Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/he

The light in the dark: In-situ biorefinement of crude oil to hydrogen using typical oil reservoir *Thermotoga* strains

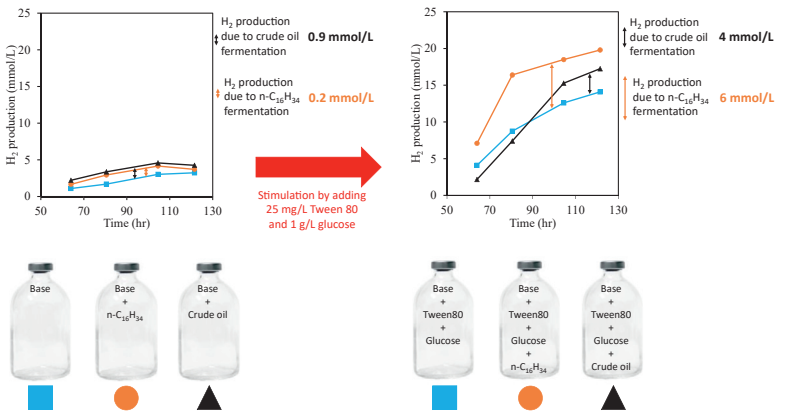
Moein Jahanbani Veshareh [a,*], *Morten Poulsen* [b], *Hamidreza M. Nick* [a], *Karen L. Feilberg* [a], *Ali A. Eftekhari* [a], *Nicole Dopffel* [c]

[a] *Danish Hydrocarbon Research and Technology Centre, Technical University of Denmark, Lyngby, Denmark*
[b] *Danish Technological Institute, Aarhus, Denmark*
[c] *Norwegian Research Center – NORCE, Norway*

HIGHLIGHTS

- *Thermotoga* strains can produce H_2 through dark fermentation of hydrocarbons.
- Tween80 and glucose boost conversion of hydrocarbons to H_2 up to 12-fold.
- Biohydrogen production rate from hydrocarbons is only a quarter of that of glucose.
- Bioconversion of hydrocarbons to H_2 is more efficient than to gasoline.
- Biorefinement is a sustainable solution for abandonment of mature oil reservoirs.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 July 2021
Received in revised form
16 August 2021
Accepted 15 November 2021
Available online 8 December 2021

Keywords:
Biohydrogen
Biorefinement
Hydrocarbon reservoirs

ABSTRACT

H_2 is a CO_2 free energy carrier that can be produced biologically through dark fermentation using specific bacteria. In general, biological production of H_2 needs a carbon source and is more efficient at higher temperatures. Mature petroleum reservoirs have the required high temperatures for H_2 production, and they contain a significant amount of organic matter in form of residual hydrocarbons. In this work, we evaluated whether indigenous microorganisms isolated from hydrocarbon reservoirs are able to biorefine hydrocarbons to H_2. We observed that two *Thermotoga* strains, *Pseudothermotoga hypogea* DSM-11164 and *Pseudothermotoga elfii* DSM-9442, are able to convert hydrocarbons to H_2. DSM 9442 produced 0.47 and 1.02 mmol H_2 per liter of growth medium from 20 mL/L of n-hexadecane or a crude oil, respectively. DSM 11164 only produced H_2 from n-hexadecane (0.94 mmol/L). Addition of 25 mg/L Tween 80, to reduce phase separation, together with 1 g/L glucose increased H_2

* *Corresponding author.*
E-mail address: moein@dtu.dk (M.J. Veshareh).
https://doi.org/10.1016/j.ijhydene.2021.11.118

Hyperthermophile
Thermotoga
Dark fermentation

production from hydrocarbons up to 12-fold. Via an energy analysis we show that bioconversion of crude oil into H_2 can be more efficient than conversion of crude oil to gasoline. Therefore, we suggest dark fermentation as a promising alternative to biorefine crude oil and unlock the energy trapped in hydrocarbon reservoirs after abandonment.

Introduction

Fossil fuels have satisfied humans' increasing demand for energy for decades. Knowing that fossil fuels are not eternal has motivated researchers to work on alternative renewable sources of energy such as biofuel [1]. However, being renewable is not the only constraint for an energy source. Climate change is another concern that makes CO_2 producing energy sources such as biodiesel, bioethanol and biogas less attractive. H_2 is a carbonless energy carrier, and it produces only water when it burns. It can be produced through water electrolysis [2], thermocatalytic reformation and biological processes [3–5].

H_2 can be produced biologically through photolysis by employing green algae, from organic acids through photofermentation by employing phototrophic microorganisms and from organic substrates through dark fermentation by utilizing anaerobic fermentative microorganisms [6–12]. Among these methods, biohydrogen production through dark fermentation is claimed to be very promising [13–17]. Dark fermentation is possible at mesophilic [18], thermophilic [19] and hyperthermophilic [20,21] temperature ranges. However, H_2 production at elevated temperatures is thermodynamically more favorable [22] and thermophilic biohydrogen production benefits from general features of high temperature processes such as lower viscosity, better mixing and higher reaction rates [23,24]. Hyperthermophilic bacteria such as *Thermotoga maritima* and *Thermotoga neapolitana* have been reported to be promising candidates for biohydrogen production, leading to an ideal H_2 yield of around 4 mol H_2 per 1 mol of glucose [25,26]. Carbohydrates and bio-oils have been mainly used as the organic matter for biohydrogen production [15–17]. To the best of our knowledge, it has not been demonstrated whether H_2 production is possible using hydrocarbons as C-source.

Hydrocarbon reservoirs are one of the key components of the deep biosphere, hosting a wide variety of microorganisms. For decades, the microbial ecology of this important component has been studied with focus on sulfate- and nitrate-reducing microorganisms [27,28] and methanogens [29]. Additionally, many studies have tried to find microorganisms that can produce bioproducts with the potential of increasing oil recovery [30,31]. Fermenting microbes such as *Thermotoga* are one genus of microorganisms that exist in petroleum reservoirs [27,32]. However, not enough knowledge exists about *Thermotoga* strains isolated from oil reservoirs, especially in regard to whether or not they can effectively produce H_2.

Many petroleum reservoirs nowadays are passing the second stage of their life in which the oil production is reducing. Depending on the type of a reservoir and applied development policies, a great portion of initial oil in place (e.g. 60%) is still left as the residual oil phase, which is hard to produce. Current climate policies against CO_2 producing fuels, makes it less and less attractive to produce this remaining oil by employing various enhanced oil recovery technologies (e.g. Refs. [30,33]). Therefore, for these reservoirs, abandonment phase is soon to come. Exactly these reservoirs may lend themselves easily for other type of production since there is usually enough access to the subsurface formation through platforms, network of pipelines, and wells and there exists adequate knowledge regarding the subsurface behavior (thanks to variety of tests e.g. 3/4D seismic, conducted throughout reservoir development).

Even though biohydrogen production at elevated temperatures is efficient in terms of rates and higher H_2 per substrate yield, maintaining a bioreactor at high temperature requires energy. Therefore, a part of the produced H_2 needs to be used to warm up the bioreactor. Depleted petroleum reservoirs, depending on the subsurface temperature gradient and their depth, can have temperatures suitable for thermophilic or hyperthermophilic biohydrogen production. Moreover, depleted oil reservoirs as mentioned earlier contain a significant amount of organic matter in the form of residual oil. Provided that fermenting microbes could consume hydrocarbons as the sole substrate to produce H_2, one could exploit the left-over energy in depleted oil reservoir to produce a clean energy carrier using dark fermentation. Therefore, it could be possible - with rather low investments - to produce biohydrogen from the mature hydrocarbon fields as they usually have the required space, temperature and organic matter.

