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Comparative study on *Thermotoga maritima* and *Rhodobacter meghalophilus* for hydrogen gas production using crude glycerol from Biodiesel plants

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Hydrogen gas is a clean fuel with high calorific value. The current study is focused on production of hydrogen gas using *Rhodobacter meghalophilus* and *Thermatoga maritima* through anaerobic fermentation. Crude glycerol, by-product from biodiesel plant is used as carbon substrate due to its rich organic composition. Batch experiments have been carried out to study the impact of the inoculum size (1, 2 and 4 mL/L) and crude glycerol (5,10 and 15 mL/L) on the bacterial growth and hydrogen production rates by both the organisms. Inoculum size of 2 mL/L and crude glycerol of 15 mL/L of crude glycerol in medium for fermentation by *R. meghalophilus*, is found to produce 160 mL/L of gas with hydrogen production rate at 1.163×10^{-8} m^3/kg.s and substrate conversion efficiency of 43.28%. Anaerobic fermentation by *T. maritima* is found to produce 120 mL/L of gas with 2 mL/L of inoculum and 10 mL/L of crude glycerol with hydrogen production rate of 9.4×10^{-9} m^3/kg.s and substrate conversion efficiency of 23.88%. GC analysis of gas produced by *T. maritima* and *R. meghalophilus* shows 25% (v/v) and 19% (v/v) of hydrogen respectively. Thus, *R. meghalophilus* is found to acclimatize faster and exhibit better hydrogen production rate while *T.maritima* produces higher yield of hydrogen.

Keywords: Anaerobic fermentation, Crude glycerol, Hydrogen, *R. meghalophilus*, *T. maritima*

All life forms on earth need energy for their sustenance. The universe relies on the sun as primary source of energy. The discovery of fossil reserves fueled up rapid civilization and the world has witnessed many innovations and discoveries which are energy demanding developments. Unfortunately, the exhaustive consumption of fossil fuel reserves has stamped serious impacts on earth such as the rapid depletion of fossil reserves, at a rate much higher than the rate of their production and severe ecological imbalance leading to lethal environmental damage. The result of which has enforced man's attention on search for alternate energy. Various sources such as solar, wind, geothermal, tidal and biomass energy is being harnessed to meet the massive energy demands[1]. Hydrogen has proven its capacity as a source of energy for industrial production processes[2]. While hydrogen is currently produced in large scale chemically, it can be considered as a sustainable form of energy when produced by biological routes[3]. Biological hydrogen production is advantageous due to its potential for employing waste organic effluents from process industries, eco-friendly and is a clean, high energy carrier[4]. Hydrogen can be produced by bacteria which are phototrophic, facultative anaerobes and strong anaerobes through light dependent or independent fermentation processes[5]. Anaerobic fermentation processes have been promising method for hydrogen generation employing diverse bacterial species that are mesophilic, thermophilic, and hyper thermophilic[6]. The scope of the present research work is to produce hydrogen gas by anaerobic fermentation employing crude glycerol as carbon substrate. Crude glycerol is obtained as a by-product from biodiesel plant. It can be employed as substrate in anaerobic fermentation for bio conversion thereby avoiding the necessity of its pre-treatment. *R. meghalophilus and T. maritima* have capabilities of decomposing crude glycerol to produce hydrogen gas. However, depending on their habitat and metabolic pathway, the organisms differ in hydrogen production rate, substrate conversion rate and the yield of hydrogen gas. The effect of process parameters such as inoculum size and the volume of crude glycerol in the medium is studied to analyze and compare the hydrogen production rate, substrate utilization rate and the yield of hydrogen gas by both the organisms.

Pure glycerol produced by chemical methods, have significant role as a raw material in food, pharmaceutical, cosmetics and medicinal

applications[7]. Biodiesel is one of the promising alternate sources of energy. Glycerol is also obtained from biodiesel plants as its by-product, accounting for 10% of the biodiesel capacity[8]. Due to its composition containing free fatty acids, alkali, methanol, soap etc, crude glycerol is termed as an unvalued product in biodiesel process as it needs cost intensive treatment process, to be used in any application. Crude glycerol has an advantage of being rich in organic content, which can be subjected to microbial degradation that generates products such as lactic acid, ethanol and hydrogen as its primary metabolite[10]. Microbial treatment of crude glycerol for value added products, can add economic benefits to biodiesel plants. As hydrogen is a pure form of energy, conversion of crude glycerol to hydrogen gas is being researched extensively[11].

