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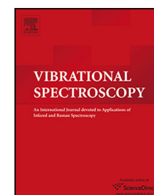

Discrimination of natural gas-related bacteria by means of micro-Raman spectroscopy

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A B S T R A C T

Methane-oxidizing bacteria (MOB) are a unique group of gram-negative bacteria that are proved to be biological indicator for gas prospecting since they utilize methane as a sole source of carbon and energy. Herein the feasibility of a novel and efficient gas prospecting method using Raman spectroscopy is studied. Confocal Raman spectroscopy is utilized to establish a Raman database of 11 species of methanotrophs and other closely related bacteria with similar morphology that generally coexist in the upper soil of natural gas. After strict and consistent spectral preprocessing, Raman spectra from the whole cell area are analyzed using the combination of principal component analysis (PCA) and Mahalanobis distance (MD) that allow unambiguous classification of the different cell types with an accuracy of 95.91%. The discrimination model based on multivariate analysis is further evaluated by classifying Raman spectra from independently cultivated bacteria, and achieves an overall accuracy of 94.04% on species level. Our approach using Raman spectroscopy in combination with statistical analysis of various gas reservoirs related bacteria provides rapid distinction that can potentially play a vital role in gas exploration.

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1. Introduction

Natural gas, as an important resource, is formed under the soil when layers of paleontology are exposed to intense heat and pressure over thousands of years [1]. It is mainly consisted of methane and a small amount of ethane, butane. Under the pressure, the light hydrocarbons leak to the upper soil where the dominant bacteria feed on these gases. For example, methane-oxidizing bacteria are a very typical group of gram-negative that has unique ability to utilize methane as a sole source of carbon and energy [2]. Thus the microorganism diversity in the upper soil of gas reservoirs will be significantly different than other places. Several studies attempt to compare the microorganism diversity between reference and natural gas area, which demonstrate that methanotrophs can be used as biological indicator for microbiological prospecting of natural gas [3,4].

With the advantages of direct and cost-effective, microbial prospecting of oil and gas (MPOG) has showed great ability in predicting the underground reservoirs and appreciated by the petroleum exploration sectors all over the world. But the distinction of bacteria, a key step in the microbial prospecting, is still carried out by the conventional method based on physiological characteristic or other methods such as polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH) and 16S RNA. However, the requirement of multiple cultivation or the needs of species-specific probes as well as the strict process make these methods a demanding technique.

Thus micro-Raman spectroscopy combined with multivariate method provides a new alternative method, which achieved label-free, culture independent, fast and reliable detection of bacteria. Raman spectroscopy, as a vibrational spectroscopic technique, detects the molecular vibrations whose frequency originated from different type of chemical bonds and atoms, which demonstrate unique vibrational spectral pattern and called fingerprint-like vibrational spectrum. Besides, the insensitivity of Raman spectroscopy to the presence of water makes it more suitable for the study of biological sample than the other vibrational method, Infrared spectroscopy.

Since the potential application of Raman spectroscopy in distinction of single cell was found by Puppels et al. [5], it has been studied furtherly by different groups the last two decades. Studies show that the differences introduced by different culture

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conditions of bacteria such as temperature, growth media and age would not affect the discrimination of bacteria [6,7]. Even the slight spectral changes in different growth phase did not obscure the interspecies discrimination [8]. Therefore, several experimental distinction of food-related [9–11], water-related [12,13], plant associated [14] and the clinical relevant bacteria [15–17] were carried out successively. Especially, identification of bacteria in real sample was applied rapidly and directly without culture step in clinical field [18]. Besides, other researches like the influences of antibiotics [19], toxic organotin compound [20] and heavy metal effects [21] were also conducted by different groups. However, the distinction of gas reservoirs related bacteria by using Raman spectroscopy has not been reported till now.

In the present study, we evaluate the feasibility of Raman spectroscopy combined with statistical method in distinction of the methane-oxidizing bacteria with other coexist bacteria in the subsurface soil right above the gas reservoirs. In the first step, a reference database of eleven bacteria has been built up and a combination of unsupervised statistical methods, principal component analysis and Mahalanobis distance (PCA–MD), was applied for training the classification model after consistent spectral preprocessing. Then, the reliability of the model was evaluated by another set of spectral data, which are collected from independently cultured bacteria.

2. Material and method

2.1. Bacteria strains, growth culture

Eleven bacterial species were selected for the study, which include *Methylomonas clara* (ATCC 31226), *Methylococcus capsulatus bath* and *Methylosinus trichosporium* (provided by professor She in Chang Jiang University), *Bacillus butanolivorans* (provided by China Agricultural University), *Bacillus cereus* (CCTCC AB 207805), *Pseudomonas arsenicoxydans* (CCTCC AB 2013630), *Flavobacterium aquatile* (CCTCC AB 2011070), *Acinetobacter lwoffii* (CCTCC AB 2014262), *Xanthomonas campestris subsp badrii Dye* (CCTCC AB 96024), *Agrobacterium rhizogenes Conn* (CCTCC AB 94020) and *Rhizobium hainanense* (CTCC AB 209172).