In this work we aim to answer the following two research questions:

1) Can indigenous microorganisms of hot petroleum reservoirs produce H_2 from ordinary organic substrates to produce biohydrogen?
2) Can these microorganisms also produce H_2 from the organic matter that exist within petroleum reservoirs?

For this, we studied the biohydrogen capability of three *Thermotoga* strains isolated from oil reservoirs by using glucose as the substrate. Furthermore, we investigated whether they can produce H_2 from n-hexadecane and an original crude oil produced from a North Sea reservoir.

Materials and methods

Microorganisms

Three *Thermotoga* strains: *Thermotoga petrophila* DSM-13995 [34], *Pseudothermotoga hypogea* DSM-11164 [35] and *Pseudothermotoga elfii* DSM-9442 [36] were ordered from DSMZ being isolated from production fluids of three different oil reservoirs.

Seed culture preparation

In order to activate ordered bacteria from DSMZ and later to prepare the inoculum the following growth medium referred to as 'seed medium' was used (g/L of water): NH_4Cl, 1.0; K_2HPO_4, 0.3; KH_2PO_4, 0.3; $MgCl_2 \cdot 6H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.1; KCl, 0.1; Na-Acetate, 0.5; yeast extract, 5.0; peptone, 5.0; Na-resazurine 0.0002; L-Cysteine-$HCl \cdot H_2O$, 0.5; glucose, 3.0; Na_2S, 0.5; $NaHCO_3$, 2.0; trace element solution, 10 mL. To prepare the seed medium, first, all the components except L-Cysteine-$HCl \cdot H_2O$, glucose, Na_2S, $NaHCO_3$ and trace element solution were mixed. After sparging with nitrogen for 30 min, L-Cysteine-$HCl \cdot H_2O$ was added, and the solution was autoclaved. $NaHCO_3$, Na_2S and trace element solution were autoclaved and added separately. Glucose was sterilized using filtration. Trace element solution contained (in g/L of water): $MnCl_2 \cdot 4H_2O$, 6.4; $CoCl_2 \cdot 6H_2O$, 0.387; $CuCl \cdot 2H_2O$, 0.035; $Na_2MoO_4 \cdot 2H_2O$, 0.033; $ZnCl_2$, 0.027; $FeCl_2$, 1.062. Fifty mL of the seed medium was filled into 113 mL serum bottles which had already been nitrogen-sparged, sealed with rubber stopper and sterilized using autoclaving. Each bottle was inoculated with 1 mL of the seed culture, placed on a magnetic stirrer at 200 rpm and incubated at 70 °C for 3 days. Note that the temperature of 70 °C was considered to represent a typical hot hydrocarbon reservoir.

Growth media for evaluating biohydrogen production

In order to evaluate biohydrogen production by different organic matter sources, two growth media were used in this work. The first medium referred to as 'base medium' had the composition of the seed medium excluding glucose. Yeast extract and peptone concentration were reduced to 2 g/L from 5 g/L. The second medium referred to as 'baseGS medium' had the composition of the base medium with 1 g/L of glucose and 25 mg/L of a surfactant (Tween 80).

Experimental design

Table 1 lists different experiments conducted in this work. Tests 1 to 3 in Table 1 were conducted for growth rate analysis using optical density measurements. Tests 4 to 6 were conducted as control tests to measure the biohydrogen production due to the presence of yeast extract and peptone in the base medium. Tests 7 to 13 were conducted to investigate the biohydrogen production by using three different carbon sources, glucose (3 g/L equivalent to 16.65 mM), n-hexadecane (20 mL/L) and crude oil (20 mL/L). Note that 20 mL of n-hexadecane is equivalent to 15.46 mol of n-hexadecane; however, solubility of n-hexadecane in water is 4.1 μM [37]. As n-hexadecane and crude oil are not miscible in the water phase, we hypothesized that addition of a surfactant such as Tween 80 (25 mg/L) may stimulate H_2 production from hydrocarbons by reducing the interfacial tension between two phases and therefore making hydrocarbons more accessible for microorganisms. Note that choosing Tween 80 rather than other surfactants was because of its relatively low toxicity [38]. Additionally, we hypothesized that an initial carbohydrate concentration (1 g/L of glucose) can assist microorganisms in degradation of hydrocarbons. Tests 14 to 19 were conducted to take into account the biohydrogen production that occurs solely due the addition of glucose and Tween 80 to the base medium. Additionally, tests 14 to 15 take into account any H_2 production increase due to possible degradation of Tween 80 or any H_2 production decrease due to the toxicity of Tween 80. Tests 16 to 19 were run to quantify whether biohydrogen production from hydrocarbons is enhanced by the addition of glucose and Tween 80. Each test in Table 1 was conducted in three replicates except tests 7 to 9 that were conducted in 4 replicates. In these tests (7–9) 3 replicates were used for sampling at different time points while 1 replicate was only sampled at the end of the experiment to evaluate the effect of sampling gas on the measured biohydrogen.

Table 1 – Various designed experiments conducted in this work.

Test number	Bacteria	Growth medium
1	DSM 11164	Seed
2	DSM 13995	
3	DSM 9442	
4	DSM 11164	Base
5	DSM 13995	
6	DSM 9442	
7	DSM 11164	Base + glucose
8	DSM 13995	
9	DSM 9442	
10	DSM 11164	Base + n-hexadecane
11	DSM 9442	
12	DSM 11164	Base + crude oil
13	DSM 9442	
14	DSM 11164	BaseGS
15	DSM 9442	
16	DSM 11164	BaseGS + n-hexadecane
17	DSM 9442	
18	DSM 11164	BaseGS + crude oil
19	DSM 9442	

Analytical methods

The composition of biogas in the headspace was analyzed using a GC-TDC (SRI 8610C, California, USA) equipped with PoraPak Q coloum installed in an oven. The operational temperatures of the injection port, the oven, and the detector were all set at 40 °C. Argon was used as the carrier gas at a flow rate of 15 mL/min. The alcohols and VFA were measured using a gas chromatograph (Thermo Scientific TRACE™ 1300, Waltham, USA) equipped with a flame ionization detector (FID) and an Agilent J&W GC capillary Column (Length 30 m, Diam. 0,530 mm, Film thickness 1,50 μm). The initial column temperature is set to 40 °C for 6 min then it rises by 20 °C/min to

200 °C. The final temperature is held for 6 min and the total run-time is 20 min with Helium as carrier gas. Further chromatographic analysis was done using an Agilent 7890 b GC with TOF-MS detection. The infrared ATR spectra of the liquid pre-concentrated samples were recorded using an Alpha II FTIR spectrometer from Bruker equipped with a platinum ATR insert. The aqueous samples were prepared by liquid-liquid extraction with dichloromethane and filtered with a 2-μm filter prior to analysis on the GC-MS. The liquid-liquid extraction is unlikely to capture the smallest VFAs as these will tend to remain in the water phase.

