [7]. Hence, hydrolytic enzymes are often the key factors for improving utilization efficiency of raw materials and biogas yield in biogas production.

Microbial serine proteases are among the most important hydrolytic enzymes and play a pivotal role in nutrient cycling in natural environments [12]. Subtilases, known as subtilisin-like serine proteases, are one of the largest superfamily of serine proteases [33]. A wide range of bacteria can produce extracellular subtilase. It is important for the degradation of exogenous proteins in many biological systems such as biogas fermentation and composting. In addition, biogas digesters are usually fed with animal manure and domestic wastewater, due to their richness in organic matter [14, 29]. In pig manure, crude proteins takes up approximately 20% of dry material [21], while proteins and lipids measures up to 70% of total organic matter in domestic wastewater [26]. It was also reported that *Bacteroidetes* group is abundant in the anaerobic digesters [18, 28], and *Bacteroidetes* were known to be proteolytic bacteria [15]. These facts suggest that subtilase may play an important role in hydrolysis of proteins in such high protein content environments. Hence, the study of subtilase gene diversity can provide important insights to the study of performance of biogas digester.

Conventional cultivation-dependent methods are typically utilized to isolate microorganisms which could produce subtilase from various environments [3, 5, 19]. However, more than 99% of microorganisms cannot be cultivated in laboratories using currently available technologies [2, 11]. This is considered a significant bottle-neck in the study and improvement of subtilase resources and utilization. In this study, a cultivation-independent method combining degenerate PCR, cloning, sequencing of clones discriminated by RFLP [25] was used to study the sublitase genes diversity in the metagenomic DNA from biogas digester microbiota.

Materials and Methods

Sample Collection

Biogas slurry was collected from an efficient 8 m^3 biogas-producing digester under lower-temperature (15°C) [6] which fed mainly on liquid pig manure and domestic wastewater in the countryside of Jiangyou, Sichuan province, China.

Metagenomic DNA Extraction and Purification

DNA was extracted according to the method of Zhou et al. [37] with some modification. In brief, five gram biogas slurry was suspended in 13.5 ml extraction buffer (100 mM Tris–HCl, pH 8.0, 100 mM EDTA, pH 8.0, 1.5 M NaCl, 100 mM sodium phosphate, pH 8.0, 1% CTAB). After brief vortexing, 50 µl of proteinase K (20 mg/ml) was added. The mixture was subjected to shake horizontally at 37°C for 30 min, and 1.5 ml of 20% SDS was added. After incubation at 65°C for 2 h with gentle inversion at every 15 min interval, the mixture was centrifuged at 6,000 $\times g$, and then transferred the supernatant to a new 50-ml tube. The precipitate was treated twice with 4.5 ml of extraction buffer, 0.5 ml of SDS (20%) and frozen in liquid nitrogen for 3 min then thawed in boiling water for 3 min. The supernatants of the three cycles were collected and extracted two times with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) then chloroform/isoamyl alcohol (24:1). Aqueous layer was precipitated with 0.6 volume of isopropanol at room temperature for 1 h. DNA pellet was obtained by centrifugation at 12,000 $\times g$ for 15 min at room temperature. After washing with cold 70% ethanol, the DNA was air-dried and dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The crude DNA was purified with Wizard Clean-up system (Promega, USA) according to the manufacturers' instructions and dissolved in TE buffer as about 100 µg/ml.

PCR Amplification of Partial Subtilase Genes and TA Cloning

Partial subtilase genes were amplified by touchdown PCR with a pair of degenerate primers [1], B2F (5′-GGCCAC GGCACCCAYGTBGCSGG-3′) and B2R (5′-CGTGAGG GGTGGCCATRSWDGT-3′). The 50 µl reaction mixture contained 300 nM of each primer, 100 ng of purified DNA, 5 µl 10 × rTaq reaction buffers, 200 µM dNTPs and 5 U *rTaq* DNA polymerase (Tiangen, China). The PCR conditions were: pre-denaturation at 95°C for 5 min; 21 cycles of 95°C for 1 min, 75°C for 1 min, and 72°C for 1 min with the annealing temperature is decreased 1°C per cycle from 75°C to a 'touchdown' at 55°C; 24 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 1 min; and a final extension at 72°C for 10 min. The reaction was performed using a EDC-810 thermal cycler (Eastwin, China). The desired PCR product (approximately 500 bp) was excised from 1.0% (wt/vol) agarose gel, purified using E.Z.N.ATM Gel Extraction Kit (Omega, USA) and ligated into pGEM-T Easy vector (Promega, USA). The ligation products were transformed into the competent *E.coli* DH 5α. Ampicillin (100 µg/ml) and a blue-white selection [31] were used to choose the required transformants.

