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Carbon isotope fractionation studies of bacterial strain *Rhodococcus rhodochrous* MTCC 291

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Abstract

Carbon isotope fractionation associated with the aerobic consumption of propane (C3) were determined using *Rhodococcus rhodochrous* MTCC 291 bacterial strain to estimate the amount of hydrocarbon oxidized using GC, fractionation of $\delta^{13}C$ carbon isotopes of propane and CO_2 using GC-C-IRMS and growth of bacteria by UV-Visible Spectrophotometer. The initial $\delta^{13}C$ isotopic value of propane was -34 ‰ and after incubation the changes of the isotopic values have been determined on 6th, 10th, 14th, and 17th days. The propane isotopic fractionation value was found to be maximum of -38.0 ‰ with an average value of -36.5 ‰ and a standard deviation of -1.22 ‰. The initial $\delta^{13}C$ isotopic value of CO_2 was -19.601 ‰. The CO_2 isotopic fractionation value was found to be maximum of -29.153 ‰ with an average value of -26.859 ‰ and a standard deviation of -28.338 ‰. The consumption of propane gas was estimated using Gas Chromatograph. The initial concentration of propane in control was found to be 53 ppm. On incubation, the consumption of the propane gas was observed to be of 26 ppm. The carbon isotope fractionation presented here may be applied to estimate the extent of C1-C4 oxidation in natural gas samples, and should prove useful in further studying the microbial oxidation of these compounds in the natural environment.

Key words

Carbon Isotope, Propane–oxidation, GC-C-IRMS, *Rhodococcus rhodocrous* MTCC 291

Introduction

Hydrocarbon gases including methane (CH_4; C1), ethane (C_2H_6; C2), propane (C_3H_8, C3), and n-butane (n-C_4H_{10}, n-C4, also C4) are produced naturally in deep subsurface environments and represent the major components of natural gas. The hydrocarbons composing natural gas are produced primarily from the decomposition of organic material. When decomposition is biologically mediated, C1 is the dominant product (biogenic gas) and higher hydrocarbons are present only at trace levels. When gas production is due to thermal decomposition (thermogenic gas), C2-C4 are more abundant, frequently comprising 10% or more of the gas (Kinnaman *et al.*, 2007). Natural gas is widely distributed in the subsurface due to its high mobility and is mined from the subsurface in areas where geologic traps have led to gas accumulation (Hunt, 1996). Microbes are known to affect the distributions of hydrocarbons, primarily through their capacity to consume hydrocarbons in the presence of a suitable oxidant (Widdel and Rabus, 2001). The microbial consumption of C1 is referred to as methanotrophy. This process has been the subject of numerous investigations due to its importance in the C1 cycle and because of the unique biochemistry involved (Hanson and Hanson, 1996). Formolo *et al.* (2004) used the isotopic composition of carbonate-associated gas-hydrates in the Gulf of Mexico to argue for the importance of C2+ hydrocarbons to carbon cycling around thermogenic gas hydrates. Few other investigations have used the isotopic composition of C2–C4 to investigate the biogeochemical cycling of these gases, and no fractionation factors are currently available for their microbial consumption (Kinnaman *et al.*, 2007).

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In deep subsurface hydrocarbon reservoirs, the production of ^{13}C depleted, biogenic C1 is a major process mediated by anaerobic methanogens, most likely involving syntrophic bacteria that also utilize higher hydrocarbons (Zengler *et al.*, 1999). The isotopic composition in C2-C4 hydrocarbon gases is routinely used to infer the conditions at which the gas was formed (Whiticar, 1990). Preference for C3 degradation followed by C4 and then C2 has been noted as well (Boreham *et al.*, 2001), with particularly high levels of biodegradation in lower temperature (<60°C) reservoirs (Wenger *et al.*, 2002). In this work, we investigate the magnitude of aerobic consumption of C3 by propane oxidizing bacterial isolate *Rhodococcus rhodochrous* MTCC 291 in order to estimate the amount of hydrocarbon oxidized, fractionation of $\delta^{13}C$ carbon isotopes of propane and CO_2 and to estimate the growth of bacteria using UV-Visible Spectrophotometer.