Biohydrogen production calculations

Before every GC measurement, serum bottles temperature and pressure were measured using a temperature-pressure measurement tool (testo 512, Black Forest, Germany). Biohydrogen production in the gas phase was then calculated by the ideal gas law equation.

Equivalent H_2

Equivalent H_2 (is the H_2 that should be produced stoichiometrically for production of VFA) production was calculated according to the following fermentation reactions (1–3):

$$C_6H_{12}O_6 \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow C_3H_7COOH + 2H_2 + 2CO_2 \quad (2)$$

$$C_6H_{12}O_6 \rightarrow 3/4C_4H_9COOH + 9/4H_2 + 9/4CO_2 \quad (3)$$

For example, 10 mM acetic acid is equivalent to 20 mM H_2.

Results and discussion

Growth rate

Fig. S1 shows the optical density measured for tests 1 to 3 (Table 1) with the seed culture as the growth medium (5 g/L of glucose as the major carbon source). Growth occurs in the first 60 h and after that, biomass concentration declines. The maximum specific growth rate for DSM 11164, DSM 9442 and DSM 13995 can be calculated to be 0.11, 0.12 and 0.14 hr^{-1}(highest slope of log(OD) vs time, Fig. S1B). Note that in Fig. S1B the OD at time equal to zero was assumed to be 50 times smaller than the OD of the initial inoculum.

Biohydrogen/VFA and alcohol production from glucose

H_2 production mainly occurs in the first 100 h, as illustrated in Fig. S2 for the three different strains studied in this work. Strain DSM 13995 and DSM 11164 have a fast early production stage while, DSM 9442 resulted in the highest total H_2 production. H_2 production for bottles, which were only sampled at the end of experiments (after 194 h), was 21.02, 29.57 and 30.00 mmol/L for DSM 11164, DSM 13995 and DSM 9442, respectively. Considering results shown in Fig. S2, the highest H_2 production rate for DSM 11164, DSM 13995 and DSM 9442 can be calculated to be 1.03, 2.79 and 0.91 mmol $L^{-1}\cdot hr^{-1}$. That is within the rate of H_2 production by *Thermotoga* species reported by Shao et al. (2020) [24]. Fig. S3 shows alcohol and VFA production. The only produced alcohol in detectable concentrations was ethanol. DSM 13995 had the highest ethanol production, but among VFA it only produced acetic acid in a detectable concentration (>2 mg/L). DSM 9442 showed the highest VFA production by producing 1275.4 mg/L compared to 931.9 and 1223.8 mg/L for DSM 11164 and DSM 13995, respectively.

Equivalent H_2 results are in agreement with H_2 production results. Note that lower measured H_2 production than equivalent H_2 production can be due to H_2 leakage from the rubber stopper, H_2 loss due to sampling for GC or pressure measurements, H_2 reactions with growth medium components and other, unmeasured metabolic products. Table S1 lists the product yield for the studied strains and for H_2, VFA and ethanol.

Table S1 illustrates that during H_2 production by the studied *Thermotoga* strains negligible amounts of butyric or iso-butyric acid is produced. The value of $(Y_{Bu} + Y_{i\text{-}Bu})/Y_{Ac}$ for DSM 13995, DSM 9442 and DSM 11164 was calculated to be 0, 0.0166 and 0, respectively. These values are much lower compared to results reported by Lin et al. (2007) [39] in which they reported the value of $(Y_{Bu} + Y_{i\text{-}Bu})/Y_{Ac}$ for four different *Clostridium* species: *C. acetobutylicum*, *C. butyricum*, *C. tyrobutyricum* and *C. beijerinckii* to be 1.54, 1.86, 0.88 and 1.82, respectively. Since more H_2 is produced from fermentation when the co-product is acetic acid rather than butyric acid, the measured $(Y_{Bu} + Y_{i\text{-}Bu})/Y_{Ac}$ shows that the studied *Thermotoga* strains are efficient H_2 producers.

Biohydrogen/VFA and alcohol production from hydrocarbons

DSM 13995 was unable to produce detectable concentrations of H_2 in the absence of glucose and presence of hydrocarbons. Therefore, here we only present the results from DSM 9442 and DSM 11164. Fig. 1 shows H_2 production for the base growth medium together with H_2 production for the base media supplemented by 20 mL (per liter of the base medium) of crude oil or n-hexadecane. DSM 9442 can produce more H_2 in experiments where base medium has been supplemented with the crude oil or n-hexadecane (Fig. 1A). The maximum rate of H_2 production for the base case was measured to be 0.72 mmol $L^{-1}\cdot hr^{-1}$ and increased to 0.85 and to 0.99 mmol $L^{-1}\cdot hr^{-1}$ with the addition of n-hexadecane and crude oil, respectively. Addition of n-hexadecane can also increase H_2 production by DSM 11164 (Fig. 1B). However, addition of crude oil leads to a higher H_2 production in the early stages of growth but the final H_2 production was measured to be lower compared to the other two cases (base and base + n-hexadecane). The maximum rate of H_2 was measured to be 0.94 mmol $L^{-1}\cdot hr^{-1}$ for the base case and increased to 1.28 mmol $L^{-1}\cdot hr^{-}1$ due to the addition of n-hexadecane and reduced to 0.88 mmol l^{-1} h^{-1} by the addition of crude oil. By subtracting the H_2 production of the experiments with hydrocarbon from the experiments without the presence of hydrocarbons (base), H_2 production from hydrocarbons can be calculated (Fig. S4). For DSM 9442, the highest rate of H_2 production from hydrocarbons can be calculated to be 0.041 and 0.033 mmol l^{-1} h^{-1} for n-hexadecane and crude oil, respectively (Fig. S4A). The maximum rate of H_2