PCR–RFLP of Insert Fragments

Thirty white clones were selected randomly and the plasmids DNA were prepared. The insert fragments of these

plasmids were amplified using B2F and B2R mentioned above. The 20 μl reaction mixture contained 300 nM of each primer, 5 ng plasmids DNA, 2 μl 10× *rTaq* reaction buffer, 200 μM dNTPs and 1.25 U *rTaq* DNA polymerase (Tiangen, China). The PCR conditions were: pre-denaturation at 95°C for 2 min; 25 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 8 min. The PCR products were digested by restriction enzymes *Alu*I, *Msp*I, respectively, in a total reaction volume of 20 μl according to the manufacturer's instructions (Takara, Japan). The bands were separated on 3% (wt/vol) standard agarose gel.

Partial Subtilase Genes Sequencing

DNA sequencing was performed using ABI PRISM kits and an ABI PRISM 3730 DNA Analyzer (Perkin Elmer) in Sangon Biotech Co. (Shanghai, China). The obtained nucleotide sequences of partial subtilase genes are available from GenBank(accession numbers in parenthesis) C1(HQ189536), C2(HQ189537), C6(HQ189538), C7(HQ189539), C8(HQ189540), C10(HQ189541), C11(HQ189542), C18(HQ189543), C19(HQ189544), C21(HQ189545). The similarity analysis was conducted on NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast/tools).

Phylogenetic Analysis

Using MEGA version 4.0 [35], an unrooted neighbor-joining [30] phylogenetic tree was constructed after the alignment by using CLUSTAL W 2.0 [17]. The statistical significance of the tree branches was evaluated by bootstrap analysis [10] of 1000 trees. The tree was constructed based on the catalytic domain amino acids sequences of subtilases [33] and the sequences of this study. All the sequences were derived from GenBank database.

Results and Discussion

PCR–RFLP Analysis of the Partial Subtilase Gene Sequences

Touchdown PCR was used to amplify the partial subtilase sequences because of its higher specificity and sensitivity in PCR amplification with the complexed metagenomic DNA [8, 16]. Consensus-degenerate primers B2F and B2R were selected as reported before [1], and by using the metagenome DNA sample from biogas digester as a template, about 500 bp DNA fragments were obtained by PCR (Fig. 1), which is in good coincidence with the length between the catalytic His and the catalytic Ser of subtilase gene sequences used for primer design [1]. There was also

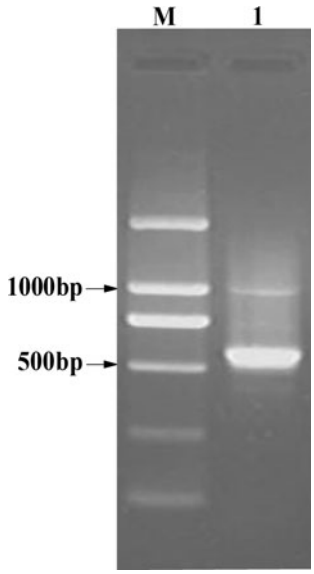

Fig. 1 PCR products of partial subtilase gene. M: 100 bp DNA ladder; *Lane 1*: PCR products

a faint band found at 1000 bp. The authors thought it may be a nonspecifically band which is a common phenomenon in PCR with metagenomic DNA using the degenerate primers. The targeted DNA was purified from gels, and was cloned into TA vector. Of the thirty white clones selected randomly, twenty-eight contained insert fragment. The positive clones were used in PCR–RFLP of the partial subtilase genes with two different restriction enzymes. Thirteen distinguished patterns were identified from the PCR–RFLP analysis (Fig. 2). The other 15 clones had patterns that matched one of the patterns found amongst the thirteen clones that were further analyzed (Table S1). The result suggested that the partial subtilase genes isolated from the metagenome DNA of biogas slurry may be diverse. In order to evaluate subtilase diversity the authors covered, a rarefaction curve about the unique RFLP patterns was drawn. As shown in Fig. S1, the unique RFLP

