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Laboratory Investigation of *Thermoanaerobacter brockii* Subsp. *Lactiethylicus* Strain 9801^T for Possible Utilization in Microbial Enhanced Oil Recovery

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Abstract

Thermoanaerobacter brockii subsp. *lactiethylicus* strain 9801^T originally isolated from a deep reservoir environment was evaluated for potential use in microbial enhanced oil recovery (MEOR). Investigation was conducted for finding the optimum environmental parameters for growth (temperature, salinity and pH) and production of metabolites desired for microbial enhanced oil recovery. There was growth in the media with pH from 6-9.5 and salinity range of 0.5-3.5% (w/v) and temperature of 50-60°C. The optimum growth occurred at about pH of 7 and temperature of 55°C. The fermentable substrates included molasses, reinforced clostridia medium, thioglycolate and crude oil with major fermentation end products being lactic acid, volatile fatty acids and some amount of gas that included carbon dioxide. The concentration of lactic acid reached 11.7 g/l with a corrosive effect on core chalk samples that led to dissolution and release of calcium ions approximately 38 times greater than the background value. Additionally, this strain also produced biomass with a yield of between 0.0034-0.052 g/h per 100 ml of broth. Result of biosurfactant production assay indicated an oil displacement area of 3.5 cm^2 and fermentation of crude oil indicated a significant reduction in concentration of long chain alkanes in both light and heavy oils. Finally this strain compared favorably well in terms of metabolites production with other known bacteria strains employed in MEOR thus confirming the potential of *Thermoanerobacter brockii* subsp *lactiethylicus* 9801^T for utilization in microbial enhance oil recovery.

Keywords: Thermoanaerobacter; Fermentation; Degradation; Oil recovery; Metabolites

Introduction

Microbial enhanced oil recovery (MEOR) is an enhanced oil recovery technique that uses bacteria in oil reservoirs through production of specific metabolic processes that can lead to enhance oil recovery. The concept of using microorganisms to enhance oil recovery was first proposed by Beckman [1]. However, it was not until the after the work of ZoBell [2] that the research on MEOR gained wider attention. Since that time multiples of microbiological enhanced oil recovery projects have been carried out in different parts of the world. MEOR is a tertiary oil recovery process, where bacteria are supported via injection of nutrients. Some processes involved injecting fermentable carbohydrates into the reservoir. Other reservoirs require inorganic nutrients and oxygen to be introduced into the oil bearing strata in quantities that allow the microbes to release the residual oil into the water phase and substrates for cellular activities.

The mechanisms by which MEOR processes work can be highly complex. In general, the mechanisms of MEOR's action are most probably due to multiple effects of the microorganisms on the environment and oil. These mechanisms include gas formation, and pressure increases, acid production and degradation of carbonate matrices, reduction in oil viscosity and interfacial tension by biosurfactant, solvent production, plugging by biomass accumulation and degradation of large molecules in oil resulting in enhanced oil recovery [3-5].

The actual mode of MEOR in a particular reservoir will be very dependent on the characteristics of the reservoir and the types of indigenous microbes present in a particular reservoir. MEOR differs from chemical enhanced oil recovery through the method by which enhancing products are introduced into the reservoirs. In MEOR, microbes produce all the necessary chemicals in-situ but generally, the application, conditions and cultures can be targeted to meet a particular oil recovery situation [6].

Although MEOR has many advantages compared to other enhanced oil recovery technologists successful application requires the selection of microorganisms producing effective quantities of desired metabolites, processes which permits injection and dispersion of these microorganisms, and their supporting nutrients, predictable proliferation, and metabolic activity and an ability for the new ecosystem to persist for periods consistent with economic profitability [7]. The most active applications of the MEOR process are single well simulation treatments for removal of near well bore formation damage or oil mobilization in the region around wellbore [8], use of microbial systems for permeability modifications to improve water flooding sweep efficiency [9], use of microorganisms to produce gas, surfactants, acids and alcohols useful for enhanced oil recovery [10]. Although the use of microorganisms has been studied and tested for many years, the success of its application can be limited by the extreme reservoir conditions.

Thus, finding the right bacteria candidate for MEOR to fulfill the environmental conditions normally encountered in the petroleum reservoirs can be very challenging. This has made the search for the bacteria that will perform the work of releasing residual oil in the reservoir environments a continuous process. One of such group of bacteria targeted for application in microbial enhanced oil recovery are members of the order Thermoanerobacteriales within the Firmicutes

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which are commonly encountered in oil fields and include isolates belonging to the genera *Thermoanarobacter* [11,12]. This is because they are conducive for growth in deep seated environment such as oil bearing reservoirs [11]. *Thermoanaerobacter brockii* is one of the thermophilic bacteria under current investigation for evaluation of the potential of these organisms for production of metabolites that can enhance oil recovery. Such metabolites include gas and acid production, surfactants, biomass etc. Their high growth temperature offers process advantage in high temperature oil reservoirs and their broad substrate spectrum may allow utilization of inexpensive carbon sources available such as molasses.

The main goal of this study is to choose thermophilic anaerobic bacteria, *Thermoanaerobacter brockii* subsp. *lactiethylicus* strain 9801^T for laboratory investigation of its potential for production of metabolites needed in microbial enhanced oil recovery processes. This strain of bacteria have been studied previously in terms of its characterization and genetic properties [11,12] but little is known about its potential for enhance oil recovery purposes. It is believe that initial outcome from this study can be specific in determining the mechanisms by which this strain could enhance or improve oil recovery before planning physical simulation experiments and also to determine if adaptation to reservoir conditions such as high salinity will be required.

Materials and Methods

Microorganisms

The microorganism, *Thermoanaerobacter brockii* subsp. *lactiethylicus* strain 9801^T was purchased from the German Culture Collection (DSM) and it was the same strain that was originally isolated from a deep subsurface French oil well at a depth of 2100 m where the temperature was 92°C and now deposited at DSM. The details of the oil strata and reservoir conditions have been described [11]. The cells are gram-positive, straight motile rods (0.5 by 2 to 3 microns) with flagella uniformly distributed over the entire surface of the cell. During cultivation, optimum growth occurred between 55 and 60°C, the fermentable substrates include glucose, fructose, galactose, mannose, cellobiose, maltose sucrose etc. The products of fermentation of glucose were lactate, acetate, ethanol, hydrogen and carbon dioxide and hydrogen while the DNA base composition is 35 mol% G + C.