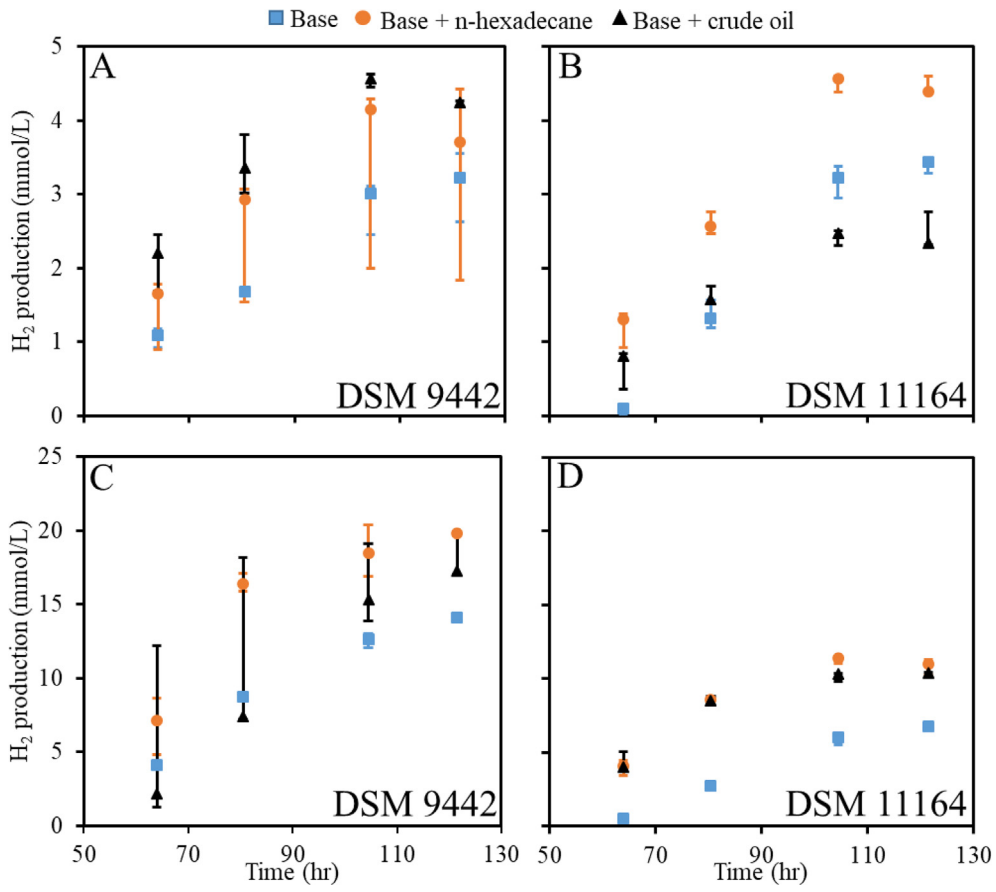


Fig. 1 – A, B) H_2 production using base medium; C,D) baseGS medium with or without the addition of hydrocarbons, at 70 °C for two *Thermotoga* strains DSM 9442 and DSM 11164. Symbols show the median of the three replicates and the error bars show the minimum and maximum.

production from n-hexadecane by DSM 11164 is 0.0027 mmol $L^{-1}{\cdot}hr^{-1}$ (Fig. S4A). Therefore, the rate of H_2 production from hydrocarbons is one or two order of magnitude lower than H_2 production from glucose. Fig. 2 shows the VFA analysis of the liquid phase corresponding to the H_2 measurements at the end of experiments shown in Fig. 1 (after 121.5 h). In the absence of glucose, neither DSM 9442 nor DSM 11164 were able to produce ethanol. Acetic acid showed to be the main VFA, about an order of magnitude higher than other VFAs. The equivalent H_2 results, calculated based on measured VFAs concentrations (Fig. 2), is in agreement with the measured H_2 production results shown in Fig. 1. Screening analysis was carried out to investigate fatty acid production due to fermentation of crude oil, and hexadecanoic acid due to fermentation of n-hexadecane. The samples originally containing crude oil showed a large range of medium-to large-sized fatty acids. These compounds are naturally present in crude oil, but our qualitative assessment indicates that they were present in higher quantities after fermentation compared to the original crude oil. Fatty acids were also identifiable in the FT-IR spectrum of these samples by absorption in the carbonyl stretching region (1720 cm^{-1}). The fact that the carboxylic acid band was detectable in the IR (Fig. 3) spectra without pre-concentration indicates that the concentrations were notably higher than naturally occurring in crude oil, where the carbonyl absorption band from naturally occurring acids is generally weaker and below the detection limit. While no hexadecanoic acid production was observed due to the activity of DSM 11164, trace of hexadecanoic acid was observed in DSM 9442 cultures. Fig. 1C demonstrates that addition of glucose and surfactant (together) increased H_2 production from hydrocarbons for both DSM 9442 and DSM 11164. Comparing Fig. 1A and C reveals that addition of glucose and surfactant increases H_2 production from hydrocarbons around 12- (from 0.47 to 5.7 mmol/L) and 3-times (from 1.019 to 3.16 mmol/L) for n-hexadecane and crude oil, respectively. Similarly, for DSM 11164 the addition of glucose and surfactant increased H_2 production from hydrocarbons around 4-fold (from 0.94 to 3.35 mmol/L for n-hexadecane and from 1.1 to 4.23 mmol/L for crude oil). By comparing the highest slope of H_2 production in Fig. S4B with that of Fig. S4A, it can be concluded that addition of glucose and Tween 80 increase the maximum rate of H_2 production from hydrocarbons by DSM 9442 around 6-fold (from 0.04 to 0.28 mmol $L^{-1}{\cdot}hr^{-1}$ for n-hexadecane and from 0.03 to 0.17 for the crude oil). For DSM 11164 addition of glucose and Tween 80 increased the maximum H_2 production rate from n-hexadecane around 400-fold (from 0.0027 to 0.14 mmol $L^{-1}{\cdot}hr^{-1}$), and it enabled production of H_2 from crude oil with the maximum rate of 0.14 mmol $L^{-1}{\cdot}hr^{-1}$.