Materials and Methods

Bacterial strain MTCC 291: The microorganism used in this study *Rhodococcus rhodochrous* MTCC 291 bacterial strain, was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh, and maintained on Nutrient agar slants by sub-culturing at regular intervals. Single strength ammonium mineral salts solution (Ronald and Lawrence, 1996) was prepared and 3ml of this salts solution was added to the 11ml capacity vials according to the experimental design, after addition of inoculum (50 µl, 1.0 OD cells). The vials were sealed with Teflon-lined butyl septa and aluminum crimp rings and partially vacuumed. To these vials 4 ml of hydrocarbon gas mixture (methane, ethane, propane, butane, n-butane) and 4 ml Zero air (purified atmospheric air) was added using gas tight syringe (Hamilton, Switzerland), these gases were obtained from Bharat Oil and Gas Corporation Ltd. India. The vials were incubated at 37°C in dark on an orbital shaker incubator set at 200 rpm. The growth parameters in the form of turbidity using U.V. Spectrophotometer (λ_{600nm}) were recorded on 6th, 10th, 14th and 17th days. The isotopic fractions were also recorded on 6th, 10th, 14th and 17th days.

Analysis of propane : The gas chromatograph analyses were carried on Varian 3380 Gas Chromatograph (GC) equipped with Flame Ionization Detector (FID). The column used was Porapak Q and the column oven temperature was operated in isothermal mode at 40°C. Injector and detector temperatures were maintained at 120° and 200°C respectively. Nitrogen was used as the carrier gas and 500 µl of gas sample extracted from the vials with Hamilton gas tight syringe and injected in to the column. The calibration of GC was carried out using an external standard with known concentrations of methane, ethane, propane, i-butane and n-butane. The quantitative estimation of propane in each sample was made using peak area measurement with respect to standard.

Analysis of light hydrocarbons: Carbon isotopic composition of light hydrocarbons ($\delta^{13}C_1$, $\delta^{13}C_2$ and $\delta^{13}C_3$) in soil samples is determined using GC-C-IRMS, which comprises of Agilent 6890 GC coupled to a Finnigan-Delta Plus^{XP} Isotope Ratio Mass Spectrometer via a GC combustion III interface. 1 ml of desorbed gas is injected to GC in splitless mode with Helium as carrier gas at fixed oven temperature of 28°C. The light hydrocarbon gases eluting from the GC column enters, combustion reactor maintained at 960°C where it gets converted to CO_2 and water. Nafion membrane tube is used to remove water, prior to the entry of CO_2 into the mass spectrometer. Reference standards were intermixed with samples to monitor instrumental performance. The carbon isotope ratio in the sample was determined by comparing isotope ratios with that of standard, NIST RM 8560 (IAEA NGS2) using ISODAT software. The carbon isotopic composition was reported in per mil (‰) relative to the PeeDee Belemnite (PDB). The precision of the isotopic analysis is ± 0.5 ‰.

Results and Discussion

The consumption of propane gas was estimated using GC analysis. The initial concentration of propane in control was found to be 53 ppm and on the growth of *R. rhodochrous* MTCC 291 strain, the propane consumption was observed. Further, on incubation, the consumption of the propane gas was observed to be of 26 ppm. The microbial consumption of C2–C4 hydrocarbons has been investigated previously, though not to the same extent as methanotrophy. C2–C4 alkane oxidation seems to be limited to a group of gram-positive organisms in the *Corynebacterium–Nocardia–Mycobacterium–Rhodococcus* group (Ashraf *et al.*, 1994), along with some gram-negative *Pseudomonas* species (Hamamura *et al.*, 1999). Some C3 consuming bacteria utilize monooxygenase enzymes to oxidize alkanes to alcohols by attacking the subterminal and terminal carbon positions (Ashraf *et al.*, 1994). A terminal oxidation pathway was determined for the C4 oxidizing *Pseudomonas butanovora* (Arp, 1999). Alkane monoxygenases also demonstrate broad specificity; it has been shown that C2–C4 grown bacteria can degrade chlorinated hydrocarbons (Hamamura *et al.*, 1997). *Pseudomonas putida* was recently identified as being capable of C3 and C4 oxidation using an alkane hydroxylase enzyme (Johnson and Hyman, 2006). The present study has been focussed on the fractionation of hydrocarbon gas mixture. The initial $\delta^{13}C$ isotopic value of propane is -34 ‰ and after incubation, the changes of the isotopic values have been determined on 6th, 10th, 14th and 17th day. The propane isotopic fractionation value was found to be maximum of -38.0 ‰ with an average value of -36.5 ‰ and a standard deviation of -1.22 ‰ (Table 1).