Many organisms have been studied for their efficacy to degrade crude glycerol and produce hydrogen[12]. *Rhodobacter meghalophilus*, a mesophilic bacterium and *Thermatoga maritima*, a thermophilic bacterium has shown potential to utilize glycerol as their carbon substrate and generate hydrogen gas through anaerobic fermentation process[12-14]. *Rhodobacter megalophilus JA194*, is a gram negative, ovoid shaped photo fermentative bacterium that belongs to the family *Rhodobactereraceae*[15,16]. *Thermatoga maritima*, is a bacterial species that belong to the family *Thermotogaceae*. The bacteria is a non-sporulating, rod shaped, gram-negative bacterium, capable of growing at high temperatures of 55 - 90°C[17].

Experimental Section

The bacterial culture of *Thermatoga maritima* was procured from Japan Culture of Microorganisms, Japan. The culture of mesophilic bacteria, *R. meghalophilus*, JA 194 was procured from Jawaharlal Nehru Technological University, Hyderabad. Crude glycerol used as carbon substrate for the microorganisms in fermentation studies, was procured from Biofuel Park, Gandhi Krishi Vignyan Kendra, University of Agricultural Sciences, Bangalore. The chemicals used for nutrient media preparation were of analytical grade, procured from SRL chemicals.

Media preparation for *Thermatoga maritima*

Thermatoga maritima is an extremophile. The natural habitat of extremophiles is deep hydrothermal bed under oceans. The media for *T. maritima* was prepared according to ATCC medium 43589, designed to facilitate its growth in an artificial sea environment. The following is the composition of the artificial sea water defined by ATCC medium 43589: NaCl (24.32 g/ L), $MgCl_2$ (5.14 g/L), $CaCl_2$ (1.14 g/L), KCl (0.69 g/L), $NaHCO_3$ (0.2 g/L), KBr (0.1g/L), H_3BO_3 (0.027 g/L), $SrCl_2$ (0.026 g/L), NH_4Cl (0.0064 g/L), NaF (0.003 g/L). To enhance the metabolic activity of the bacteria, the medium also defines the formulation of Wolfe's mineral solution, composed of $C_6H_9NO_6$ (1.5 g/L), $MgSO_4.H_2O$ (3 g/L), $MnSO_4.H_2O$ (0.5 g/L), NaCl (0.1 g/L), $NiCl_2.H_2O$ (0.1 g/L), $CaCl_2$ (0.1 g/L), $ZnSO_4$ (0.1 g/L), H_3BO_3 (0.01 g/L), $Na_2MoO_4.2H_2O$ (0.01 g/L).The artificial sea water was diluted with distilled water in the ratio 1:3 and the following nutrient supplements were added: soluble starch (5 g/L), K_2HPO_4 (0.5 g/L), $NiCl_2$ (2.0 g/L), NaCl (20.0 g/L), Yeast extract (0.5 g/L), Wolfe's mineral solution (30 mL/L) and resazurin (1 mg/L).

Media preparation for *Rhodobacter meghalophilus*

The nutrient medium for the mesophilic bacteria, *Rhodobacter meghalophilus* was prepared according to DSMZ medium 27 with yeast extract (0.3 g/L), C_2H_3O (0.5 mL/L), $C_4H_4Na_2O_4$ (1 g/L), $C_2H_7NO_2$ (0.5g/L), 0.1% of $C_6H_5FeO_7$ solution (5 mL/L), KH_2PO_4 (0.5 g/L), $MgSO_4.7H_2O$ (0.4 g/L), NaCl (0.4 g/L), NH_4Cl (0.4 g/L), $CaCl_2.2H_2O$ (0.05 g/L), vitamin B_{12} solution (0.4 mL/L), trace element solution (1 mL/L). The trace element solution consisted of $ZnSO_4.7H_2O$ (0.1 g/L), $MnCl_2.4H_2O$ (0.03 g/L), H_3BO_3 (0.3 g/L), $CoCl_2.6H_2O$ (0.2 g/L), $CuCl_2.6H_2O$ (0.01 g/L), $NiCl_2.6H_2O$ (0.02 g/L), $Na_2MoO_4.2H_2O$ (0.03 g/L).

