Using metagenomics tool to evaluate the enrichment efficiency of methanogens in marine sediment in Truong Sa archipelage, Khanh Hoa province, Vietnam

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Abstract. Methanogens (archaea bacteria that produce methan) play an important role in the anaerobic digestion of organic waste, however, in marine environment, the low density of methanogens makes anaerobic digestion very slow. In this study, we used methagenomics tool to evaluate the enrichment efficiency of methanogens communities in marine sediments as a basis for building a anaerobic microbial formulation to treat organic waste in marine environment. The results of determination of methanogen density by MPN method have shown that methanogen has been accumulated with higher density (up to $3.2.10^7$ MNP/ml) through 2 times of enrichment in artificial seawater with CH_3COONa 10 mM substrate. In addition, metagenomics data have also shown a decrease in the number of archaea species through each enrichment, indicating that methanogenic species have gradually dominated the microbial community. This is the first study on using metagnenomics tools to evaluate the enrichment of methanogens in marine sediment samples in Truong Sa archipelago, Vietnam. Metagenomics data help provide more reliable evidence in microbial studies, especially in obligate anaerobes such as methanogens.

1 Introduction

Human activities in Truong Sa archipelago have discharged a large amount of domestic waste into the ocean. In seawater, this waste degrades slowly due to the effects of high salinity and lack of suitable microbial resources [1]. Therefore, it is necessary to find a microbial formulation comprising of halophilic microorganisms capable of degrading organic waste in seawater to treat organic waste in Truong Sa archipelago. Compared with aerobic treatment, anaerobic digestion of organic waste has advantages such as: simple, does not require energy, generates less sludge [2]. Therefore, anaerobic digestion is a suitable solution for organic waste treatment in Truong Sa archipelago - where seawater has high salinity and limited energy resources.

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Anaerobic digestion is a methanogenic process in which methanogens are the main group of microorganisms. [3]. Currently many halophilic methanogenic strains have been isolated by enrichment method , but there is no report on evaluating enrichment efficiency through comparison of microbial communities using metagenomics tool [2].

Metagenomics is an effective tool in microbial diversity assessment. There have been many studies using metagenomics to assess microbial diversity in extreme environments such as geographical poles, very arid deserts, volcanoes, deep ocean trenches, [4]. In addition, metagenomics data can also be used to compare microbial communities in the same sample under different culture conditions or through different enrichments to assess the impact of different factors in culture to microbial communities [5].

2 Materials and methods

2.1 Materials

2.1.1 Sample

05 marine sediment samples were collected from different locations in Truong Sa Dong island (belonging to Truong Sa archipelago, Vietnam). Samples are mixed together to form a homogeneous sample (Pic 1.).

Pic 1. The location of Truong Sa archipelago in Vietnam (left) and Sampling locations at Truong Sa Dong island (right)

2.1.2 Media

The materials that were used in this study included:

Artificial seawater with CH_3COONa 10 mM substrate [6]

Zymo Research Kit (USA).

2.2 Methods

The study used the following methods:

Methanogen enrichment method: Methanogens in marine sediment samples were enriched by inoculating serum flasks containing artificial seawater with 10 mM CH3COONa substrate (ratio 10% w/v). Incubate statically in an incubator at 30°C. Subsequent enrichment was performed every 7 days during which 10% of the culture volume was transferred to a new serum flask. The methanogen density and the methane ratio are the evaluation basis for the enrichment process [3].

Counting the number of of methanogen by Most Probable Number (MPN) [7].

DNA extraction: the experiment was carried out using Zymo Research Kit (USA).

Metagenomics: V3 - V4 region metagenomics data used to compare microbial communities between enrichments.

3 Results and discussions

The efficiency of methanogen enrichment in marine sediments was directly evaluated through the methanogen density and the methane ratio of the enriched samples. In addition, the results demonstrating the presence of methanogen in the enrichment samples by metagenomics have provided high reliability assessments.

3.1 Features of the enrichment samples

Methanogen density and methane ratio were measured continuously for 7 days at each enrichment. The values were recorded in Table 1.