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Different pure cultures can show great variations in fractionation for similar biological processes. Morasch *et al*. (2002) found varying levels of carbon isotope fractionation during aerobic toluene degradation for bacterial strains with different enzyme mechanisms. Hall *et al*. (1999) concluded that the observed variation in fractionation during aerobic degradation of phenol and benzoate was due to differences in bacterial metabolic pathways and growth rates. Conflicting reports of the amount of fractionation occurring for polycyclic aromatic hydrocarbons (PAH's), summarized by Abrajano *et al.* (2004) imply that enzymes catalyzing similar reactions in different organisms display dissimilar fractionation factors.

Further, the carbon isotopic fractionation studies for $\delta^{13}C$ isotope have been studied as the end product of the carbon metabolism of propane oxidizing bacteria (*R. rhodochrous* MTCC 291) is CO_2. So it is crucial to study the carbon isotopic fractionation of CO_2 and also the amount of CO_2 evolved during the growth of *R. rhodochrous* MTCC 291 bacterial isolate. The initial $\delta^{13}C$ isotopic value of CO_2 was -19.601 ‰. The carbon dioxide isotopic fractionation value was found to be a maximum of -29.153 ‰ with an average value of -26.859 ‰ and a standard deviation of -28.338 ‰ (Table 1). The consumption of hydrocarbons and oxygen and the production of CO_2 all change the quantity of bulk gas and thus the mixing ratio of residual hydrocarbons. The consumption of oxygen and hydrocarbon are partially offset by the production of carbon dioxide (Kinnaman *et al.,* 2007).

The growth plot of *R. rhodochrous* MTCC 291 using UV-Visible Spectro-photometer is shown in Fig. 1. The growth parameters were measured using U.V. Spectrophotometer (λ_{600nm}), on 6th, 10th, 14th and 17th days. From the growth plot it was observed that the growth in the form of turbidity (O.D) of bacterial strain *R. rhodochrous* MTCC 291 was maximum on 10th day and gradual decrease in the growth rate for the consecutive days. *P. angusta* MTCC-225 was tested for gas mixture utilization. GC analysis showed that only propane was utilized in all the tested trials.

Table 1 : Values of carbon isotopic fractionation for propane and CO_2 in bacterial strain *Rhodococcus rhodochrous* MTCC 291

Days	δ C^{13} propane	δ C^{13} CO2
0	-35.5	-19.601
6	-36.0	-28.694
10	-38.0	-29.153
14	-35.1	-28.471
17	-37.6	-26.899
Average	36.57± -1.22 (-35.1 to -38)	-26.859 ± -3.98 (-19.601 to -29.153)

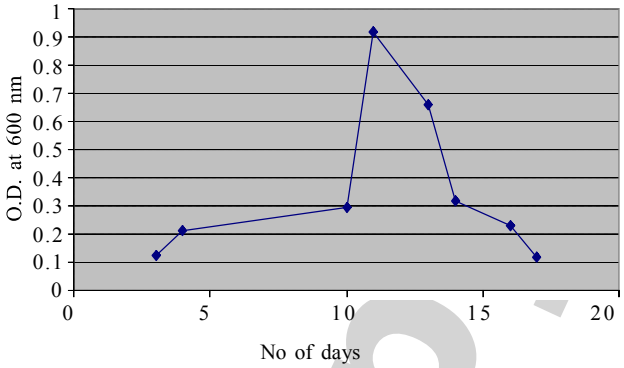

Fig. 1 : Plot showing the growth of *Rhodococcus rhodochrous* MTCC 291 using UV-Vis-Spectrophotometer

The propane-utilizing strains were more useful as standard strains in geomicrobial hydrocarbon explorations (Sreenivas *et al.*, 2005). Details regarding the microbes responsible for oxidation of C2–C4 in seep environments are lacking, though microbes capable of growth on C_2, C_3, and C_4 (Hamamura *et al.*, 1999) have been isolated from other environments. The present study reports preliminary findings of the bacterial oxidation of hydrocarbons and carbon isotopic fractionation studies. The study presents an excellent methodology for carrying out bacterial fractionation studies in various environmental conditions especially in seepage related areas and also in marine sediments. The carbon isotope fractionation presented here may be applied to estimate the extent of C1–C4 oxidation in natural gas samples and should prove useful in further studying the microbial oxidation of these compounds in the natural environment.

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