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Molecular Diversity of Methanogen in Moderate Temperature Anaerobic Fermentation Process of Water Hyacinth

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It is to collect the biogas slurry in the moderate temperature anaerobic fermentation process of water hyacinth during the peak biogas generation period for extracting total genomic DNA, amplify 16S rRNA gene in total genome with 16S rDNA specific primer of methanogen, establish 16S rDNA library and analyze 16S rDNA in the library with RFLP method. The results indicated that some 1.2 Kb of target bands may be obtained by amplifying the extracted genome with 16S rDNA specific primer of methanogen; HaeIII was used to conduct restriction enzyme analysis about 16S rDNA fragments and through comparative statistics of its restriction enzyme mapping, we have obtained 23 kinds of RFLP.

Keywords: Water hyacinth, Peak fermentation period, 16S rDNA, Incision enzyme HaeIII.

INTRODUCTION

Methanogen is a strict anaerobic archaea and due to its strict anaerobic feature and long culture cycle, it is very difficult to analyze the community structure of methanogen with the traditional culture and separation technology and it cannot completely and accurately reflect the community structure of methanogen and its diversity in the system[1]. With the development of modern molecular biology, analyses based on specific amplification, cloning and base sequence of 16S rDNA has been widely applied in microbial ecology studies, making it possible to study the community structure biodiversity in the anaerobic system in a fast, complete and accurate manner[2]. In this article, through PCR, we selectively amplified 16 SrDNA of methanogen in materials during peak biogas fermentation period with water hyacinth as the raw material, established 16S rDNA library and selected HaeIII restriction endonuclease to conduct RFLP to 16S rDNA in the library and analyzed the community structure of methanogen biogas slurry during the peak biogas generation period. It provided bases for diversity of methanogen during the peak biogas generation period.

EXPERIMENTAL

Collecting samples: We have collected the biogas slurry samples during the peak biogas generation period in the moderate temperature anaerobic fermentation process of water hyacinth and it is to adopt the method of sampling at multiple locations, so that the samples taken can represent the communities of mathanogen in the entire reactor. It is to fill the bottle with samples taken, screw the cover tightly and collect the sample *via* centrifugation (12000 g, 40 C, 10 min) and store at -20 °C till extraction of total DNA.

DNA extract: 100 mmol *tris*·HC1 (pH 8), 100 mmol of EDTA·Na (pH 8), 100 mmol solution of sodium phosphate (pH 8), 1.5 mol NaCl, 10 g/L CTABo

1 × Homogenate buffer: 100 mmol *tris*·HC1 (pH 8), 100 mmol EDTA·Na (pHB.0), 200 g/L SDS, 10 g/L CTABo

Washing buffer: 0.33 mol *tris*·HCl (pH 8), 1 mmol EDTA·Na (pH 8)

Lysis buffer: 100 mmol *tris*·HCl (pH 8), 100 mmol EDTA·Na (pH 8), 1.5 mol NaCl, 10 g/L CTAB; lg/L sodium pyrophosphate; 200 g/L SDS; Protease K (20 mg/mL); lysozyme (50 mg/mL).

Chloroform:isoamyl alcohol (v:v = 24:1); isopropanol; sodium acetate (pH 5.2).

Nucleic acid electrophoresis buffer: 1 × TAE buffer.

Method for extraction of DNA from biogas slurry: We followed Huang *et al.*[3] and Zhang *et al.*[4] method. Weigh about 20 g biogas slurry sample, add 50 mL precooled aquae sterilisata, mix evenly and homogenize in a homogenizer. During the process of homogenization, there should be time intervals to prevent temperature from being excessively high, then it is to supplement 2 × blending buffer, adequately oscillate and mix evenly; centrifugate for 10 min at low speed (2,000-3,000 g) under room temperature, collect the supernatant and transfer to another centrifugal bottle. Precipitate and then wash and