Experimental procedure for batch fermentation studies

Batch experiments were carried out employing thermophilic bacteria, *T. maritima* and mesophilic bacteria, *R. meghalophilus*, for hydrogen gas production by anaerobic fermentation process. To study the potential of these two bacteria to generate hydrogen gas using crude glycerol as carbon substrate, parameters such as the inoculum size and the volume of crude glycerol in the growth medium were varied, while the temperature and medium *p*H were maintained constant in accordance with the bacterial growth conditions. As *T. maritima* is an extremophile, experimental batch trials were carried out at a temperature of 80°C and at medium *p*H of 6.8.*R. meghalophilus* being a mesophilic bacterium, batch trials were carried out at room temperature and the medium *p*H at 6.8. The parameters that were

subjected to study in the present work are the bacterial inoculum size and the volume of crude glycerol in the growth medium. Inoculum size for the fermentation process by both *T. maritima* and *R. meghalophilus* was varied as 1, 2 and 4 mL/L, while the crude glycerol volume added to the growth medium was varied as 5, 10 and 15 mL/L respectively. Their impact on the bacterial growth phase, volume of hydrogen gas generation, substrate conversion rate and percentage of hydrogen in the gas generated was analyzed. The experimental set up and the fermentation process by *T. maritima* and *R. meghalophilus*, is discussed below.

The batch scale experimental trials on *T. maritima* were carried out in a 3 neck jacketed fermenter of 0.5 L capacity. A hot plate with magnetic stirrer was used to maintain the fermentation temperature at 80°C. A J type thermocouple with an ON/OFF controller was used to monitor and control the temperature. 0.25 L of the growth medium, defined according to ATCC medium 45389, was prepared and the varying volume of crude glycerol was added accordingly for each batch trial. The prepared medium was autoclaved for 15 min at 121°C. The medium was cooled and inoculated with varying inoculum size of *T. maritima*, for different experimental batches. As the process is anaerobic, nitrogen was flushed through a tube in one of the necks of the fermenter to displace out the residual oxygen before each trial. The gas produced during the process was drawn out through another tube, inserted through the third neck of the fermenter, and collected in a measuring cylinder by water displacement method. To prevent vaporization of the medium components at process temperature of 80°C, cold water was circulated as coolant in the outer jacket of the fermenter.

Fermentation experiments using *R.meghalophilus* were carried out in a 0.5 L Buchner flask. As *R.meghalophilus* is a photo fermentative bacteria, LED bulb was used as light source in the batch photo fermentation process. The mouth of the Buchner flask was closed with a stopper. The stopper had provisions for nitrogen flushing to displace out oxygen trapped in the system and to introduce a LED bulb in the system. A pipe was fitted to outlet of the flask to withdraw the gas and collect it in the inverted cylinder by volume displacement method. A magnetic stirrer was used to ensure the components in the medium are thoroughly mixed during the process of fermentation. The schematic representation of the experimental set up for fermentation process by *R. meghalophilus* is shown in Fig. 1. DSMZ (0.25 L) medium 27 was prepared to which crude glycerol in varying volume percentage was added as key carbon substrate. The medium contents were autoclaved for 15 min at 121°C. The fermentation medium was then cooled and inoculated with varying inoculum size of *R. meghalophilus*. Nitrogen gas was flushed in the reactor to displace out any trapped oxygen prior to photo fermentation. The gas produced during fermentation by *R. meghalophilus* was collected in a measuring cylinder by water displacement method.

The concentration of hydrogen in the collected gas samples was analyzed by Gas Chromatography with TCD detector. The effect of inoculum size and the crude glycerol on bacterial growth, the substrate conversion rate, and the volume of hydrogen gas generation by the two bacteria were analyzed. To