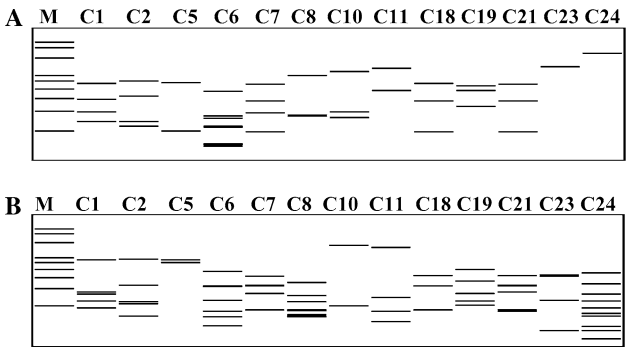

Fig. 2 Electronic electrophoresis results after digestion with the restriction enzymes Alu I and Msp I. M: 50 bp DNA Ladder 1000, 750, 500, 300, 250, 200, 150, 100, 50 bp

Table 1 Similarity analysis of partial subtilase ammino acid sequences

Clone no.	Length (bp)	Closest NCBI match for:			
		Enzyme accession no. organism identity (%)			
C1	549	Peptidase S8 and S53 subtilisin, kexin, sedolisin	YP_003641066	*Thermincola* sp.	39
C2	546	Subtilisin-type proteinase	ZP_01170337	*Bacillus* sp.	46
C6	552	Extracellular alkaline serine protease	YP_002247839	*Coprothermobacter proteolyticus*	53
C7	510	Peptidase families S8 and S53 domain protein	ZP_05058875	*Verrucomicrobiae bacterium*	49
C8	477	Subtilisin Carlsberg	YP_002368297	*Bacillus cereus*	56
C10	516	Serine protease	YP_003558468	*Shewanella violacea*	61
C11	564	Extracellular alkaline serine protease	YP_002247839	*Coprothermobacter proteolyticus*	49
C18	423	Peptidase families S8 and S53 domain protein	ZP_05058875	*Verrucomicrobiae bacterium*	47
C19	528	Peptidase S8 and S53 subtilisin, kexin, sedolisin	YP_847029	*Syntrophobacter fumaroxidans*	44
C21	552	Peptidase families S8 and S53 domain protein	ZP_05058875	*Verrucomicrobiae bacterium*	50

pattern number showed no increase with the increase of clones analyzed. This meant that the authors have captured the majority of the subtilase genes which can be amplified by using the B2F and B2R.

Partial Subtilase Gene Sequences Analysis

Thirteen insert fragments with distinguished PCR–RFLP patterns were sequenced (about 500 bp). BLAST search analysis of the nucleotide sequences revealed that none of the partial genes the authors identified showed significant similarity against the non-redundant Nucleotide database of NCBI. Blastp search analysis of the deduced amino acid sequences revealed that with the exceptions of C5, C23, and C24, ten out of the thirteen sequences were related to serine protease, which were named as C1, C2, C6, C7, C8, C10, C11,C18, C19, C21 (Table 1). None of the deduced amino acid sequences of C5, C23, and C24 showed significant similarity against the non-redundant database (data not shown). The deduced amino acid sequences of the remaining ten showed moderate identities to the existing members of subtilases family, ranging from 31 to 61%. As shown in the Table. 1, five out of the ten (C1, C7, C18, C19, and C21) showed sequence similarity with peptidase families S8 and S53 domain protein from *Thermincola* sp., *Verrucomicrobiae bacterium* or *Syntrophobacter fumaroxidans*(39, 49, 47, 44, 50% identity, respectively), which are common in spring [34], anaerobic granular sludge [13] or marine environments [36]; C6 and C11 showed similarity with extracellular alkaline serine protease from *Coprothermobacter proteolyticus* (53, 49% identity, respectively), which was isolated firstly from a methanogenic enrichment [22, 24]; C2 and C8 showed similarity with subtilisin from *Bacillus* sp. (46, 49% identity, respectively); C10 showed higher similarity with a serine protease from *Shewanella violacea* (61% identity), which was isolated from sea sediment [20]. In order to evaluate the similarity between these genes, a multiple sequence alignment was conducted. As shown in the Fig. S2, all the ten identified sequences contained the B2F and B2R. Although, Figs. 2 and 3 suggest that the sequences of C7, C18, and C21 are very similar; they are actually different in length and nucleotide composition.

