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Stimulation of an indigenous thermophillic anaerobic bacterial consortium for enhanced oil recovery

Short Title: Potential of indigenous bacterial consortium for EOR

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Abstract

Microbial enhanced oil recovery (MEOR) is potentially useful in incremental oil recovery from oil reservoirs beyond primary and secondary recovery operations using microorganisms and their metabolites. In the present study, anaerobic, thermophilic methanogenic consortium TERIL63 was developed from high temperature oil reservoir of India. TERIL63 was identified with strains of *Methanothermobacter thermoautotrophicus* and *Thermoanaerobacter* sp. It can grow in the range of 60 – 100 °C temperature, 0 – 5 % salinity (NaCl) and 5.0 – 9.0 pH in methanogenic medium. The novelty of the consortium is to produce gases (14.74 mmol L^{-1}), volatile fatty acids (2037 mg L^{-1}), acetone (1.95 g L^{-1}) and butanol (0.63 g L^{-1}) in 10 days through optimized recipe, which facilitates the displacement of oil towards the surface. The generated data was successfully exploited as a proof of oil recovery by performing core flood, where TERIL63 showed 15.49 % enhanced oil recovery. Thus non-toxic, non-virulent TERIL63 is a promising candidate for economically sustainable and environmentally safe solution for MEOR application.

KEYWORDS

Microbial enhanced oil recovery; coreflood; methane; volatile fatty acids (VFAs)

1. INTRODUCTION

Biotechnology has an increasing application in petroleum engineering. Microbial enhanced oil recovery (MEOR) is one of the biological efforts, which has substantial impact on the petroleum industry under the current energy shortage scenario. Crude oil production, under the recovery percentage by both primary and secondary methods is approximately 30-50 % [1-2]. The current tertiary recovery, or enhanced oil recovery (EOR), allows another 5-15 % residual oil to be recovered [3].

MEOR is a petroleum biotechnology process for manipulating function and /or structure of microbial environment existing in oil reservoirs for prolonged exploitation of the largest source of energy. MEOR is an environment friendly method posing several advantages over other enhanced oil recovery. MEOR is a less expensive process when compared with other EOR methods (such as chemical, thermal, polymer and surfactant) because microorganisms can synthesize useful products by fermenting low-cost substrates or raw materials [3]. Also, Kobayashi et al. [4] stated that MEOR is considered to be more cost effective and environmentally friendly, since the nutrients (and microbes) are biodegradable and relatively less expensive than the chemicals. In this technology, addition of nutrient allows the development of microorganism and the production of metabolites such as gases, solvents, acids, biopolymers, biosurfactants, enzymes and biomass which could be utilized to extend the life of the oil reservoirs [5]. Biomass can modify the permeability of porous media by selective plugging in high permeable zones and redirecting the oil flow in the reservoir [6]. The fermentation of carbohydrate produces gases such as carbon dioxide, methane and hydrogen, which contribute in repressurizing the reservoir[7].

Another benefit of fermentative activity is the production of volatile fatty acids (VFAs), for example acetic acid, propionic acid and the production of solvents such as ethanol, acetone and butanol [4]. Acids can reduce the pH of the environment and can alter fluids and solid surfaces, which can improve the flow behaviour of oil [8].

A variety of aerobic and mesophillic microorganisms has been reported for the microbiological recovery processes. However, studies have established that anaerobic, thermophillic, halotolerant indigenous population are most appropriate for the MEOR process [9]. Since the mechanism of MEOR depends on the microorganisms and characteristic of the oil it is reasonable to assume that indigenous

microbes remains metabolically active in the reservoir i.e., their native environment, during the MEOR operation [10]. In MEOR the nutritional components for microbial growth plays an important role in developing biological processes for enhanced oil recovery. However, nutrients are the largest expense in the MEOR processes where fermentation medium can represent almost 30% of the cost for a microbial fermentation [11]. According to Al- Sulaimani et al. [11] media optimization is very important since the types of bioproducts that are produced by different types of bacteria are highly dependent on the types, concentrations and components of the nutrients provided. Therefore, in the present study, optimization of nutrient recipe was taken into consideration to reduce the cost of nutrient injection in MEOR to make the overall process more economical and efficient.

Thus, in the present study, onshore Linch, Kalol and Nandasan oil fields of Gujarat, India were chosen as main reservoirs belonging to Kalol sands of middle Eocene age. Currently, these oil fields are partially depleted and characterized by high overall water cut (~90 %). Thermophillic anaerobic indigenous microbes were isolated from formation fluid samples and screened for anaerobic growth and metabolite (gases, VFA, and solvents) production in order to obtain viable solution for MEOR application. The unique combination of novel consortium and optimized nutrient recipe was successfully evaluated by performing core flood study for MEOR application.

2. Materials and methods

2.1. Sample collection

The formation fluids were collected from well head of different oil wells (Linch, L#63; Kalol, K #529; K#243; K#152 and Nandasan, N #60) into 100 ml anaerobic pre-sterilized serum bottles containing 1 ml of 2% Na_2S during February, 2012 as described by Lavania et al. [12]. Oil wells were situated in 70 Km periphery of Ahmedabad (Gujarat), India, at longitude 23.22 °N 72.68 °E with the elevation of 81 m (266 ft).

The average annual rainfall is around 803.4 mm (31.63 in). The climate is semi-arid with the maximum 42 °C and minimum 14 °C temperature. The *in situ* bottom hole temperature of the reservoirs were in the range of 70-100 °C. Formation fluids were transported at ambient temperature

to the laboratory within 24-48 h, stored at 4 °C and processed immediately for activity measurements and microbial analysis.