resuspend with 100 mL 1 × blending buffer, centrifugate and take the supernatant and combine with the previous time. Centrifugate the obtained supernatant under room temperature at high speed (10,000 g) for 0.5 h to recover somatic cells. The obtained cells are to be sequentially washed with 150 mL 1 % sodium pyrophosphate and 100 mL chrombath buffer, centrifugate to obtain sediment, resuspend and precipitate in 8 mL lysis buffer, add 160 μL lysozyme (50 mg/mL) and 20 μL protease K (20 mg/mL), water bath for 0.5 min at 37 °C; Add 1 mL 200 g/L SDS, water bath for 2 h at 65 °C, during which it is to gently turn upside down for even mixing every 15-20 min; Once reaction is completed, it is to extract with chloroform-isoamyl alcohol and precipitate with isopropanol; It is to recover, precipitate and wash, blow dry and dissolve in 200 μL sterilized ultrapure water.

PCR amplification of 16S rRNA gene

Primer: Met86F (5'-GCTCAGTAACACGTGG-3'), Met1340R (5'-CGGTGTGTGCAAGGAG-3').

The amplification system is as follows:

10 × Taq polymerase reaction buffer	2.5 μL
Distilled NTP (20 mmol/L)	2.5 μL
5' end primer (25 pmol/μL)	0.5 μL
3' end primer (25 pmol/μL)	0.5 μL
Mg^{2+} (25 mmol/L)	2.5 μL
Biogas slurry total DNA	1.0 μL
Taq DNA polymerase (5U/μL)	0.3 μL
Double distilled water	16.7 μL
Total volume	25 μL

Reaction conditions: first circle, pre-denaturalize for 3 min at 94 °C; denaturalize for 30 s at 94 °C, renaturize for 30s 58 °C, extend for 90s at 72 °C, 30 circles in total and finally extend for 10 min at 72 °C.

It is to repeatedly amplify 10 tubes of biogas slurry DNA samples, mix the amplified products evenly to eliminate the bias of single amplification.

Purification recovery of PCR amplified products: It is to be conducted in accordance with the operating instructions on the gen purification recovery reagent box.

Preparation of highly competent cells: It is to cut a loop of *E. coli* DH5a from the strains preserved at -70 °C, culture overnight at 37 °C, pick out 2-4 colonies with diameter being about 2 mm, vaccinate to 50 mL SOB culture media, violently oscillate in 18 °C and culture till OD_{600} is 0.6. It is to put triangular flask on ice, cool for 10 min, centrifugate at 40 °C 2500 × g for 10 min, precipitate in 20 mL TB buffer, add DMSO to final concentration of 7 %, put on ice for 10 min, put in 1.5 mL eppendorf tubes by 1 mL each, put into liquid nitrogen and store for over 48 h and it will be highly competent cells.

T/A cloning of PCR products[5]: It is to mix 1.2 Kb 16SrDNA amplified fragments (with concentration being about 12-14 ng/μL) obtained from purification recovery with pMDlB-T Vector by 2:1 mole ratio and under the effects of T4 ligase, it is to stay the night at 16 °C. The enzyme linked system is as follows:

pMD18-T Vector	1 μL
PCR products	4 μL
Ligation solution (Including T4DNAligase)	5 μL
Total volume	10 μL

Conversion of enzyme linked products: It is to take 200 μL *E. coli* DH5a highly competent cells preserved in liquid nitrogen, transfer to sterilized centrifugal tubes, add 2 μL enzyme linked product in each tube, gently rotate to mix the components evenly, put in ice for 0.5 min, put the centrifugal tube into circulating water bath preheated to 42 °C, put there 90s exactly and not shake the centrifugal tube. It is to quickly transfer the centrifugal tube to the ice bath, cool the cells for 1-2 min, add 800 μL SOC culture media in each tube, heat the culture media to 37 °C with water bath, then transfer centrifugal tube to 37 °C shaking table, incubate for 45 min to resuscitate the bacteria and express plasmid-encoded antibiotic resistance gene.