To better understand the subtilase genes diversity and their phylogenetic relationships among the subtilase superfamily members, a phylogenetic tree was constructed based on the deduced amino acid sequences between the catalytic domains of subtilases (Fig. 3). The ten deduced

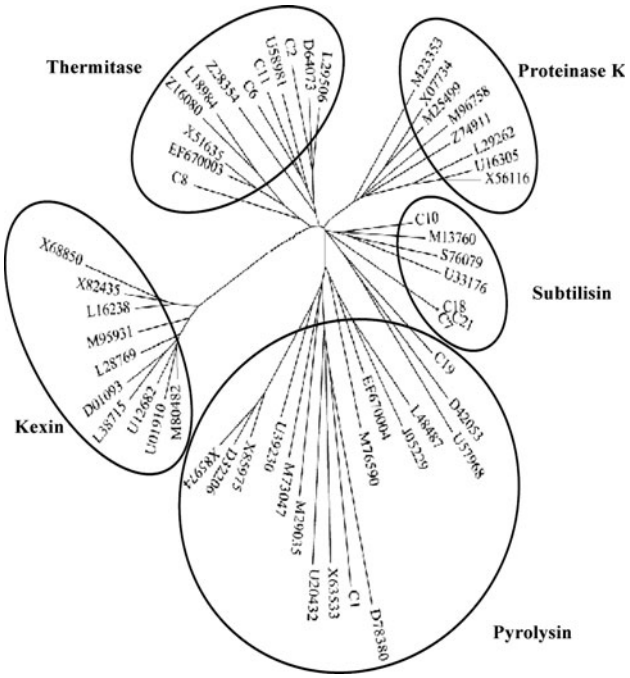

Fig. 3 Phylogenetic tree of subtilase genes based on partial sequences of amplified subtilase genes and closely related sequences. Different families of the subtilase superfamily were represented by Pyrolysin, Subtilisin, Kexin, Thermitase, Proteinase

amino acid sequences of the new partial subtilase genes clustered into three subtilase families. C2, C6, C8, C11 were clustered in the family of Thermitase; C7, C10, C12, C18 were grouped into the family of Subtilisin; C1, C19 were clustered in the family of Pyrolysin.

It was obtained ten distinguished partial subtilase gene sequences, which distributed in three subtilase families. These results indicate the great diversity of subtilase genes in the biogas digester. Based on the partial subtilase gene sequences obtained in this study, it is quite possible that we obtain the complete subtilase gene sequences by use of genome walking PCR [1]. So this study laid a good foundation for the application of subtilase in processs of biogas fermentation.

Specificity and Universal Application of Subtilase Gene Primers

These results demonstrated that the degenerate primers the authors used in this study for partial subtilases gene amplification is an efficient tool for molecular detection of subtilase genes from metagenomic DNA. Although ten out of thirteen partial sequences the authors isolated were related to the subtilase genes, some of subtilase genes may not be amplified with just only one pair of degenerated primers. It has been reported that besides the classic catalytic Ser/His/Asp triad, there are some nonclassic variations to the catalytic triad, such as Ser/His/Glu, Ser/His/His, Ser/Glu/Asp, Ser/Lys, and Ser/His [9, 23, 32]. A greater diversity of subtilase genes could be discovered if a series of degenerate primers specific for different subtilase catalytic triad were designed and used.

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