Determination of most suitable nutrient broth for growth of Thermoanaerobacter brockii 9801-T

All nutrients broths were prepared by weighing the accurate amount specified on the products bottles and mixed in a volumetric flask to 1000 ml. After all salts were completely dissolved they were divided into 100 ml portions in serum bottles flushed with nitrogen and autoclaved at 121°C for 20 min. The nutrient broths were later inoculated with 0.5 ml and incubated for 42 h at 50°C.

Optimization of pH in the substrate

Clostridia nutrient medium and thioglycolate broth with resazurin was prepared according to the given instructions on the bottles and divided into portion where the pH was adjusted with the help of a pH Meter and a calibrated pH electrode and NaOH and HCl. For clostridium medium, the pH was adjusted to 5.00, 6.00, 6.60 (which is the original pH of the base) 7.00, 8.00, 9.00, 10.00, 11.00 and12.00 while for thioglycolate broth, the pH was adjusted to 5.00, 6.00, 7.00 (which is the original pH of the base), 8.00, 9.00, 10.00, and 11.00. 100 ml of each nutrient broth was dispensed into serum bottles, purged with nitrogen for 2 min and autoclaved at 121°C. All the serums bottles were inoculated with 0.5 ml culture with a sterile needle and syringe and incubated in an anaerobic jar at 50°C for approximately 42 h, and OD was measured. There were 4 replicates of each pH solution. Graphs were drawn over the average results (standard deviation =0.08).

Effect of temperature on growth

Clostridia media and thioglycolate broth with resazurin were used in this experiment. The media were dispensed into 100 ml serum bottles, purged with nitrogen and autoclaved at 121°C for 2 min. The pH was adjusted to 6.60 and 7.10 for both media. All the serums bottles were inoculated with 0.5 ml culture with a sterile needle and syringe and incubated in an anaerobic jar at 50, 55, 60, 65, 70, and 75°C for 42 h. OD was measured and graphs were drawn over the average results of 4 replicates (standard deviation =0.1). The temperature of the incubation oven was monitored with a calibrated digital thermometer.

Effect of salinity on growth

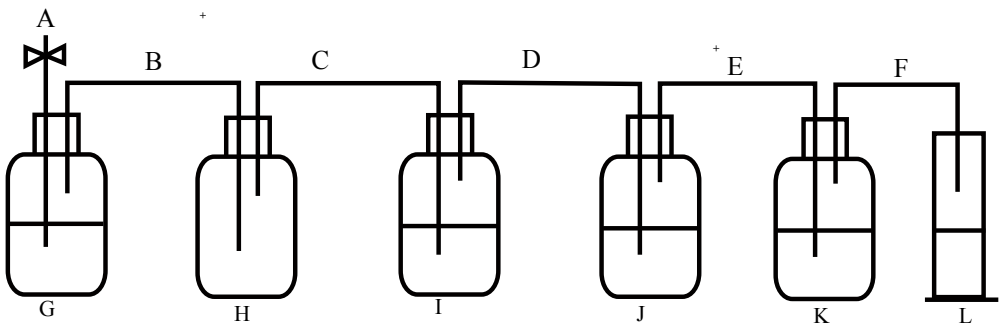
Clostridia media was prepared according to specifications and the broth were adjusted to salt concentration of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6 7 and 10 % (w/v) respectively. 100 ml was dispensed into serum bottles, autoclaved and inoculated with 0.5 ml culture with a sterile needle and syringe and incubated in an anaerobic jar at 50°C for 42 h. The optical density (OD) was measured and graphs drawn over the average of 4 results (standard deviation =0.12). The temperature of the incubation oven was monitored with a calibrated digital thermometer.

Screening for metabolites production

2½ litres of Clostridial nutrient medium (Fluka 27546) was prepared and autoclaved at 121°C according to the given instructions. The pH electrode and temperature sensor were calibrated prior to start of the experiment. The fermentation cultivation vessel, tubing, temperature sensor, pH electrode and all other utensils were sterilized following standard procedure. After all components were sterilized the reactor system was assembled and the medium was aseptically poured into the vessel and was purged with nitrogen so it was anaerobic and inoculated with 10 ml of a 42 h old culture. The temperature was set at 55°C and start pH at 7.28. 4 gas wash bottles were connected to the outlet; the first bottle was empty used as a safety bottle number 2 was with the saturated $Ba(OH)_2$, bottle number 3 was with the 0.1 M $Cu(NO_3)_2$ the last bottles was with water from which a tube ran over to a graduated cylinder (it is very important that the system is 100% anaerobic). These wash bottles were used for qualitative detection of gases. Carbon dioxide when passed through $Ba(OH)_2$ gives a white precipitate of $BaCO_3$, similarly when hydrogen sulphide is passed through $Cu(NO_3)_2$ it gives a reaction which result is a precipitate of CuS. The gas wash bottle with water was used for the purpose of the occurrence of other types of gas which could be detected by the displacement of water. The process was started and samples were taken for OD analysis and VFA at convenient intervals. The fermentation was continued for 52 h and at the end of the fermentation ethanol and lactic acid was determined.

Cell biomass determination

Triplicate 5 ml samples (fermentation broth) were put in 100 mm centrifuge tubes. The samples were centrifuged for 15 min at 5000 rpm. The supernatant was decanted. The precipitate was washed twice with 10 ml distilled water. The final residue was poured into a tarred aluminum weighing dish which had been previously dried in the oven for 24 h. The dishes were placed in an oven at 105°C and dried to constant weight. The aluminum dishes were cooled in a dessicator before weighing. The biomass was determined by calculating the difference in the weight of



A=sample outlet with valve, B,C,D,E,F=gas outlet, G=fermentation bottle, H= safety bottle, I=barium hydroxide bottle, J=copper nitrate bottle , K= water for gas displacement, L=measuring clinder

Figure 1: Schematic of substrate fermentation process.

the full and empty dishes. The final cell biomass concentrations were reported as g of dry biomass per 100 ml of fermentation broth.