Constitution, examination and preservation of 16S rRNA gene library: It is to pick out the Leukoplakia transformants cloned by 16S rRNA gene in the course of fermentation, pick out 120 spots and plant on antibiotic plate, culture at 37 °C till colonies are grown. It is to randomly select some transformants for small extraction of plasmid and it is to examine the insertion of extraneous fragments. The cultured plates will be preserved at 4 °C for future use.

PCR amplification of cloned and inserted fragments of the library: In the experiment, it is to directly take thallus transformants as the template, with universal primer of pMD18-Vector directly PCR amplifying 1.2 kb external source inserting into fragments. It is to amplify its inserted fragments with 120 clones in each library as the template.

Primer:

BcaBEST Primer RV-M
5'-GAGCGGATAACAATTTCACACAGG-3'

BcaBEST Primer M13-47
3'-CGCCAGGGTTTTCCCAGTCACGAC-3'

The amplifying reaction system is as follows:

10 × Taq polymerase reaction buffer	2.5 μL
Distilled NTP (20 mmol/L)	2.5 μL
BcaBEST Primer RV-M (25 pmol/μL)	0.5 μL
BcaBEST Primer M13-47 (25 pmol/μL)	0.5 μL
Mg^{2+} (25 mmo1/L)	2.5 μL
Thallus	Trace (Picking out with sterilized toothpick)
Soil temperature 20 (10 %)	2 μL
Taq DNA polymerase (5U/μL)	0.3 μL
Doubled distilled water	14.2 μL
Total volume	25 μL

Reaction conditions: First circle, pre-denaturalize for 5 min at 94 °C; denaturalize for 30 s at 94 °C, renaturize for 30 s at 58 °C, extend for 1 min at 72 °C, 35 circles in total and finally extend for 20 min at 72 °C.

16S rDNA Fragment of Restrictive Enzyme Digestion in the library: It is to conduct enzyme digestion analysis with incision enzyme HaeIII.

Enzyme digestion system:

HaeIII	0.3 μL
10 × L buffer	1 μL
PCR products	5 μL
Double distilled water	3.7 μL
Total volume	10 μL

37 °C water bath enzyme digestion for 3 h

It is to conduct 2 % agarose gel electrophoresis to HaeIII enzyme digestion products and it is to conduct statistical analysis after staining with ethidium bromide.

RESULTS AND DISCUSSION

Extraction of biogas slurry DNA: After electrophoresis, the vast majority of DNA extracted from biogas slurry are high molecular, with the fragmental length being greater than 23 Kb and very few of which are broken into small fragments (refer to Fig. 1).

16S rDNA amplification: It is to dilute the extracted DNA by 50 times with sterilized water, amplify with methanogen16S rDNA specific primer and obtain some 1.2 Kb target bands (Fig. 2).

Type of 16S rDNA PCR RFLP maps: It is to conduct restriction enzyme analysis about 16S rDNA fragments with Hae III, remove part of the unclear or uncut bands and select 100 cloned Hae III restriction enzyme maps of 16S rDNA. Through comparative statistics of cloned Hae III restriction enzyme maps, we obtained 23 types of RFLP (Table-1). Different

TABLE-1
HAEIII RESTRICTION ENZYME MAP TYPES AND STATISTICS OF 16S rDNA

Sample No.	Type of Restriction Enzyme Map HaeII	Type Statistics	Sample No.	Type of Restriction Enzyme Map HaeII	Type Statistics
1	a	1	51	q	17
2	b	2	52	j	10
3	c	3	53	d	4
4	b	2	54	f	6
5	b	2	55	e	5
6	d	4	56	r	18
7	e	5	57	q	17
8	a	1	58	q	17
9	d	4	59	s	19
10	f	6	60	i	9
11	b	2	61	b	2
12	g	7	62	t	20
13	h	8	63	t	20
14	b	2	64	g	7
15	e	5	65	l	12
16	f	6	66	t	20
17	i	9	67	r	18
18	j	10	68	s	19
19	k	11	69	p	16
20	d	4	70	e	5
21	l	12	71	n	14
22	j	10	72	m	13
23	j	10	73	u	21
24	l	12	74	l	12
25	l	12	75	e	5
26	l	12	76	b	2
27	m	13	77	a	1
28	m	13	78	n	14
29	f	6	79	s	19
30	h	8	80	v	22
31	j	10	81	p	16
32	e	5	82	f	6
33	n	14	83	h	8
34	a	1	84	h	8
35	g	7	85	u	21
36	m	13	86	v	22
37	c	3	87	r	18
38	e	5	88	s	19
39	o	15	89	w	23
40	a	1	90	s	19
41	d	4	91	c	3
42	g	7	92	w	23
43	p	16	93	s	19
44	o	15	94	t	20
45	p	16	95	f	6
46	h	8	96	a	1
47	f	6	97	w	23
48	e	5	98	v	22
49	b	2	99	s	19
50	b	2	100	j	10