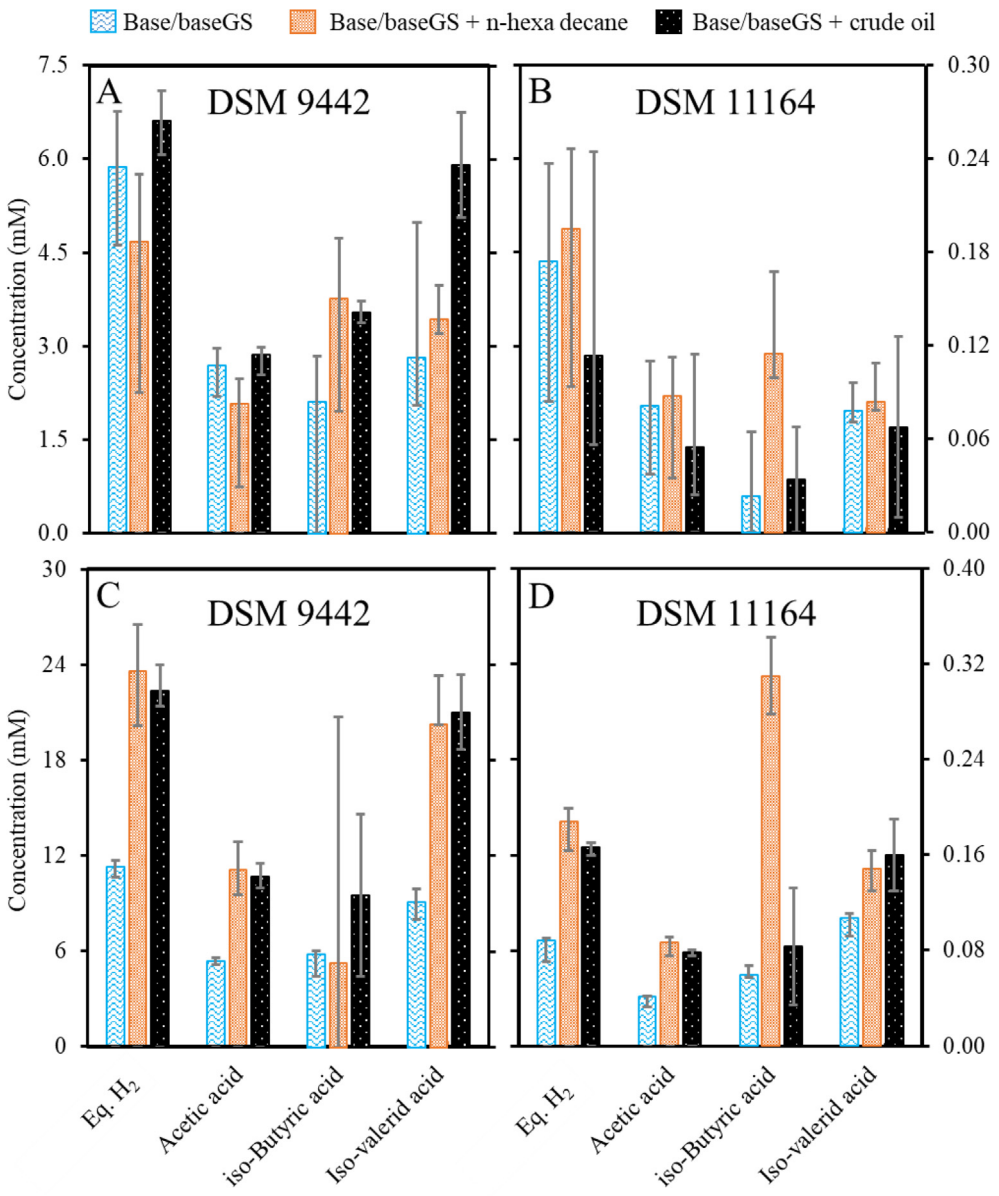


Fig. 2 – A, B) VFA and ethanol production using base medium; C,D) baseGS medium with or without the addition of hydrocarbons, at 70 °C after 121.5 h for two *Thermotoga* strains DSM 9442 and DSM 11164. Equivalent hydrogen is the hydrogen that should be produced stoichiometrically for production of VFAs. Equivalent hydrogen and acetic acid concentrations are read from the left vertical axis while other concentrations are read from the right vertical axis. Bars show the median of the three replicates and the error bars show the minimum and maximum.

VFAs measurements (Fig. 2C and D) are in agreement with H_2 production data, as a similar trend is observed for the calculated equivalent H_2 shown in Fig. 1. Our qualitative approach did not prove any enhancement in crude oil fermentation due to the addition of glucose and surfactant, as no difference was detectable between cultures with or without glucose and surfactant. However, no traces of hexadecanoic acid were observed for DSM 111464 and DSM 9442, whereas some hexadecanoic acid was detected when glucose and surfactant were added, indicating that the addition of glucose and Tween 80 has enhanced n-hexadecane fermentation.

Overall, the highest H_2 amount is produced from glucose (Fig. 4). The total H_2 production from glucose is at least 19- and 12-times higher compared to the values from hydrocarbons for DSM 9442 and DSM 11164, respectively. Addition of small amounts of glucose and surfactant stimulates H_2 production from hydrocarbons significantly, which enables the strains to reach production rates, which are only around 3- to 5-times smaller than H_2 production from pure, high-concentrated glucose (Fig. 4). We assume that the initial supply with glucose provides enough energy for the strains to start the energy-demanding degradation of the supplied hydrocarbons. Furthermore, the surfactant enables a better hydrocarbon accessibility. Note that according to our experiments, we cannot conclude if the H_2 production increase from hydrocarbons is due to addition of surfactant or glucose or the

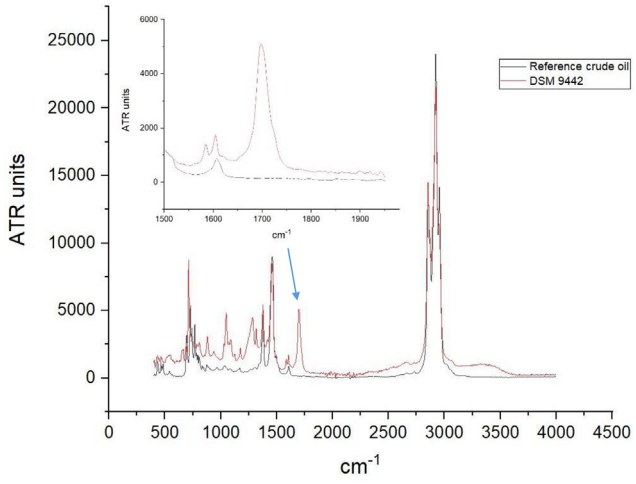


Fig. 3 – ATR-FTIR spectra of the extract of the aqueous sample containing crude oil compared to the original crude oil. The C=O stretching band at 1720 cm^{-1} is not visible in the original oil, but clearly present after the sample treatment.

combination thereof. In future experiments, these and other stimulating additives should be tested separately and in combination to understand the single and synergistic effects. Also, future research should focus on optimizing the concentration of additives for increasing H_2 production from the supplied hydrocarbons.

Increase in H_2 production by addition of surfactants has been also observed by previous researchers. Elsamadony et al. (2015) showed that two non-ionic surfactants (Tween 80 and polyethylene glycol) substantially increase H_2 production yield from municipal solid waste [40]. Non-ionic surfactants have been also shown to increase H_2 production yield from palm oil mill effluent [41,42]. The impact of surfactants on increasing biohydrogen production is not limited to dark fermentation. Fan et al. (2021) showed that addition of Rhamnolipid and Tea Saponin increase H_2 production yield from corncub in photofermentation process [43]. The increase of H_2 production yield due to addition of Rhamnolipid has been also shown by Zhang et al. (2021) [44]. Modifying substrate structure so that it is more accessible for enzymes [45], positively affecting enzyme substrate interaction for example by facilitating desorption of enzymes from substrate [46], and enhancing mass transfer [47] are three mechanisms reported in the literature for H_2 yield increase due to the addition of surfactants.

Efficacy of biohydrogen production from hydrocarbons

To compare the energy efficiency of biohydrogen production from hydrocarbons, we can compare the energy efficiency of converting a unit volume of a crude oil to gasoline or to biohydrogen. We consider the North Sea crude oil reported in the work of Schmidt et al. (2005) [48] with a molecular weight of 140.63 g/mol and a density of 790 g/L. We assume that the yield of gasoline production from the crude oil is equal to 43%.