Determination of biosurfactant activity and surface tension

A qualitative method to determine the displacement of oil by biosurfactant was employed [13].The produced biosurfactant was separated from the culture media by centrifugation to get culture supernatant. 50ml of distilled water was pipetted on a petridish (18 cm diameter) followed by addition of 20 µl of crude oil to the water surface, and finally 10 µl of culture broth supernatant were gently put on the centre of the oil.The displacement pattern was observed. A clear circle was visible under light. The area of the circle was measured and calculated for oil displacement area (ODA) by the equation (1).

$$ODA = 22/7\ (radius)^2\ cm^2 \quad (1)$$

Fermentation of light and heavy oil

Fermentation processes was carried using light and heavy oil with and without molasses. 1500 ml of seawater, 5% thin oil, and 0.5% molasses was put in a 2000 ml blue cap media storage bottle and a second solution consisting of 1500 ml of seawater, 5% thin oil without molasses was put into another 2000 ml bottle. The two set ups were completed with two outlets on the cap from which samples were taken and the other outlet for gas exhaustion. Both oil solutions were sterilized at 120°C for 20 min. Wash bottles were attached to the exhaust outlets with the help of silicone tubing. Like in the screening experiment, the first wash bottle was a safety bottle, the second was with saturated $Ba(OH)_2$ that could precipitate any carbon dioxide produced, the third wash bottle contain 0.1M $Cu(NO_3)_2$ to absorb any H_2S and the last bottle was with water to detect any other gas produced through displacement of water (Figure 1). The oil mixtures were then inoculated with 10 ml of 48 h culture and both bottles were incubated in a water bath at 55°C. The pH and temperature were monitored with the help of a data logger. Samples were taken at intervals for analysis and also to monitor the culture under microscope. A similar experiment as was carried out with heavy oil for comparison.

Degradation of different alkanes

9 ml aliquot of seawater was dispensed into 8 serum bottles purged with nitrogen and sterilized. Prior to inoculation, 100 µl of vitamins, and 200 µl minerals were injected from sterile stock solutions and 100 µg of different alkanes were put in the serum bottles respectively and identified by a clear label. Repeated experiments were carried out by replacing seawater with reinforced clostridia media to evaluate the effect of additional nutrient on the degradation process.

Bacteria solution in chalk sample

A chalk core obtained from a Danish oilfield with a diameter of 2.1 cm was cut into a piece of 5 cm long. It was sterilized and placed in a sterile core holder and attached to a sterile blue cap bottles with an assembly spout and 200 ml clostridia nutrient media at each end. Before all experimental runs, the flowing tubing and fittings were disinfected with 70% IPA spirits and then completely flushed with sterile 0.9% brine before each start-up. It is very important to be very sure that there is no leaking of media from one chamber to another by ensuring that the core fits tightly. The system was flushed with nitrogen to ensure anaerobic condition. The only way the bacteria and broth can reach the other chamber is to migrate through the pores of the chalk core. One of the bottles was inoculated with 10 ml 48 h culture and incubated at 55°C. The apparatus was monitored for leaks and the system proved to be leak proof. The experiment was carried out twice. The system fulfilled all the requirements for rapidly and effectively identifying a migration of a growing culture which showed potential of being candidates for further testing when time permits [14].

The second experiment with chalk samples was to determine the effect of acid produced by the strain on carbonate rock. Four identical chalk samples with diameter of 2.0cm and length of 2.5 cm were used for the experiment. Duplicate samples were immersed in flasks containing100 ml bacteria solution for 72 h. Duplicate samples were also immersed in flasks containing100 ml demineralized water for the same period to serve as control. Final result was average of two measurements (standard deviation =0.023).Background value of calcium ions measured in the reinforced clostridia media was deducted from the measured concentration after 72 h. The initial and final pH of each the media were also measured.

Analytical methods

The optical density measurements were acquired a spectrophotometer (Model: Cecil 7200). The concentration of volatile fatty acid (VFA) and the ethanol produced during fermentation was analyzed using GC Varian 3800 model with column; Varian 25 m, 0.32 mm ID. The carrier gas was helium/10 psi. The lactic acid was determined using bio-analysis kit for lactic acid (Cat. No 10 139 084 035) from Food Diagnostics. The biogas yield was measured by using a lure lock syringe and a needle. Varian GC-MS (Model Varian 3800) was used for chromatographic test of alkanes and crude oil. Viscosity of the

Media	Optical Density (650 nm)
Glucose based medium with 0.2 ml trace elements and 0.2 ml vitamin solution	0.328
Glucose based medium with 0.2 ml trace elements without vitamins	0.156
Glucose based medium without trace elements and vitamin solution	0.051
Glucose based medium without trace elements and with 0.2 ml vitamin solution	0.013
Clostridial nutrient medium without vitamins	1.344
Clostridial nutrient medium with 0.2 ml vitamins	1.270
Thioglycolate broth with resazurine without vitamins	0.904
Thioglycolate broth with resazurine with 0.2 ml vitamins	1.256

Table 1: The results of the different media used for growth based on optical density measurement.

oil sample before and after bacteria inoculation was determined using a Brookfield viscometers. The surface tension measurements were taken using a modified Wilhelmy surface tensiometer and measurement taken at static mode. The fluid samples were analyzed for calcium ions concentration by atomic absorption spectra (AAS) using Perkin Elmer Optima (DV 3000).All the analyses were performed according to standard methods.