Table 1. Comparison of methanogen density and methane ratio between enrichments

Time (day)	**Methanogen density** (MPN/ml)			**Methane ratio** (%)		
	E1	**E2**	**E3**	**E1**	**E2**	**E3**
1	3	1.4.10	$1.7.10^2$	0	7	8
2	3	$1.4.10^2$	$2.9.10^3$	0	7	12
3	5	$1.8.10^2$	$2.1.10^5$	0	8	13
4	1.1.10	$2.2.10^2$	$1.1.10^6$	2	20	45
5	1.8.10	$1.1.10^3$	$1.4.10^6$	2	27	59
6	2.3.10	$2.0.10^3$	$1.5.10^7$	10	35	66
7	$1.2.10^2$	$2.2.10^3$	$3.2.10^7$	15	41	82

(E1, E2, E3 - 1st, 2nd, 3rd enrichment times)

Thus, through 3 times of enrichment in artificial seawater with a salinity of 35‰, halophilic methanogen was accumulated at a high density ($3.2 \cdot 10^7$ MPN/ml) and methane ratio accounted for 82% in the total gas produced. This result shows that methanogen is well adapted to enrichment conditions. The methanogen density and methane ratio obtained after each complete enrichment are similar to the results of Youngsukkasem S. *et al.* (2012) when enriching anaerobic sludge for encapsulation in alginate gels [3]. However, for a clearer assessment, we used metagenimics tool to compare microbial communities in each enrichment.

3.2 Comparison of microbial communities among enrichment samples based on metagenome data

The metagenome data of the enrichment samples were compared via: Evaluation and purification of data, species diversity, taxonomy.

3.2.1 Evaluation and purification of data

Sequencing quality was evaluated by FastQC1 tool, the results showed that both forward and reverse read segments were of good quality, with an average quality score of over 30. The length distribution showed most read passages are 220 bp in length. Short reads (less than 200 bp) will be removed using trimmomatic with MINLEN = 200. After-purification data were presented in Table 2. Sample E2 had the highest retained reads (78%), sample E1 had the lowest retained reads (63%). Thus, on average over 60% of the data were retained for the following analyses. Compared with similar Uritskiy G. *et al.* (2019) study on purification of metagenome data for halophilic microbiome [5], this retention rate is even lower, however, above 60% is a common rate in the purification of metagenomics data, which shows that the data used in the analysis have high reliability.

Table 2. Statistics of original data and after-purification data

Samples	Pre-reads	Length	After-reads	Retained Reads
E1	94.818	52-220	60.037	63%
E2	109.316	54-220	84.892	78%
E3	78.971	1-220	53.398	68%

3.2.2 Species diversity

Rarefaction curves are graphs that represent the number of OTUs observed as a function of the subsample size. The higher the slope of the rarefaction curve, the higher the diversity [4].

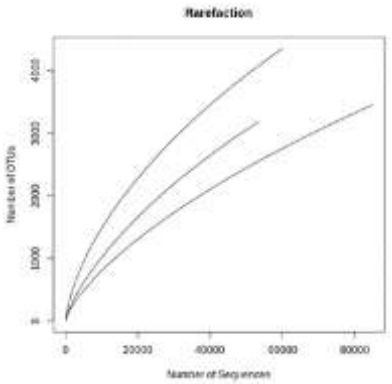

Fig 1. Rarefaction curves of samples E1, E2, E3 in order of top down

Figure 1 has shown that the slopes of the curves decrease in the order of E1, E2, E3, indicating that the diversity has decreased gradually through each enrichment.

3.2.3 Taxonomy

The total number of sequences decreased after each enrichment, indicating that microorganisms not adapted to the enrichment conditions were eliminated. In addition, the number of sequences in each taxonomy also differs between enrichment times. Compared with sample E1, sample E3 has about 27% reduction in total sequences, while the total number of archaeal sequences of E3 has increased 7 times compared to E1. The sequence number of bacteria decreased, while the sequence number of archaea increased, indicating

that halophilic methanogenic group was well accumulated through the enrichment times. Besides, the group of acidobacteria (which is the intermediate group that creates organic acid sources for the methanogen group) also thrived in the first and second enrichment times (E1, E2) and then decreased in the third time (E3) because at this time the methanogen group was dominant. (Table 3.).

Table 3. Sequence numbers in each taxonomy

Taxon	E1	E2	E3
Total	4353	3448	3180
Archaea	16	17	112
Bacteria	4336	3432	3086
Acidobacteria	285	208	79

4 Conclusions

Combining metagenomics data with an assessment based on methanogen density and methane ratio, the effect of halophilic methanogen enrichment from marine sediments has been elucidated. Methanogens obtained after the 3rd enrichment (E3) are the main source of microorganisms to create microbial formulation capable of decomposing organic waste in seawater.

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