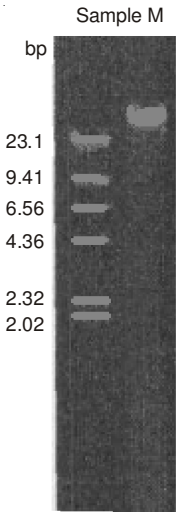

Fig. 1. Biogas slurry total DNA electrophoretogram

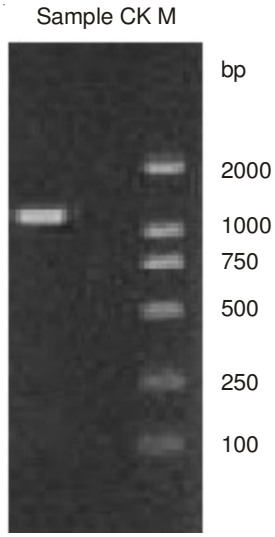

Fig. 2. Methanogen 16S rDNA electrophoretogram

kinds of restriction enzyme electrophoretograms are shown in Fig. 3.

Conclusion

Through electrophoresis, it was found that the fragmental length of DNA extracted from biogas slurry is greater than 23 Kb; By amplifying extracted genome DNA with mathanogen 16S rDNA specific primer, we may obtain about 1.2 Kb of

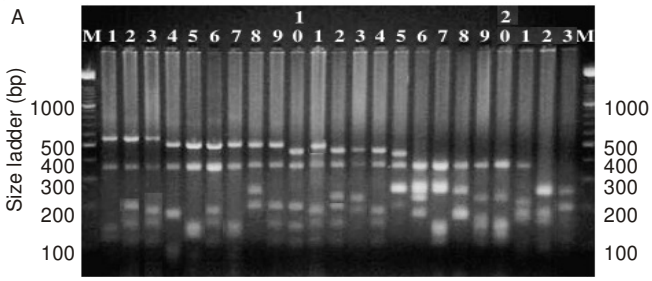

Fig. 3. Hae III restriction enzyme electrophoretogram

target bands; It is conduct restriction enzyme analysis to 16S rDNA fragments with Hae III and through comparative statistics of its restriction enzyme maps, we obtained 23 kinds of RFLP.

By reflecting diversity of methanogen communities in biogas slurry with cloning of mathanogen 16S rDNA, we may avoid deviations due to culture of the traditional culture method. It is particularly noteworthy that for such strictly aerobatic microbes as mathanogen, it may truly and conveniently reflect the structural composition and diversity of mathanogen in biogas slurry in normal position.

With total DNA of biogas slurry as the template and by amplifying 16S rDNA with PCR, it may somehow deviate from the truth due to deviations of PCR amplification. In the course of experiment, it was to repeatedly amplify and evenly mix the PCR products, which can minimize such deviations. Due to diversity of mathanogen in biogas slurry and in light of relevant literatures, it is to select about 110 clones to reflect the diversity of mathanogen in biogas slurry during the experiment. In this article, the author only conducted an analysis about diversity of methanogen in water hyacinth during peak biogas generation period of moderate temperature anaerobic fermentation, which cannot reflect the changes to diversity of methanogen in the whole fermentation process.

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