Results

Growth in nutrients

The ability to utilize various substrates was tested and given as a function of optical density (OD) (Table 1). The OD of the growth showed that the highest growth occurred in the clostridial nutrient media with and without vitamins. The use of thioglycolate broth with vitamins resulted in a better growth than thioglycolate broth without vitamins. The glucose based medium with or without vitamins showed little or no growth. All the results were obtained after 42 h of incubation, the glucose medium when incubated for a period of 4 days longer showed only a minimal extra growth. These results suggest that both clostridial and thioglycolate can be a better substrate than glucose based medium for cultivation of *Thermoanerobacter brockii* subsp. *lactiethylicus* 9801^T as they provide enough nourishment for the bacteria to survive with a reasonable growth rate. Thus, the two media were selected for evaluation of the optimum pH for growth. Figure 2 showed the phase contrast micrographof *Thermoanerobacter brockii* subsp. *Lactiethylicus* 9801^T.

Optimization of pH in the substrate

An overlay of the results of the pH in different media is shown in Figure 3. The result shows that there is optimal growth at a pH interval of 6.5-8.5 for clostridia medium with and without vitamins. This suggests there is no need to add vitamins as the growth seem to be the same. When compared to the thioglycolate broth with and without vitamins, it shows that the thioglycolate with vitamins has growth at pH of 7.5-8.7 and thioglycolate without vitamins has an optimal growth at an approximate pH of 6.5-7.5. Combining all the results, it can be deduced that this strain can grow at a pH of 6-9.5.

Effect of temperature on growth

As shown in Figure 4, growth was observed at a temperature range of 45-70°C for the two substrates. The OD was highest at 55°C for reinforced clostridia media; however for the thioglycolate the OD was slightly lower at 55°C. When the two lines for the broths were statistically compared by running an F-test, both averages was found to be the same. It can be concluded that given a pH of 6.6 affords the best optimal temperature for this strain to be deduced in a substrate. Since the clostridial nutrient medium gives the best result for optimum temperature, further experiments conducted uses this substrate as growth media. It is time saving and affords the opportunity to concentrate entirely on a single substrate.

Effect of salinity on growth

The effect of salt concentration on *Thermoanerobacter brockii*

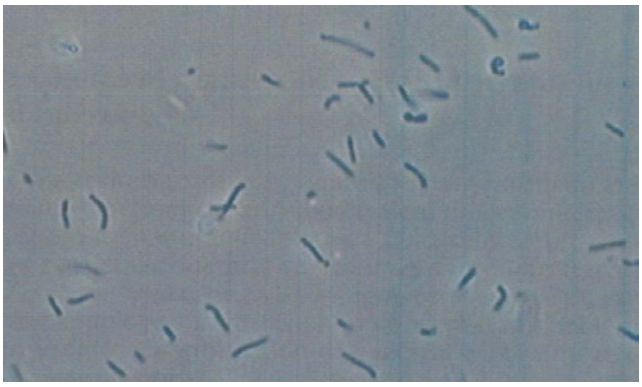


Figure 2: Phase-contrast micrograph of *Thermoanerobacter brockii*subsp. *lactiethylicus* 9801^T (x 40).

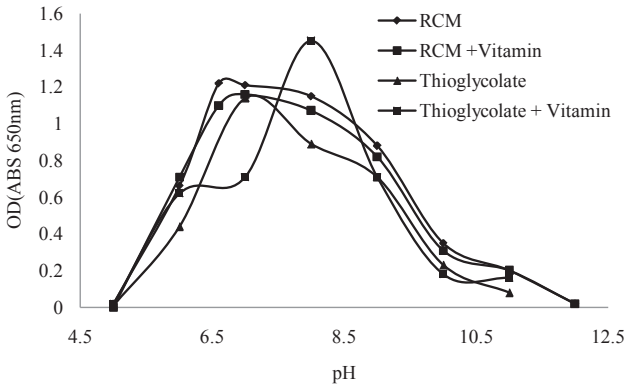


Figure 3: Optimum pH for growth in different media.

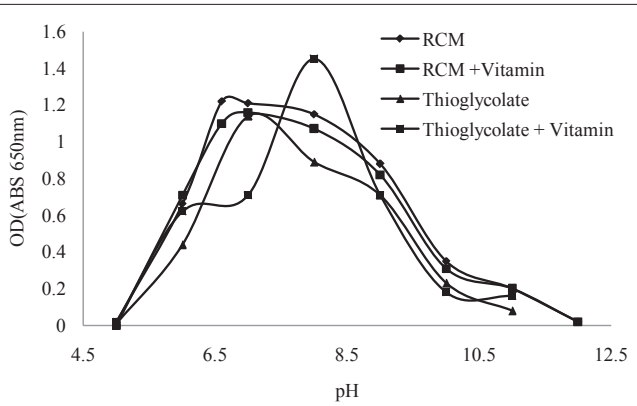


Figure 4: Temperature optimum after 24 h incubation.

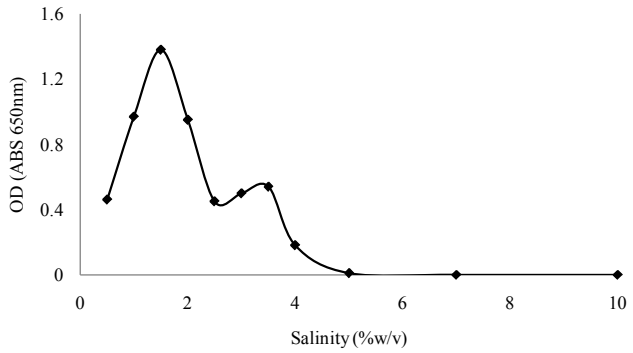


Figure 5: Growth of *Thermoanaerobacterbrockii* 9801^T at different salt concentration.

