Madras Agric. J., 2019; doi:10.29321/MAJ 2019.000310

RESEARCH ARTICLE

Hydrogenotrophic activity of strain RWL1 similar to *Methanothrix soehngenii*

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Received : 07th November, 2019
Revised : 12th November, 2019
Accepted : 13th November, 2019

ABSTRACT

Methanogens are strictly anaerobic organisms that produce methane. Based on the nature of substrate utilization, they are classified into acetoclastic, hydrogenotrophic, and methylotrophic methanogens. *Methanothrix soehngenii* is a methanogen believed to be acetoclastic. A strain RWL1, similar to *M. soehngenii* with hydrogenotrophic activity, has been isolated, characterized and identified based on 16S rRNA sequencing. The strain RWL1 utilizes H_2+CO_2 (4:1) and has recorded methane production of 74.82%, thereby opening a new gate for substrate diversity studies. This study reveals the hydrogenotrophic nature of strain RWL1 clustered with *M. soehngenii*.

Keywords: *Anaerobe, methanogen, aceticlastic, hydrogenotrophic, substrate.*

INTRODUCTION

Carbon sequestration is an essential phenomenon for ecological balance. Life on earth is impossible without this phenomenon (Marris, 2006). Methanogenesis is one such process exhibited by a special group of organisms called methanogens. Methanogens are of three types, namely acetoclastic, hydrogenotrophic and methylotrophic methanogens which are classified and named after their nature of substrate utilization. Acetoclastic organisms use acetate as their carbon source for methane production whereas hydrogenotrophs utilize H_2+CO_2 and/or formate as their carbon source. Methylotrophic methanogens utilize methylated compounds for methane production (Enzmann *et al.*, 2018). *Methanothrix soehngenii* is an acetoclastic organism which was called as "fat rod" and found mostly in anaerobic digesters. Söhngen first isolated the organism in 1906 from anaerobic sludge (Söhngen, 1906). This was later characterized after three decades by Bryant in 1974 which has been further studied by Zhender *et al* (1980). Hitherto, studies were conducted and revealed that *M.soehngenii* is exclusively an acetoclastic organism. The study focuses on strain RWL1, isolated from the rice field, and found similar to *M. soehngenii* utilizing H_2+CO_2 (4:1) as substrate.

MATERIAL AND METHODS

Sample collection and isolation

Soil samples were collected from rice field (A1 plot) wetland, Tamil Nadu Agricultural University, Coimbatore (11°.005'13" N, 76°.93'045" E) under anoxic condition. Isolation was done by Hungate's roll tube technique using Modified Hungate's medium (Hungate, 1969) and incubated for 15 days in an anaerobic jar (Hi media laboratories) of 3.5 litres capacity. The anaerobic condition inside the jar has been maintained by anaerobic gaspak (Hi media laboratories).

Catalase activity

The colonies formed in the roll tube were tested for catalase activity. In this assay, a colony was picked from the roll tube using a sterile toothpick and placed on a clean dried slide. Over this colony, a drop of 3% hydrogen peroxide has been added. A rapid bubble formation within 5 to 10 seconds indicates positive results and the absence of bubble formation indicates negative (Reiner, 2010).

Enrichment of the cultures in hydrogenotrophic media

Then the cultures were enriched in H-complex media. The colony formed in the roll tube were picked using a hypodermic sterile syringe connected with a lumbar needle under anoxic condition by purging oxygen-free N_2 gas into the roll tubes. Then the colony was transferred to 120mL serum bottles containing 50 mL sterile H-complex media under a N_2 environment by Hungate's roll tube technique (Hungate, 1969). The anoxic condition inside the bottles was maintained by purging O_2-free N_2 gas. Enrichment was done in triplicate in 120 mL serum bottles. The headspace of the serum bottle occupies 58% of the total volume, in which 25% (15cc) of

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headspace was replaced with H_2+CO_2 (4:1). The ratio H_2+CO_2 (4:1) was brought by collecting both H_2 and CO_2 in the same bladder (size 5 latex bladder) at 1psi pressure and different periods. The time taken to fill the bladder at 1psi pressure is 40 seconds. Hence, the bladder was filled with H_2 for 32 seconds and CO_2 for 8 seconds respectively. Here, as H_2+CO_2 (4:1) was used as a substrate, 0.4% sodium bicarbonate was also added to enhance the reduced condition (Morii *et al.*, 1983).

Examining for growth and methane production

The cultures were assayed for their growth at 12 hr interval using Cary UV-vis spectrophotometer (Agilent technologies) at 660nm. Methane production of the isolates was assessed by passing 1cc of headspace gas to Nucon 5765 gas chromatograph with porapak-Q column of 2 mm diameter (mesh range of 60-80) and furnished with FID detector. Methane production was calculated by the formula (Eqn.1)

$$\text{Quantity in sample} = \frac{\text{Peak area of the sample}}{\text{Response factor}}$$

Where,

$$\text{Response factor} = \frac{\text{Peak area of the standard}}{\text{Quantity of the standard}}$$

CO_2 reduction capacity

The strain was also assessed for its CO_2 reduction capacity by growing in 50 mL H-minimal medium under different concentrations *viz*., 15%, 30% and 60% of H_2+CO_2 (4:1) in the headspace of 120 mL serum vials.