Methanotrophs were cultivated in NMS media, 1 L NMS media include $MgSO_4$ 0.4886 g, $CaCl_2{\cdot}2H_2O$ 0.1153 g, KNO_3 1.0 g, EDTA Fe 0.004 g, trace element 0.5 mL, KH_2PO_4 0.272 g, $Na_2HPO_4{\cdot}12H_2O$ 0.717 g, 1 L H_2O with 1 mL methanol (pH 6.8).

Bacillus butanolivorans was cultivated in PY2 media. 1 L PY2 media include K_2HPO_4 1.55 g, NaH_2PO_4 0.85 g, NH_4Cl 2.0 g, $MgCl{\cdot}6H_2O$ 0.075 g, $(NH_4)\ _2SO_4$ 0.1 g, $ZnSO_4{\cdot}7H_2O$ 4.4 mg, $CaCl_2$ 1.108 mg, $MnCl_2 \cdot 4H_2O$ 1.012 g, $FeSO_4{\cdot}7H_2O$ 0.998 mg, $(NH_4)\ _2MoO_4{\cdot}4H_2O$ 0.22 mg, $CuSO_4{\cdot}5H_2O$ 0.341 mg, $CoCl_2{\cdot}6H_2O$ 0.322 mg with 5 mL butanol.

Other soil bacteria were cultivated in LB media and R2A media (Becton, Dickinson and Company, USA).

2.2. Sample preparation

The single bacteria should not be destroyed during the sample preparation for Raman measurement. So, one milliliter of bacteria sample was fixed with 20% formaldehyde about 20 min and centrifuged for 5 min at 3500 rpm with sterilized distilled water for three times. The supernatant was discarded and the resulting pellet was suspended in 1 mL of sterilized distilled water and kept in 4 °C. Before Raman measurement, 2 μL of centrifuged bacterial suspension was dropped on aluminum-coated slides and air-dried at room temperature for 15 min. Each aluminum-coated slide was washed with deionized water and methanol to remove any residue from slides.

2.3. Raman microspectroscopy

All Raman spectroscopic measurements were performed with Jobin Yvon HR 800 confocal Raman spectrometer (French). He–Ne laser at 632.8 nm was used for excitation. By using 100× object lens (NA 0.9, Olympus MPlan, Japan), the laser beam was focused on the sample with a laser power of approximately 3 mW. 600 lines/mm grating with thermoelectrically cooled CCD camera was applied. The pinhole of 200 μm was chosen to get better signal of single bacteria. Accumulation time of 30–60 s and a wavenumber range of 400–3200 cm^{-1} were set for a single bacterial

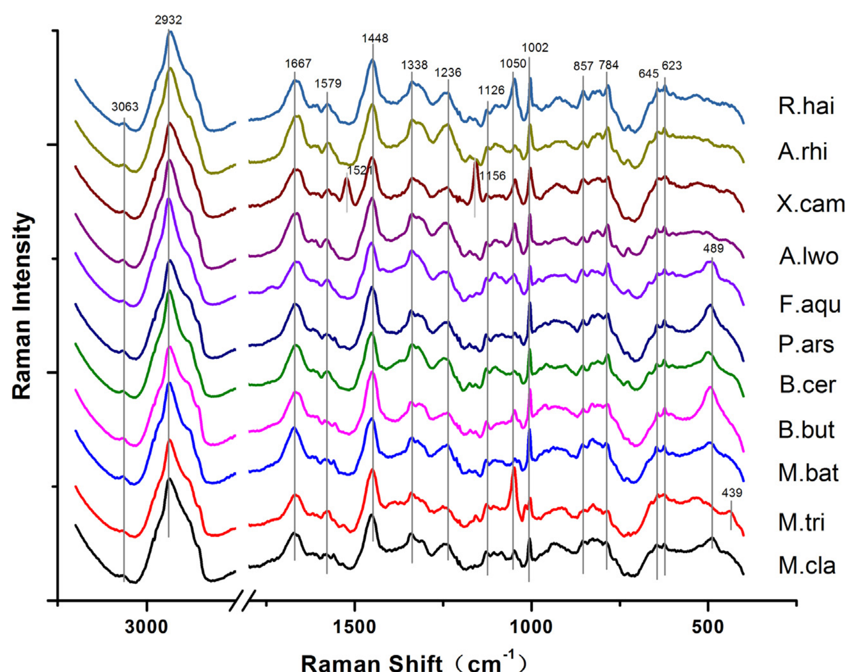

Fig. 1. Processed and normalized mean spectra of all bacterial species.

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