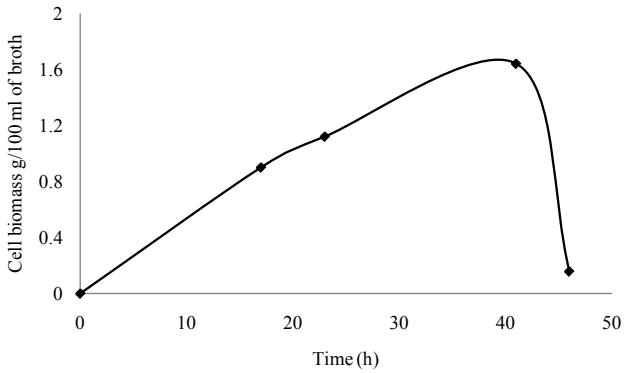


Figure 6: Biomass per 100 ml substrate over 46 h of fermentation.

subsp. *lactiethylicus* 9801^T is shown in Figure 5. The bacteria appear to grow reasonably well in salt concentrations from 0.5% to 3.5% (w/v) which is the concentration of seawater. The optimum growth was found at 1.5% (w/v) concentration. When the concentration becomes greater, the growth slows down, and subsequently results in death of the bacterium culture. Further experiments showed that if this strain is grown repeatedly at 4% (w/v) concentration, they become adapted and subsequent increased by 1% (w/v) of salt concentration at time causes growth till 5% (w/v) of salinity.

Cell biomass determination

The result of cell biomass determination is shown in Figure 6. The final cell biomass concentrations are reported as g of dry biomass per 100 ml of fermentation broth. If we consider the cell biomass production rate over time, the cell biomass rate decrease from 0.052 $g.h^{-1}$ in 17 hours to about 0.0034 $g.h^{-1}$ in 46 h. The decrease in the cell biomass rate can be related to decrease in growth rate over time as less and less nutrient is available and suggest that less cell biomass can be harvested as time increase.

Fermentation of medium

Table 2 shows the summary of metabolite products from the fermentation process using RCM as substrates. It was observed that a good amount of CO_2 was produced as indicated by precipitate of barium carbonate. The volume produced was not measured. No hydrogen sulphide was detected during the fermentation process, but there was about 100 ml of water displaced which suggests that there was a production of another type of gas, probably methane.

The pH fell from 6.8 to 4.48 in the fermentation flask suggesting the productions of acids. Analysis of the samples showed the presence of acetic acid, isovaleric acid, lactic acid and ethanol. The average concentration of lactic acid produced during the fermentation process was 11.7 g/l. The volatile fatty acids (VFA) that turned out to be acetic acid and of isovaleric acid had average concentrations of 2.5 g/l and 0.08 g/l respectively. The concentration of the ethanol produced during fermentation was in average about 2.4 g/l.

Furthermore, the effect of ionic strength of the media on gas production per 100 ml of substrate after 48 h was investigated. The results (Figure 7) showed that the specific salt concentration that was present at the optimal growth also were involved in the highest gas production, i.e. 1.5% (w/v) with the exception of 0.5% (w/v) which gives almost the same amount. The volume of gas produced was between 10-40 ml and it decreases with increasing ionic strength.

Biosurfactant production and surface tension measurement

The result of surface tension measurement for the culture grown on different salt concentration is shown in Table 3. The measurement of surface tension gives a rough idea about the concentration of the biosurfactant in the broth. The surface tension measurement showed almost constant surface tension independent of salinity or culture growth and very similar to the value of pure water at room temperature. Therefore, this method was not able to show increase in concentration of biosurfactant at the relatively high salinities.

Contrary to this, the bio surfactant activity monitored by oil displacement showed a very distinctive effect visible under light (Figure 8).It can be seen that there was a very distinctive displacement of oil when the culture broth supernatant was put on top of it; an indication of that biosurfactant possibly reduces the surface activity resulting in the formation of a clear zone on the drop of oil. The area of the circle as calculated by equation (1) was 3.5cm^2 representing about 10% of area of the oil spread. No such effect was observed when distilled water

Products	Lactic acid (g/l)	Ethanol (g/l)	Acetic acid (g/l)	Iso-valeric acid (g/l)	CO_2	H_2S	Other Gas (ml)
Amount	11.7		2.5	0.08	P	N.P	100

P=produced, N.P=not produced

Table 2: Metabolites produced during fermentation process with reinforced clostridia medium.

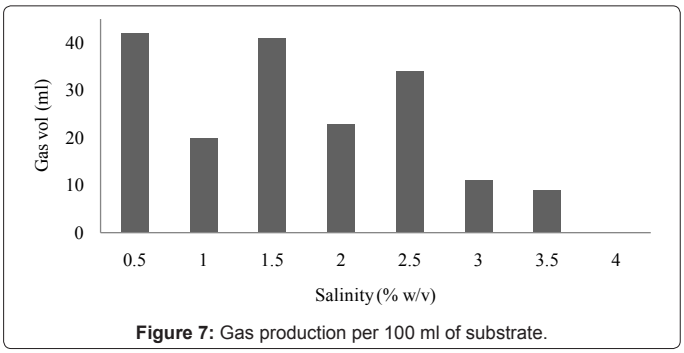


Figure 7: Gas production per 100 ml of substrate.

NaCl (% w/v)	0.5	1.0	1.5	2.0	2.5	3.0	3.5
Initial surface tension (N/m)	0.057	0.059	0.057	0.059	0.057	0.057	0.056
Final surface tension (N/m)	0.054	0.055	0.053	0.053	0.056	0.058	0.057

Table 3: The result of surface tension measurement for culture grown on different salt concentration.

Figure 8: Oil displacement by biosurfactant. The area displaced can be seen as whitish halo within the oil spread.

Components	Acetic acid	Butyric acid (mg/l)	Ethanol
Thin oil			
Initial conc.	0.0	0.0	0.0
Final conc.	4869.0	795.3	0.30
Heavy Oil			
Initial conc.	0.0	0.0	0.0
Final conc.	700.97	0.0	0.17

Table 4: VFA and ethanol production in heavy and thin oil after fermentation.

was drop on the surface of the oil. The blank experiment showed that there is no clearing and the drop of oil stayed intact. This test indicated that biosurfactant was produced by *Thermoanerobacter brockii* subsp. *lactiethylicus* 9801^T.