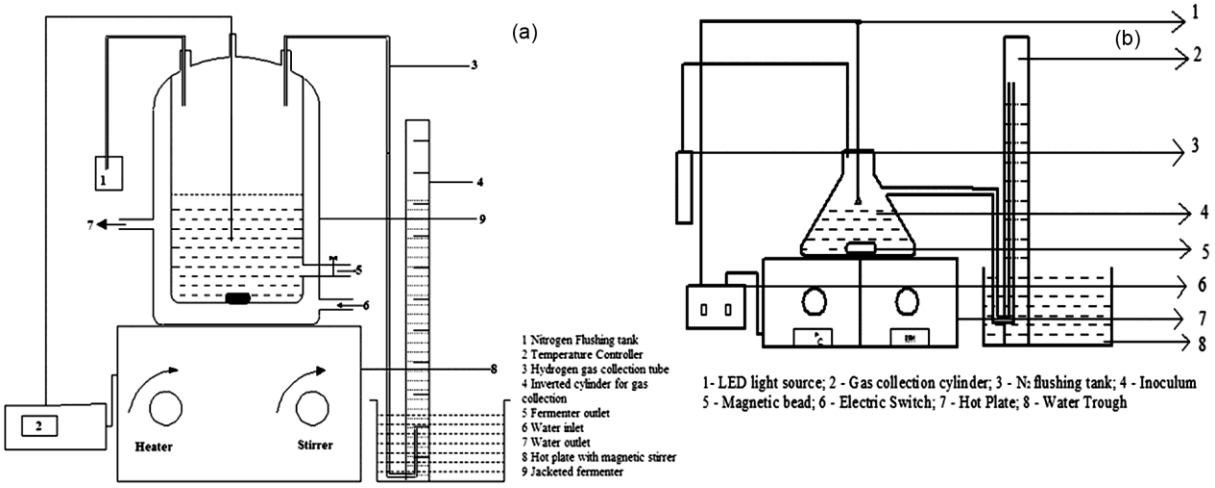

Fig.1 — (a) Experimental setup for anaerobic fermentation and (b) —Experimental setup for anaerobic fermentation for hydrogen gas generation by *T.maitima*. for hydrogen gas generation by *R. meghalophilus*.

calculate the substrate conversion rate, the amount of free glycerol in the medium before and after the fermentation process was determined by ortho toluidine test.

Results and Discussion

Study on anaerobic fermentation process by *T. maritima* and *R. meghalophilus* showed the potential of both the bacteria to degrade crude glycerol and produce hydrogen gas. The effect of varying the inoculum size and the volume of crude glycerol in the growth medium on the bacteria under study are discussed.

Effect of inoculum size

All bacteria exhibit lag phase initially in their growth cycle. But, as the lag phase elongates, it delays the bacteria's exponential growth phase that subsequently affects the yield of the primary metabolite. As hydrogen is a primary metabolite, exponential period in the growth cycle of the bacteria is a vital parameter to enhance its production rate. As inoculum size defines the exponential period in bacterial growth cycle, its effect on *T. maritima* and *R. meghalophilus* is observed as shown in Figs. 2a and 2b respectively. For T. maritima, increase in the inoculum volume showed gradual reduction in the length of the lag phase. For inoculum size of 1 mL/L, the lag period extended up to 5 days. As the inoculum size was gradually ramped up to 4 ml/L, the lag phase was shortened to 1 day. Significant difference in the inoculum size also affected the volume of gas generated. With inoculum size of 2 mL/L, the increase in gas volume was 2.4 times more than that with inoculum size of 1 mL/L. However, the increase in gas volume slowed down to 1.5 times as the inoculum size was stepped up to 4 mL/L.

The effect of inoculum size on *R. meghalophilus*is is shown in Fig. 2b. Studies on impact of inoculum size on *R. meghalophilus* bacteria showed that this bacteria has faster acclimatization to the growth medium with crude glycerol as the lag period was significantly shorter irrespective of the inoculum size. Also, the bacterium was observed to have shorter growth cycle. However, the variation in the volume of gas generated by this bacterium was similar to the observations made for *T. maritima*, for inoculum size of 1, 2, and 4 mL/L. The gas volume doubled with increase in inoculum size from 1 to 2 mL/L, but slowed down to 1.5 times for inoculum size of 4 mL/L.