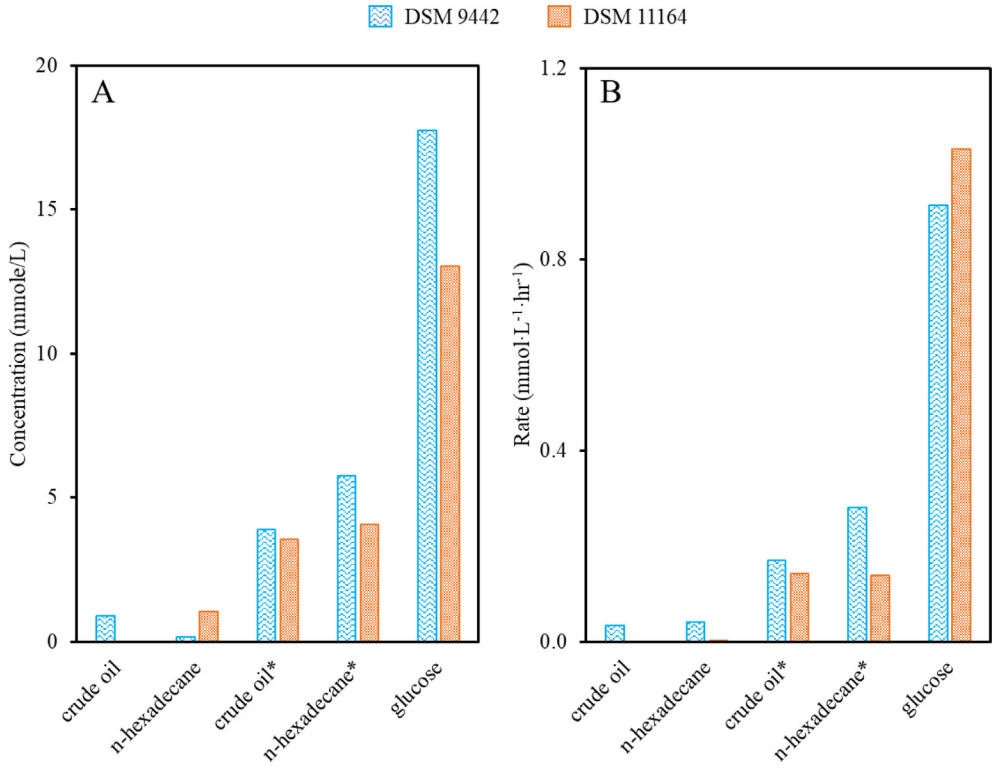


Fig. 4 – A) H_2 production and B) maximum H_2 production rate within 121.5 h at 70 °C by two *Thermotoga* strains DSM 9442 and DSM 11164. Carbon sources with * have been supplemented by glucose and surfactant. Note that the values are the difference calculated to the base or baseGS results.

Gasoline has a molecular weight of around 100 g/mol, and therefore can be represented with heptane [49]. This means, 1 L of crude oil produces 0.43 L of gasoline. Taking into account heptane density (684 g/L) and molecular weight (100.21 g/mol), 0.43 L of gasoline is equivalent to 2.93 mol of heptane. Considering heptane heat of combustion (4817 kJ/mol), 1 L of crude oil produces 14100 kJ by conversion to gasoline.

Given the molecular weight of 140.63 g/mol, we assume that the North Sea crude oil can be represented by decane. Biohydrogen production from decane can be represented by the following reaction (4) (details in Supplementary materials):

$$C_{10}H_{22} + 9.20H_2O + 0.22\,HCO_3^- + 0.14\,NH_4^+ \rightarrow 0.7\,CH_{1.8}O_{0.5}N_{0.2} + 3.27H^+ + 4.76\,CH_3COO^- + 10.48\,H_2 \quad (4)$$

Considering the crude oil density, a liter of the crude oil contains 5.55 mol of decane, which can result in production of 58 mol H_2. Considering H_2 heat of combustion (286 kJ), 1 L of crude oil produces 16588 kJ by conversion to H_2. Therefore, in terms of energy, conversion of crude oil to H_2 is more efficient than conversion to gasoline.

In order to evaluate the dynamic aspect of energy production by biohydrogen, we can determine the time required to produce H_2 equivalent of 1 L of gasoline in a standard reactor. One liter of heptane is 6.82 mol resulting in 32851 kJ energy. To produce 32851 kJ from H_2, 115 mol of H_2 are required. If we assume that the rate of H_2 production from decane is equal to 0.28 mmol $L^{-1}\cdot hr^{-1}$(the maximum H_2 production rate from hydrocarbons observed by DSM 9442 and DSM 11164), and if we assume that the rate of H_2 production can be sustained at maximum in a 1000-Liter reactor, then it requires 17 days to produce biohydrogen equivalent to a liter of gasoline. Note that by addition of glucose this time would be smaller (4.6 days). Therefore, producing biohydrogen in commercial amounts requires significant time and large reactors. Depleted hydrocarbon reservoirs could provide exactly these required organic matter sources, volumes and temperatures for biohydrogen production.

Challenges and opportunities in transition from lab scale to field application

There are several important considerations before employing dark fermentation in mature reservoirs. One of the challenges of fermentation processes is the co-production of organic acids and the consequent decrease in pH that can inhibit microbial growth [50]. Presence of carbonate minerals in depleted petroleum reservoir rocks offer a significant buffering capacity [51] and may help sustaining the fermentation process in a high rate. Additionally, even though H_2 is a green energy, dark fermentation produces significant amount of CO_2, i.e. the dark fermentation process is not entirely green. According to reactions 1 to 3, for each mole of H_2, 1 or 2 mol of CO_2 can be produced. However, dark fermentation in a depleted petroleum reservoir may have lower or insignificant CO_2 footprint for two reasons. First, the produced CO_2 may react with reservoir rock minerals such as feldspars [52] and produce carbonate minerals. Second, the solubility of CO_2 in water is significantly higher compared to H_2 (e.g. 14 times at 50 atm and 75° C), leading to an expected higher H_2 concentrations in the produced gas phase compared to CO_2 (for an equal total mole fraction in the system).

Despite abovementioned advantages, dark fermentation in depleted reservoirs may suffer from co-existence of other metabolisms such as hydrogenotrophic sulfate reduction that can turn H_2 into H_2S. However, hydrogenotrophic sulfate reduction is restricted to presence of sulfate in the reservoir fluids. Sulfate can be either initially present in some reservoir brines [53] or it can be introduced to the reservoir during seawater flooding [54]. Sulfate also can be introduced to the formation brine by dissolution of sulfate minerals such as anhydrite [55], which could be triggered by the dark fermentation process. Therefore, hydrogenotrophic sulfate reduction should be prevented by selecting a reservoir that (i) has a sulfate-free formation brine and (ii) does not contain sulfate minerals. Souring can also be mitigated by various methods such as nitrate and perchlorate treatment [56]; however, such mitigation methods may inhibit the activity of fermenting microbes [57] as well as activating other microorganisms that can couple nitrate and perchlorate reduction to H_2 oxidation [58]. Hydrogenotrophic methanogenesis and homoacetogenesis are other metabolisms that can occur and turn the produced H_2 into CH_4 or acetate, respectively, if (i) methanogens/acetogens are present and (ii) mineral fixation of CO_2 does not remove the produced CO_2 during biohydrogen production. As methanogens are non-sporulating organisms, various inoculum pre-treatment methods such as heat shock, repeated aeration, acidifying and alkalifying have been employed to minimize methanogenesis during H_2 production in on-land fermenters. Many fermenting microbes tolerate these pretreatments by forming spores. While such pretreatment methods can inhibit bioconversion of H_2 into CH_4, it cannot inhibit conversion to acetate as some homoacetogens are spore forming [59]. However, ammonia treatment has shown to suppress H_2 consumption by homoacetogenesis [60]. Another challenge for dark fermentation in mature reservoirs is whether suitable fermenting microbes are present indigenously, as we also observed that one tested *Thermotoga* strain did not show any activity on hydrocarbons. In case active fermenting microbes are not present in the reservoir, the reservoir needs to be inoculated, in which case the ability of the inoculated fermenting microbe to tolerate harsh reservoir environmental condition such as salinity will be of paramount importance and must be tested carefully beforehand.

It is unclear whether these methods can be transferred and used for reservoirs, as manipulation of reservoir conditions and communities are difficult. Field realistic tests using original brine, crude oil and cores using relevant pressures and temperatures will elucidate, whether dark fermentation rates are sufficiently high under the given thermodynamic conditions and whether H_2 consumption can be avoided. Further studies are required to evaluate the overall efficiency and potential of biohydrogen production through dark fermentation in depleted reservoirs by taking into account above mentioned challenges and opportunities.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The research leading to these results has received funding from the Danish Hydrocarbon Research and Technology Centre under Radical Innovation Sprint program. Christian Holst Fischer and Lone Tang are thanked for their helps in regard with the experimental work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijhydene.2021.11.118.