Characterization and identification

Morphological characters of the cultures were studied under bright field microscope. Gram staining was done using modified Hucker orBurke's method (Hucker, 1921). The DNA extraction was done by the CTAB method. The extracted DNA was amplified using methanogen specific primers Met 86f (5'-GCTCAGTAACACGTGG-3') and Met 915r (5'-GTGCTCCCCCGCCAATTCCT-3') as forward and reverse primers respectively (Zhou and Hernandez-Sanabria, 2009). The sequences of the amplified DNA were retrieved which were run in BLASTn to retrieve the similar sequences. Then the sequences were aligned using CLUSTALW and the phylogenetic tree was constructed using Maximum Parsimony at the bootstrap value of 1000 (Hall, 2013).

Growth in sodium acetate

Then the strains were grown in minimal media (Westermann *et al*., 1989) having 50 mM and 100mM sodium acetate. The strains were then assessed for their growth and methane production, as described above.

RESULTS AND DISCUSSION

Diversity in habitat

M. soehngenii was mostly reported in anaerobic digesters (Ten Brummeler *et al.*, 1985). Strain RWL1 was isolated from the rice field which extended not only its normal habitat but also its substrate diversity.

Diversified substrate utilization

The strain was grown in H-minimal media supplemented with H_2+CO_2 (4:1) as well as sodium acetate in different concentrations and aided in methane production. This reveals the nature of the substrate diversity of the strain RWL1.

H_2+CO_2 (4:1) utilization

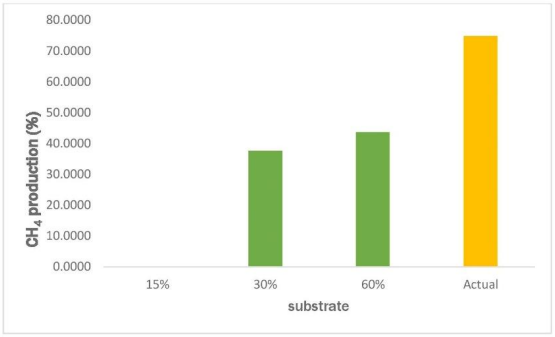

Figure 1. CH_4 production on different concentration of H_2: CO_2 (4:1) in the headspace

The strain exhibited good growth in H-complex media which was exclusive for hydrogenotrophs.

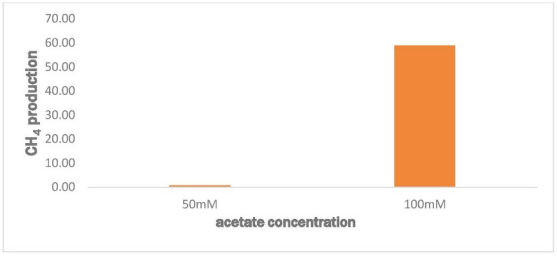

Figure 2. Methane produced at different concentrations of acetate

When grown in minimal media with different concentration (15%, 30%, 60%) of H_2+CO_2 (4:1) in headspace (Morii *et al.*, 1983), strain RWL exhibited methane production of 37.66% and 43.65% when supplemented with 30% and 60% of headspace gases respectively which was lesser than its methane production capacities under

Acetate utilization

When grown in 50mM and 100mM sodium acetate, the strain exhibited 10.80% and 58.96% of methane production respectively (Fig 2.). This condition reveals the substrate diversity nature of the strain. (Li *et al.*, 2013. This has to be studied

further, as it opens a new gate for substrate diversity and evolutionary information.

Relation with Methanothrix soehngenii

The strain is a long rod and responded negatively to the catalase test and gram stain (Huser *et al.*, 1982). The DNA of strain RWL1 was amplified under methanogen specific primer as described above. The phylogenetic tree (Fig.3.) reveals that the strain is closely related to *M. soehngenii*, an acetoclastic methanogen.

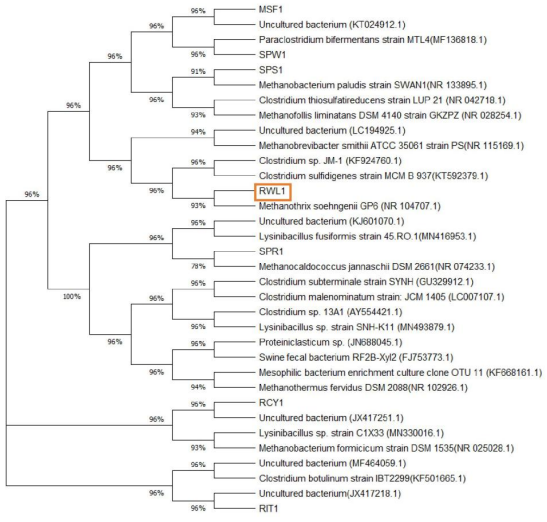

Figure 3. Evolutionary significance

M. soehngenii, has a filament-like structure, also called 'fat rod' whereas strain RWL1 has a characteristic rod structure. On the other hand, *M. soehngenii*, was proposed as a strict acetate utilizer whereas strain RWL1 utilizes H_2+CO_2 (4:1) as well as acetate for methane production. This condition adds essence to the metabolic diversity of *M. soehngenii*.

CONCLUSION

The slight modification in the shape of the cell as well as diversity, suggests that the strain could be exploited for hydrogenotrophic substrates under acetate limited conditions or there may be some metabolic adaptations unrevealed. Hence, the reason behind substrate diversity in terms of physiological as well as evolutionary relationships should be studied. This study also opens a new gate for interaction studies between acetoclastic and hydrogenotrophic methanogens. A new era of substrate diversity has been revealed by strain RWL1, isolated by rice field, similar to *M.soehngenii,* and this shift for substrate regains more significant physiological as well as evolutionary significance which is to be studied further.

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