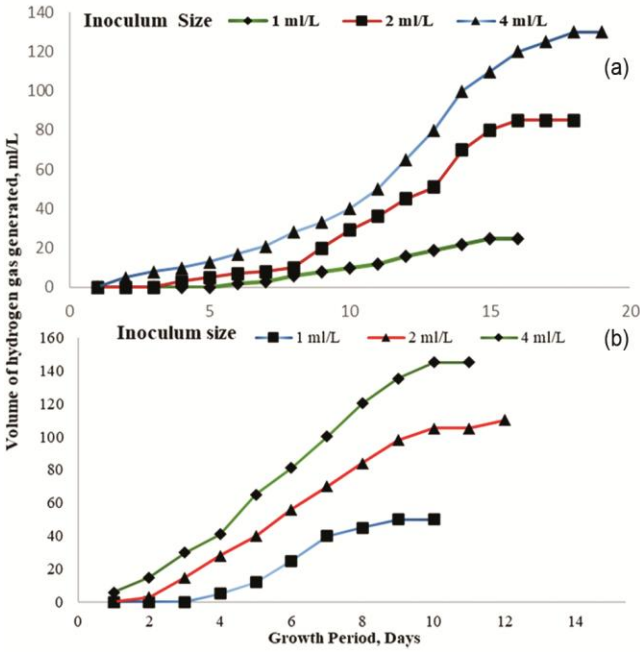

Figure 2 — (a) Effect of inoculum size on *T.maritima* and (2b) Effect of inoculum size on *R.meghalophilus*

Inference: The size of inoculum had influence on both the bacteria. Increase in culture volume was helpful to gradually overcome the lag phase for *T. maritima* but for *R. meghalophilus*, the inoculum size had neutral effect. As the microbial population increased, with inoculum size, the rate of nutrient consumption was also rapid,leading to early depletion of key substrate as well as faster aging of microbial population. As higher inoculum size had a diminishing impact on the yield of hydrogen gas, the inoculum size of 2 mL/L was chosen as optimum for anaerobic fermentation to generate hydrogen gas by both the bacteria.

Effect of crude glycerol

Crude glycerol was used as the key carbon substrate, replacing sucrose, for both *T. maritima* and *R. meghalophilus* in the respective medium prepared. The effect of volume of crude glycerol on hydrogen gas generation by both bacterial cultures is shown in Figs. 3a and 3b.

Effect of crude glycerol volume on fermentation by *T. maritima*

It was noticed that as the volume of crude glycerol increased in the nutrient medium, acclimatization of the bacteria was faster as evident with shortening of the lag phase. However, variation in the volume of crude glycerol had an impact on the bacterial growth

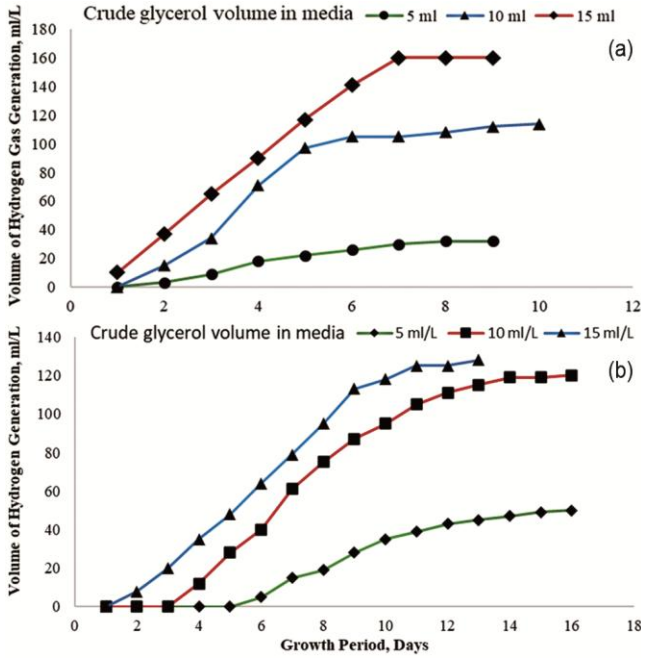

Fig. 3 — (a) Effect of crude glycerol volume on *T.maritima and* (b) Effect of crude glycerol volume on *R.meghalophilus*

and the volume of gas produced. With 5 mL/L of crude glycerol in the medium, *T. maritima* showed a noticeable delay in its growth up to 5th day after inoculation, as no water displacement was noticed in the gas collection cylinder. On increasing the crude glycerol volume to 10 and 15 ml/L, displacement in water levels was noticed from 4th and 2nd day of inoculation respectively, with the volume of gas produced as 120 and 128 mL/L. As, there was no significant difference in gas volumes observed between 10 and 15 mL/L of glycerol concentration in the nutrient medium, 10 mL/L was considered as carbon substitute in the nutrient medium for *T. maritima*.

Effect of crude glycerol volume on fermentation by *R.meghalophilus*

Figure 3b shows that the lag phase of *R. meghalophilus* was considerably shorter. It was observed that *R.meghalophilus* is responsive to crude glycerol even at lower concentrations indicating its faster rate of acclimatization to crude glycerol. The increase in gas volume was also substantial with increase in crude glycerol in growth medium, producing 160 mL/L of gas for medium with 15 mL/L of crude glycerol.