REFERENCES

[1] Raheem A, Prinsen P, Vuppaladadiyam AK, Zhao M, Luque R. A review on sustainable microalgae based biofuel and bioenergy production: recent developments. J Clean Prod 2018;181:42–59.
[2] Ursua A, Gandia LM, Sanchis P. Hydrogen production from water electrolysis: current status and future trends. Proc IEEE 2011;100(2):410–26.
[3] Levin DB, Pitt L, Love M. Biohydrogen production: prospects and limitations to practical application. Int J Hydrogen Energy 2004;29(2):173–85.
[4] Usman M, Kavitha S, Kannah Y, Yogalakshmi K, Sivashanmugam P, Bhatnagar A, Kumar G. A critical review on limitations and enhancement strategies associated with biohydrogen production. Int J Hydrogen Energy 2021;46(31).
[5] Nabgan W, Abdullah TAT, Nabgan B, Jalil AA, Nordin AH, Ul-Hamid A, Hassan NS, Hussain I, Coelho A, Amin A. Catalytic biohydrogen production from organic waste materials: a literature review and bibliometric analysis. Int J Hydrogen Energy 2021;46(60).
[6] Urbaniec K, Bakker RR. Biomass residues as raw material for dark hydrogen fermentation–A review. Int J Hydrogen Energy 2015;40(9):3648–58.
[7] Sarma SJ, Pachapur V, Brar SK, Le Bihan Y, Buelna G. Hydrogen biorefinery: potential utilization of the liquid waste from fermentative hydrogen production. Renew Sustain Energy Rev 2015;50:942–51.
[8] Ghimire A, Frunzo L, Pirozzi F, Trably E, Escudie R, Lens PN, Esposito G. A review on dark fermentative biohydrogen production from organic biomass: process parameters and use of by-products. Appl Energy 2015;144:73–95.
[9] Das D, Veziroglu TN. Advances in biological hydrogen production processes. Int J Hydrogen Energy 2008;33(21):6046–57.
[10] d'Ippolito G, Dipasquale L, Vella FM, Romano I, Gambacorta A, Cutignano A, Fontana A. Hydrogen metabolism in the extreme thermophile Thermotoga neapolitana. Int J Hydrogen Energy 2010;35(6):2290–5.
[11] Chen CI, Reinsel MA, Mueller RF. Kinetic investigation of microbial souring in porous media using microbial consortia from oil reservoirs. Biotechnol Bioeng 1994;44(3):263–9.
[12] Ren N-Q, Cao G-L, Guo W-Q, Wang A-J, Zhu Y-H, Liu B-f, Xu J-F. Biological hydrogen production from corn stover by moderately thermophile Thermoanaerobacterium thermosaccharolyticum W16. Int J Hydrogen Energy 2010;35(7):2708–12.
[13] Show K, Lee D, Tay J, Lin C, Chang J-S. Biohydrogen production: current perspectives and the way forward. Int J Hydrogen Energy 2012;37(20):15616–31.
[14] Chen C-C, Chuang Y-S, Lin C-Y, Lay C-H, Sen B. Thermophilic dark fermentation of untreated rice straw using mixed cultures for hydrogen production. Int J Hydrogen Energy 2012;37(20):15540–6.
[15] Valdez-Vazquez I, Ríos-Leal E, Esparza-García F, Cecchi F, Poggi-Varaldo HM. Semi-continuous solid substrate anaerobic reactors for H2 production from organic waste: mesophilic versus thermophilic regime. Int J Hydrogen Energy 2005;30(13–14):1383–91.
[16] Tawfik A, El-Qelish M. Continuous hydrogen production from co-digestion of municipal food waste and kitchen wastewater in mesophilic anaerobic baffled reactor. Bioresour Technol 2012;114:270–4.
[17] Varrone C, Rosa S, Fiocchetti F, Giussani B, Izzo G, Massini G, Marone A, Signorini A, Wang A. Enrichment of activated sludge for enhanced hydrogen production from crude glycerol. Int J Hydrogen Energy 2013;38(3):1319–31.
[18] Bernal AP, de Menezes CA, Silva EL. A new side-looking at the dark fermentation of sugarcane vinasse: improving the carboxylates production in mesophilic EGSB by selection of the hydraulic retention time and substrate concentration. Int J Hydrogen Energy 2021;46(24):12758–70.
[19] Lovato G, Augusto IMG, Júnior ADNF, Albanez R, Ratusznei SM, Etchebehere C, Zaiat M, Rodrigues JAD. Reactor start-up strategy as key for high and stable hydrogen production from cheese whey thermophilic dark fermentation. Int J Hydrogen Energy 2021;46(54):27364–79.
[20] Weide T, Peitzmeier J, Wetter C, Wichern M, Brügging E. Comparison of thermophilic and hyperthermophilic dark fermentation with subsequent mesophilic methanogenesis in expanded granular sludge bed reactors. Int J Hydrogen Energy 2021;46(57):29142–59.
[21] Pradhan N, Dipasquale L, d'Ippolito G, Panico A, Lens PN, Esposito G, Fontana A. Hydrogen and lactic acid synthesis by the wild-type and a laboratory strain of the hyperthermophilic bacterium Thermotoga neapolitana DSMZ 4359T under capnophilic lactic fermentation conditions. Int J Hydrogen Energy 2017;42(25):16023–30.
[22] Verhaart MR, Bielen AA, Oost Jvd, Stams AJ, Kengen SW. Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: mechanisms for reductant disposal. Environ Technol 2010;31(8–9):993–1003.
[23] Wiegel J, Ljungdahl LG, Demain AL. The importance of thermophilic bacteria in biotechnology. Crit Rev Biotechnol 1985;3(1):39–108.
[24] Shao W, Wang Q, Rupani PF, Krishnan S, Ahmad F, Rezania S, Rashid MA, Sha C, Din MFM. Biohydrogen production via thermophilic fermentation: a prospective application of Thermotoga species. Energy 2020;197:117199.
[25] Van Ooteghem SA, Beer SK, Yue PC. Hydrogen production by the thermophilic bacterium Thermotoga neapolitana. Appl Biochem Biotechnol 2002;98(1–9):177–89.
[26] Schröder C, Selig M, Schönheit P. Glucose fermentation to acetate, CO 2 and H 2 in the anaerobic hyperthermophilic eubacterium Thermotoga maritima: involvement of the Embden-Meyerhof pathway. Arch Microbiol 1994;161(6):460–70.