Fermentation of hydrocarbon

The results from fermentation of heavy and thin oil showed different characteristics. The viscosity of thin oil did not change during the period of fermentation. The initial viscosity measured was 23 cP at the start of experiment; the final viscosity measured at the end after approximately 130 days is 24 cP indicating almost no change. However, for the thick oil, the viscosity was reduced from initially value of 70 cP to 8 cP, representing an about 9 times reduction in viscosity. Acetic acid, butyric acid and ethanol were also produced as shown in Table 4 during fermentation of crude oil in seawater.

The result of the alkane degradation is shown in Figure 9. The results from the GC/MS runs were calculated by taking the top height of the chromatogram from the alkanes and dividing it with the top height of the internal standard. When the results are analyzed as shown in Figure 6, it can be concluded that the ratio of alkanes falls during the period of incubation. C_{15}, C_{16}, C_{18}, C_{20} and C_{28} all show a drastic fall in concentration, while for carbon chains C_{10}, C_{12} and C_{13}, the fall in concentration was minimal. When the degradation of heavy oil in seawater and in the reinforced clostridium media (RCM) were compared (Figure 10 and 11), the oil in seawater showed a slower degradation than oil in the reinforced media. This suggests there was better metabolism for this strain in the reinforced media and oil as carbon source than with oil and seawater.

Bacteria solution in chalk sample

The result of bacteria solution in chalk core showed that bacteria migration through chalk core occurred after 3 days of incubation. There was growth in the media which was not inoculated with culture suggesting a migration of bacteria through chalk core from the inoculated part had taken place which was indicated by change of turbidity of the not inoculated fluid. However, after further incubation for another week, it was observed when examined under the microscope that there was an aggregation of the cells on the surface ends of the chalk (Figure 12). The result of the corrosive effect of the acid produced on chalk samples is shown in Table 5. The measured concentration of calcium ions in the fermentation medium of *Thermoanerobacter brockii* subsp. *lactiethylicus* 9801^T was 354.8 g/l compared to 3.1 g/l when chalk samples were immersed only in de-mineralized water. The background

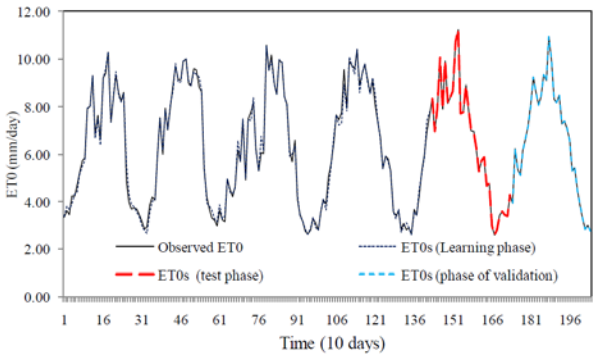


Figure 9: The result of the degradation of alkanes.

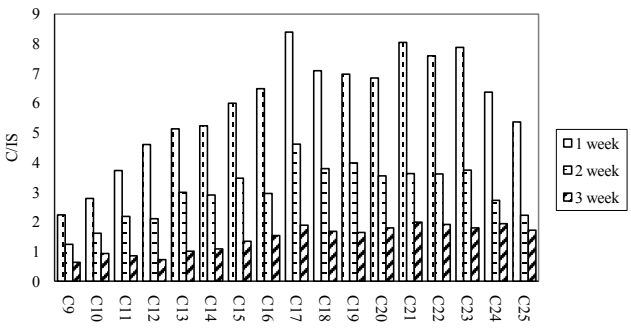


Figure 10: Growth in heavy oil with seawater as substrate.

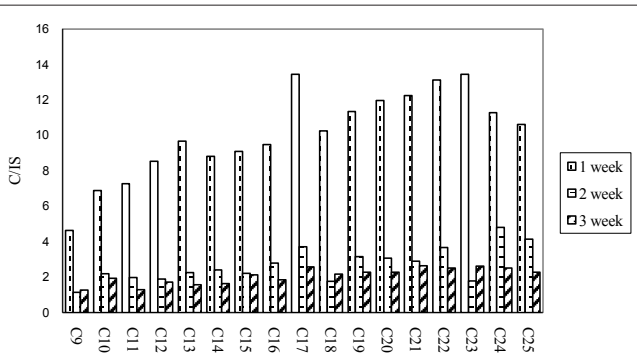


Figure 11: Growth in heavy oil with reinforced clostridia media as substrate.

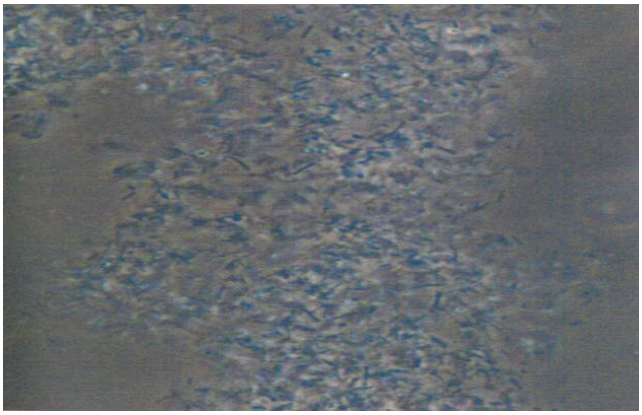


Figure 12: Aggregated cells of at one end of chalk core.

Experiment	Medium	Initial pH	Final pH	[Ca^{2+}] (mg/l)
1	Chalk + bacteria solution	6.80	6.15	354.8
2	Chalk + demineralized water	6.34	6.30	3.12
3	Bacteria solution without chalk sample	6.80	5.12	9.43

Table 5: Comparison of chalk dissolution in bacteria medium and demineralized water.

value of calcium ions in the bacteria media without chalk samples was only 9.4 g/l an indication that the acid effect increased the calcium ions concentration by a factor of 38.