Inference: Growth period of T. maritima was reported to be 14.3 days for 10 mL/L of crude glycerol as carbon substrate, producing 120 mL/L of hydrogen gas. *R. meghalophilus* exhibited the growth period of 9 days for 15 mL/L of crude glycerol in the medium, producing 160 mL/L of hydrogen gas. Thus, *T. maritima* exhibited a prolonged growth phase due to its slower rate of acclimatization to crude glycerol than *R. meghalophilus*. These observations showed that with higher acclimatization rate to crude glycerol, *R. meghalophilus* exhibited faster growth indicating its good potential to generate hydrogen gas by anaerobic fermentation.

Rate of hydrogen gas generation

The amount of crude glycerol consumed during the fermentation process was determined by ortho toluidine test to analyze the effective utilization of crude glycerol as substrate for hydrogen gas production by both the bacteria. The ability of the bacteria to act on crude glycerol and liberate hydrogen gas was calculated according to the formula given below.

$$Specific\ hydrogen\ production\ rate = \frac{Volume\ \ of\ \ hydrogen\ \ produced}{Mass\ \ of\ \ substrate\ \ *time}, \frac{m^3}{kg.s} \quad \ldots(1)$$

The specific hydrogen production rate by *T. maritima* was found to be 9.4 × 10^{-9} m^3/kg.s for medium with 10 mL of crude glycerol. *R. meghalophilus* showed the higher specific conversion rate at 1.163 × 10^{-8} m^3/kg.s for medium with 15 mL of crude glycerol. Comparing the two bacteria, *R. meghalophilus* was found to have higher production rate than *T. maritima*. This is attributed to the observation that *T. maritima*, with slower acclimatization rate showed a slower growth period, thereby reducing the rate of hydrogen gas production.

Substrate conversion and Hydrogen yield

The glycerol content of crude glycerol in nutrient medium was determined to be 0.67 g/mL. The residual glycerol in the nutrient medium at the end of fermentation trials with 5, 10 and 15 mL of crude glycerol/ L of nutrient medium was found to be 0.58, 0.51 and 0.49 g/mL respectively for *T. maritima* and 0.54, 0.46 and 0.38 g/mL respectively for *R.meghalophilus*. The substrate conversion efficiency of microorganisms was calculated according to the formula:

$$Substrate\ conversion = \frac{Amount\ \ of\ substrate\ \ utilized}{Amount\ \ of\ substrate\ \ suppl\ ied} * 100 \quad \ldots(2)$$

Accordingly, higher substrate conversion efficiency was reported for *R. meghalophilus* with 43.28 % at 15mL/L of crude glycerol in its nutrient medium. *T. maritima* reported 23.88% for 10mL of crude glycerol added to its nutrient medium. The gas generated from the anaerobic fermentation process by both the bacteria was analyzed by gas chromatography with thermal conductivity detector to determine the concentration of hydrogen. GC analysis of gas produced by *T. maritima* and *R. meghalophilus,* showed hydrogen content as 25% (v/v) and 19% (v/v), respectively. The yield of hydrogen in gas generated by *T. maritima* was higher than that of *R. meghalophilus.*

Inference: Anaerobic fermentation at 80°C helped to reduce the viscosity of crude glycerol leading to its better mixing in the medium and uptake of nutrients by *T. maritima*. *R. meghalophilus* acclimatized faster to crude glycerol as observed with its higher substrate conversion rate. The process carried at room temperature would have resulted in formation of other metabolic products like ethanol, resulting in rapid depletion rate of substrate and reduced yield of hydrogen in gas produced by *R. meghalophilus*.

Conclusion

Both the bacterial culture could acclimatize to crude glycerol and produce hydrogen gas. The inoculum size and the crude glycerol volume showed to impact the lag period, volume of gas produced and the yield of hydrogen. The yield of hydrogen in the gas produced by *T. maritima* is higher than that by *R. meghalophilus,* but the process is energy intensive. *R.* me*ghalophilus* grows at room temperature and does not require any additional energy to be supplied. In order to increase the yield of hydrogen, *T. maritima* can be subjected to pre inoculation to reduce the lag period and similarly, the anaerobic fermentation by *R. meghalophilus* can be carried out in fed batch system to avoid rapid substrate depletion.

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