[27] Vigneron A, Alsop EB, Lomans BP, Kyrpides NC, Head IM, Tsesmetzis N. Succession in the petroleum reservoir microbiome through an oil field production lifecycle. ISME J 2017.
[28] Gittel A, Kofoed MV, Sørensen KB, Ingvorsen K, Schramm A. Succession of Deferribacteres and Epsilonproteobacteria through a nitrate-treated high-temperature oil production facility. Syst Appl Microbiol 2012;35(3):165–74.
[29] Shelton JL, Andrews RS, Akob DM, DeVera CA, Mumford A, McCray JE, McIntosh JC. Microbial community composition of a hydrocarbon reservoir 40 years after a CO2 enhanced oil recovery flood. FEMS Microbiol Ecol 2018;94(10):fiy153.
[30] Veshareh MJ, Azad EG, Deihimi T, Niazi A, Ayatollahi S. Isolation and screening of Bacillus subtilis MJ01 for MEOR application: biosurfactant characterization, production optimization and wetting effect on carbonate surfaces. J Petrol Explor Prod Technol 2018:1–13.
[31] Veshareh MJ, Ayatollahi S. Microorganisms' effect on the wettability of carbonate oil-wet surfaces: implications for MEOR, smart water injection and reservoir souring mitigation strategies. J Petrol Explor Prod Technol 2019:1–12.
[32] Marietou A, Kjeldsen KU, Røy H. Physicochemical and biological controls of sulfide accumulation in a high temperature oil reservoir. Appl Microbiol Biotechnol 2020;104(19):8467–78.
[33] Taheriotaghsara M, Hosseinzadehsadati S, Nick HM. The impact of spatially correlated heterogeneity and adsorption on modified salinity water in carbonates. ACS Omega 2020;5(46):29780–94.
[34] Takahata Y, Nishijima M, Hoaki T, Maruyama T. Thermotoga petrophila sp. nov. and Thermotoga naphthophila sp. nov., two hyperthermophilic bacteria from the Kubiki oil reservoir in Niigata, Japan. Int J Syst Evol Microbiol 2001;51(5):1901–9.
[35] Fardeau M-L, Ollivier B, Patel B, Magot M, Thomas P, Rimbault A, Rocchiccioli F, Garcia J-L. Thermotoga hypogea sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. Int J Syst Evol Microbiol 1997;47(4):1013–9.
[36] Ravot G, Magot M, Fardeau M-L, Patel B, Prensier G, Egan A, Garcia J-L, Ollivier B. Thermotoga elfii sp. nov., a novel thermophilic bacterium from an African oil-producing well. Int J Syst Evol Microbiol 1995;45(2):308–14.
[37] Mirgorod YA. The solubilities of propylbenzene and hexane in water as functions of temperature. Russ J Phys Chem C/C Zh Fiz Khim 2002;76(10):1608–11.
[38] Yeh DH, Pennell KD, Pavlostathis SG. Toxicity and biodegradability screening of nonionic surfactants using sediment-derived methanogenic consortia. Water Sci Technol 1998;38(7):55–62.
[39] Lin P-Y, Whang L-M, Wu Y-R, Ren W-J, Hsiao C-J, Li S-L, Chang J-S. Biological hydrogen production of the genus Clostridium: metabolic study and mathematical model simulation. Int J Hydrogen Energy 2007;32(12):1728–35.
[40] Elsamadony M, Tawfik A, Suzuki M. Surfactant-enhanced biohydrogen production from organic fraction of municipal solid waste (OFMSW) via dry anaerobic digestion. Appl Energy 2015;149:272–82.
[41] Leaño EP, Babel S. The influence of enzyme and surfactant on biohydrogen production and electricity generation using palm oil mill effluent. J Clean Prod 2012;31:91–9.
[42] Singh L, Siddiqui MF, Ahmad A, Rahim MHA, Sakinah M, Wahid ZA. Application of polyethylene glycol immobilized Clostridium sp. LS2 for continuous hydrogen production from palm oil mill effluent in upflow anaerobic sludge blanket reactor. Biochem Eng J 2013;70:158–65.
[43] Fan X, Li Y, Zhu S, Zhang H, Ai F, Zhang Q, Zhang Z. Role of surfactant in affecting photo-fermentative bio-hydrogen production performance from corncob. Bioresour Technol 2021;333:125173.
[44] Zhang Z, Fan X, Li Y, Jin P, Jiao Y, Ai F, Zhang H, Zhang Q. Photo-fermentative biohydrogen production from corncob treated by microwave irradiation. Bioresour Technol 2021:125460.
[45] Kaar WE, Holtzapple MT. Benefits from Tween during enzymic hydrolysis of corn stover. Biotechnol Bioeng 1998;59(4):419–27.
[46] Eriksson T, Börjesson J, Tjerneld F. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzym Microb Technol 2002;31(3):353–64.
[47] Singh A, Van Hamme JD, Ward OP. Surfactants in microbiology and biotechnology: Part 2. Application aspects. Biotechnol Adv 2007;25(1):99–121.
[48] Schmidt KA, Quiñones-Cisneros SE, Kvamme B. Density and viscosity behavior of a North Sea crude oil, natural gas liquid, and their mixtures. Energy Fuel 2005;19(4):1303–13.
[49] Xu R, Saggese C, Lawson R, Movaghar A, Parise T, Shao J, Choudhary R, Park J-W, Lu T, Hanson RK. A physics-based approach to modeling real-fuel combustion chemistry–VI. Predictive kinetic models of gasoline fuels. Combust Flame 2020;220:475–87.
[50] Yang P-B, Tian Y, Wang Q, Cong W. Effect of different types of calcium carbonate on the lactic acid fermentation performance of Lactobacillus lactis. Biochem Eng J 2015;98:38–46.
[51] Elderfield H. Carbonate mysteries. Science 2002;296(5573):1618–21.
[52] Rochelle CA, Czernichowski-Lauriol I, Milodowski A. The impact of chemical reactions on CO2 storage in geological formations: a brief review. Geol Soc Lond Spec Publ 2004;233(1):87–106.
[53] Schovsbo NH, Holmslykke HD, Kjøller C, Hedegaard K, Kristensen L, Thomsen E, Esbensen KH. Types of formation water and produced water in Danish oil-and gasfields: implications for enhanced oil recovery by injection of 'smart'water. Geol Surv Den Greenl Bull 2016;35:43–6.
[54] Veshareh MJ, Nick HM. A sulfur and nitrogen cycle informed model to simulate nitrate treatment of reservoir souring. Sci Rep 2019;9(1):7546.
[55] Hemme C, Van Berk W. Hydrogeochemical modeling to identify potential risks of underground hydrogen storage in depleted gas fields. Appl Sci 2018;8(11):2282.
[56] Veshareh MJ, Kjeldsen KU, Findlay AJ, Nick HM, Røy H, Marietou A. Nitrite is a more efficient inhibitor of microbial sulfate reduction in oil reservoirs compared to nitrate and perchlorate: a laboratory and field-scale simulation study. Int Biodeterior Biodegrad 2020;157:105154.
[57] Bundhoo MZ, Mohee R. Inhibition of dark fermentative bio-hydrogen production: a review. Int J Hydrogen Energy 2016;41(16):6713–33.
[58] Sahu AK, Conneely T, Nüsslein K, Ergas SJ. Hydrogenotrophic denitrification and perchlorate reduction in ion exchange brines using membrane biofilm reactors. Biotechnol Bioeng 2009;104(3):483–91.
[59] Pendyala B, Chaganti SR, Lalman JA, Shanmugam SR, Heath DD, Lau PC. Pretreating mixed anaerobic communities from different sources: correlating the hydrogen yield with hydrogenase activity and microbial diversity. Int J Hydrogen Energy 2012;37(17):12175–86.
[60] Wang D, Duan Y, Yang Q, Liu Y, Ni B-J, Wang Q, Zeng G, Li X, Yuan Z. Free ammonia enhances dark fermentative hydrogen production from waste activated sludge. Water Res 2018;133:272–81.