Discussion

In this study, *Thermoanaerobacter brockii* subsp. *lactiethylicus* strain 9801^{T}ananaerobic, rod shaped, gram positive and thermophilic was found to ferment a variety of carbohydrates and produce lactate ethanol, acetic, CO_2 and other gases. This matches earlier descriptions [12]. Also the strain utilizes thiosulphate as an electron acceptor to produce sulphide but does not utilize sulphate. Sulphide production makes strain 9801^T a potential bio-corrosive agent in oil wells because of the presence of thiosulphate as it has been mentioned that the *Thermoanaerobacter* species have the ability to reduce thiosulphate during growth on carbohydrates [9, 12].

On the basis of its optimum temperature for growth and sodium chloride requirement, *Thermoanaerobacter brockii* subsp. *lactiethylicus* 9801^T did not grow at temperature above 70°C although the in-situ temperature of the oil well from which it was isolated was 92°C indicating that this microbe may have colonized in the cooler parts of the reservoir. The optimum growth occurred at temperatures of 55°C. This falls within the optimum temperature range of between 55-60°C reported by [11,12]. Even though many oil reservoirs have initial temperature of greater than 75°C but due to continuous injection of water for oil recovery over time, the temperature in the reservoirs is much less than the initial reservoir temperature as the field matures. MEOR application is usually targeted for matured water flooded reservoir, therefore there is a potential for this strain to be utilized in MEOR if other properties such as acids, gases and biosurfactant production for improve oil recovery are fulfilled. Compared to mesophilic bacteria such as *Clostridium tyrobutyricum* and *Bacillus* that normally grows at optimal temperature of 37°C, this strain has a potential for application in high temperature reservoirs.

There was growth in the media with pH from 6-10 and salinity range of 0.5-3.5% (w/v). The optimum growth was found ata salinity of 1.5% (w/v). However, further experiments showed that if this strain was grown repeatedly at 4% (w/v) concentration of NaCl, they become adapted, but no growth occurred when the concentration was further increased to 7% and 10% (w/v) salt concentration, respectively. It is evident that the current salinity range at which growth occurred for this strain is low compared to salinity condition for many oil wells. For example *Bacillus licheniformis* strain, JF-2 from an oil filled water injection was found to grow and produced biosurfactant at salinity up to 8% and temperatures up to 45°C [16]. Experience have shown that with careful planning and repeated sub-culturing, it is possible that this strain can grow in higher salt concentrations normally encountered in the reservoirs, given that salinity gradient can vary from low to high, however we did not investigate this further in the current study.

The fermentable substrates include molasses, thioglycolate, RCM and crude oil. However the strain showed little or no growth in glucose medium with or without vitamins which is opposite to earlier observation reported by different workers [11,12]. The reason for this cannot be fully understood, and raises the question whether the lower pH is actually causing the reduction in growth rate observed in the presence of glucose. It was also found that *Thermoanaerobacter brockii* subsp. *lactiethylicus* strain 9801^T can grow in media containing oil supplemented with either reinforced clostridia medium, molasses or seawater. At a temperature of 55°C the strain was capable of producing biogas and bioacids. Analysis of the samples for VFA's showed the presence of acetic acid, isovaleric acid, lactic acid and ethanol. The average concentration of lactic acid produced during the fermentation process was 11.7 g/l. The volatile fatty acids (VFA) that turned out to be acetic acid and of isovaleric acid had average concentrations of 2.5 g/l and 0.08 g/l respectively while concentration of the ethanol is about 2.4 g/l. Analysis at different intervals indicated an irregular pattern of rise and fall for the net VFA's produced during fermentation suggesting that bacteria can be utilizing some of the acid for metabolism. These results support the earlier findings regarding the metabolic products of *Thermoanaerobacter brockii* subsp. *lactiethylicus* 9801^T during fermentation and was comparable to the measured value of organic acids of 9.7-42 g/l reported for *Clostridium tyrobutyricum* ATCC 25755 [17] and 11.4 g/l of organic acids by another strain of *Clostridium tyrobutyricum* [18].

The full composition of the biogas formed was not determined, but it was clear that one of the major constituent was carbon dioxide as indicated by the precipitate of barium carbonate. Composition of biogas production from other bacteria strains employed in microbial enhanced oil recovery showed that *Clostridium tyrobutyricum* produces about 80% hydrogen and 15-20% of hydrogen and small amount of methane [18,19]. However the volume of biogas produced is very low. In comparison, reported range of biogas include between 250-3000 ml by adapted strain of *Clostridium tyrobutyricum* 663 [19], and 9700 ml for *Clostridium* sp. [18]. Explanation for the low volume of gas could be as a result of low volume of fermentation broth and nutrient which was only 100 ml compared to the 500 ml for the results cited. Biogas formation during a microbial oil recovery process may decrease the oil viscosity and increase reservoir pressure that can force out oil from the rock pores.

This strain was also capable of producing cell biomass. The cell biomass yield per 100 ml of broth decreased from 0.052 $g.h^{-1}$ in 17 h to about 0.0034 $g.h^{-1}$ in 46 h. The decrease in the cell biomass rate can be related to decrease in growth rate over time as less and less nutrient is available and suggest that less cell biomass can be harvested as time increase. The cell biomass yield can be said to be low for this strain as

similar experiments showed a biomass concentration of 0.11-0.15 g/g for *Clostridium tyrobutyricum* ATCC 25755 [17] and a value of 1.73 g/l for *Bacillus claussi* [20].A likely explanation for the observed low cell biomass yields is that most of the energy source consumed is diverted for the production of metabolic products. This argument is supported by the presence of high concentration of lactic acid and VFAs in the fermentation broth.

The presence of biosurfactant produced by bacteria has been shown to reduce the surface tension and interfacial tension (IFT) of the medium [21]. Result of biosurfactant production by surface tension measurement method could not clearly indicate change in surface tension measurement at relatively higher salinities (>2.5% (w/v)) for the RCM cultures. This was consistent with earlier observation that little changes can occur in the surface active properties of biosurfactant with addition of NaCl up to 2.0 mol^{-1} [22]. However, the production of biosurfactant was confirmed by oil displacement test. There was a very distinct displacement of oil when the culture is put on top it, an indication that the biosurfactant reduces the surface activity thereby making a clear zone on the drop of oil. The oil displacement area was about 3.5 cm^2 or a diameter of 1.5 cm in less than 1 h. This result is comparable to that of experiment with a biosurfactant producing *Staphylococcus* with potential application on hydrocarbon bioremediation that measured diameter of 2.5 cm for oil displacement test [23].We did not measure the concentration of the biosurfactant produced, however, the quality and quantity of biosurfactant production are affected and influenced by the nature of the carbon substrate [24,25]. Biosurfactant yield from a strain of Bacillus measured 0.015mol biosurfactant/ mol glucose [16].

The ability to produce biosurfactant can increase the oil degradation potential at a higher rate with possibilities of lowering the viscosity as shown by the results of oil degradation. The viscosity in heavy oil was approximately 10 times thinner than the original oil but no change was observed in the viscosity of thin oil under the same condition.

Moreover, the degradation of alkanes was also very significant probably from the effect of biosurfactant production. All the alkanes both thin and heavy oil form a pattern to show that *Thermoanerobacter brockii* subsp. *lactiethylicus* 9801^T can degrade alkanes. Comparison of heavy oil degradation using two different media of seawater and reinforced clostridia medium showed that the seawater had a slower degradation than the oil in reinforced clostridia media which suggests there was better metabolism for this strain in the reinforced media and oil as carbon source than with oil and seawater. It can also be concluded from the results (Figures 9 and 10) that not all the alkanes were equally reduced as a difference was recorded in each graph but it suggested interesting properties for its application in microbial enhanced oil recovery as biosurfactant is one of the most sought after bioproducts in oil and gas industry.

Evidence of bacteria transportation through the chalk core was manifested by growth in the media which was not inoculated with culture suggesting a migration of bacteria through chalk from the inoculated media. This a good property as it demonstrated that after injection, further transport of bacteria during incubation can occur by growth and mobility through the stagnant nutrient medium which fills the porous rock. However, after further incubation for another week, it was observed when examined under the microscope that there was an aggregation of the cells on the surface ends of the chalk. This can be an indication of potential plugging of the inlet surface of the core and can also suggest that this strain may be used to stimulate the in-situ growth of microorganisms in high permeable zone during selective microbial plugging process.

It was earlier shown that the average concentration of lactic acid produced during fermentation process was 11.7 g/L the effect of which was confirmed by the corrosion of the chalk samples as indicated by increased concentration of calcium ions in the media after 72 h of immersion in the fermentation fluid (Table 5). The concentration of the calcium ions was increased by a factor of 38. The increased concentration of calcium ions can only be explained by the dissolution of the chalk samples, which in MEOR application can improve chalk formation porosity and permeability. In comparison, acid and gas production by *Clostridium tyrobutyricum* increased dissolved calcium ions in carbonate chalk samples by a factor of 20 [26], *B.lichenformi*s increased dissolved calcium by 13% [27], fourfold increase of the calcium ions of the stratum water compared to the initial value was also reported [18].

Success of microbial enhanced oil recovery depends not only on the ability of selected microbes to produce desired metabolites but also on the good understanding of the reservoir conditions and whether the selected strain can proliferate and produced desired metabolites under such conditions. It can be inferred from the experimental results that this strain exhibited some desirable properties in production of metabolites such as acid production, production of biosurfactant, biomass production and ability to degrade alkanes in light and heavy oils and ability to penetrate core pores. The amounts of the metabolites produced are comparable to values cited in the literatures for other strains of bacteria commonly employed in microbial enhanced oil recovery purposes. However, what makes this strain better lies in the ability to grow at high temperature of 55^o C in comparison to many bacteria isolated and identified for microbial enhanced oil recovery purposes. Many of the bacteria strains used in MEOR usually experienced low efficiency when maintained at higher temperatures but this strain was able to exhibit good potential in production of metabolites at such high temperature. The significant production of lactic acids as well as acetic, isovaleric acid, ethanol and biomass can be an additional factor in rock modification and oil mobilization. Although, composition of gas was not specifically determined, formation of gaseous products under anaerobic condition is a positive result. Four conditions indicated to be met to achieve successful in situ bacteria growth and core plugging are: the cells must be transported throughout the rock stratum, nutrients must be transported for growth, the microorganisms must be able to grow and reduce the permeability of the rock by biomass and extracellular polymer production, the bacteria growth must not be so rapid that it results in the formation of bacterial plugs farther away from the wellbore [28,29]. The results of this study have demonstrated that *Thermoanerobacter brockii* subsp. *lactiethylicus* 9801^T meets all of these criteria and in addition was able to produce a broad spectrum of metabolites including acids, biomass, biosurfactants, gases whose production can be important in the oil recovery process. The only concern is its low growth at relatively high salinity (>5%) but it is believed that careful adaptation process can help to overcome this limitation as it has been shown that successful adaptation of bacteria to high salinity condition is possible [30].

Conclusions

Thermoanaerobacter brockii subsp. *Lactiethylicus* 9801^T has been investigated as a suitable candidate for microbial enhanced oil recovery. From this investigation, it can be concluded that *Thermoanaerobacter brockii* subsp. *lactiethylicus* 9801^T have a potential for possible utilization in microbial enhanced oil recovery. This is based on the ability of this strain to grow at an optimal temperature of 55^{o}C and production of metabolites that can be utilized for MEOR purposes. The strain was

capable of producing organic acids that can modify rock properties evident in dissolution of chalk samples. Cells were also able to migrate through pore spaces of carbonate rock sample suggesting possibility of high mobility when injected for microbial enhanced oil recovery purpose. Degradation of the alkanes was also significant. It is evident that this strain offer useful metabolic products such as biosurfactants, biogas, biomass, in addition to bio-acids for enhancing oil recovery. These bio-products can contribute to different microbial systems which can tackle specific problems of oil recovery. However, the finding indicated that the range of salinity at which this strain could grow is low compared to saline content of many oil wells but it could provide an opportunity for further research in terms of its applications in microbial enhanced oil recovery.

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