2.2. Analysis of formation water

Physio-chemical properties such as hydrogen ion concentration (pH), total dissolved solids (TDS), salinity and electrical conductivity were measured as described by Sharma et al. [13]. Heavy metal content of the formation fluids including arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), zinc (Zn), silver (Ag), nickel (Ni) and Iron (Fe) and the presence of anion (chloride, fluorides and sulphate) were also determined. The compositional analysis (carbon, hydrogen, nitrogen and sulphur) of the formation fluid was determined using IS: 1350/APHA guideline.

The amount of total petroleum hydrocarbons (TPH) recovered from oil was determined gravimetrically as described by Mishra et al.[14].

2.3. Enrichment of thermophillic consortia

To isolate thermophilic anaerobic microbes for enhance oil recovery, 10 % (v/v) of formation fluid was inoculated into pre-sterilized serum bottles containing modified MPB medium with 1 % crude oil. The modified MPB medium per litre contained 1.0 g NH_4Cl; 0.1 g $MgCl_2 \cdot 6H_2O$; 0.4 g K_2HPO_4; 0.2g KH_2PO_4; 1.0 g casein peptone; 1.0 g yeast extract; 2.0 g CH_3COONa; 2.0 g; sodium formate; 2.0 g; sodium chloride; 5.0 g [15]. During enrichment studies, experiments was performed in 67 mL serum bottles containing 30 mL of above anaerobic medium and was incubated for 30 days at the respective temperature (70-100 °C) of the bottom hole of the wells. The bottom hole temperature of the selected oil wells L#63, K#529, K#152, K#253 and N#60 were at 70°C, 90°C, 84°C, 84°C and 100°C respectively. Metabolite production such as gases (hydrogen, nitrogen, methane and carbon-dioxide) and VFAs (acetic acid, butyric acid) were quantified as the method described in the section 2.8. A control set of un-inoculated medium bottles were kept at the similar experimental conditions. The consortium from L#63 oil well showed highest metabolite production it was taken up for further study and referred to as TERIL63.

2.4. Identification and characterization of TERIL63

To identify TERIL63, total genomic DNA was extracted and PCR amplification was done with universal bacterial primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-ACG GCT TAC CTT GTT ACG CTT-3') as well as archaeal primers Met86f (5' GCT CAG TAA CAC GTG G-3') Met 915r (3'GTG CTC CCC CGC CAA TTC CT-5') as described by Lavania et al., [10.] Clones were processed for cycle sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The 16S rRNA sequences were checked for purity with Check-Chimera program (http://rdp.cme.msu.edu/). Multiple sequence alignments were performed using CLUSTAL W, version 1.8 [16]. A phylogenetic tree was constructed with the evolutionary distances using the neighbor-joining method. Tree topologies were evaluated by performing bootstrap analysis of 1000 data sets with the MEGA version 5.1 packages [17]. Morphology of TERIL63 was studied by scanning electron microscopy and cytochrome C420 was used in case of fluorescent microscopy [18].

2.5. Effect of reservoir conditions on metabolites production by TERIL63

The efficiency of TERIL63 was determined in terms of metabolite production at a range of temperature (60, 70, 80, 90 and 100 °C), pH (5, 7 and 9), and salinity (0.5, 1, 2.5 and 5 % NaCl (w/v). All experiments were performed for 30 days in 67 mL serum bottles with working volume of 30 mL of anaerobically prepared modified MPB medium with 1 % crude oil at 70 °C. Buffer for the pH experiments was used as described by Hoaki et al. [19]. Actively growing culture (cell count 10^5 cells mL^{-1}) was inoculated at 10% (v/v) by using disposable syringe. The data points are average of the triplicate ± standard deviation (less than 10 % of average).

2.6. Optimization of nutrient recipe for maximum metabolite production

Response surface methodology (RSM) using Box Behnken design was applied to optimize the five important medium components namely ammonium chloride 0.25-2 g L^{-1} (X_1), peptone 0-1 g L^{-1} (X_2), yeast extracts (YE) 0-1 g L^{-1} (X_3), sodium acetate 0-2 g L^{-1} (X_4), sodium formate 0-2 g L^{-1} (X_5) favouring maximum metabolites production by TERIL63 using Minitab 16 as described in Table S1

(Supplementary data). In order to find the most appropriate combination of these five components, forty six (46) sets of experiments (also known as Run Orders) were generated. The contour plots were generated to understand the interaction of various variables and were used to find the optimum concentration of the medium components affecting the response. Experiments were performed in 67 mL serum bottles containing 30 mL modified MPB medium with 1% crude oil at 70 °C with 10% (v/v) bacterial inoculum (cell count 10^5 cells mL^{-1}). The parameters analysed were gas and VFAs production.

2.7. Core flood assay

The core flood experiment was conducted at simulated oil reservoir condition using oilfield-injected water and crude oil under similar temperature condition to the oil reservoir. The set up has been demonstrated in Fig.1. The Linch core of Chatral sand was used in the study as described in Table 1. In core apparatus the core plugs were placed inside a stainless steel core holder. Molten Cerro metal was poured in the gap between the core plug and core holder at 160 °C temperature.

Fluids (brine, oil, and microorganisms with nutrients) were injected using an injection pump. Core flood studies were performed in duplicate at 70 °C by using brine (10.4 g L^{-1} NaCl) at atmospheric pressure. Brine was injected until core saturation. Subsequently, the core plugs were saturated with oil. Water flooding was carried out by brine injection till the stage where no oil was coming out. Thereafter, optimized nutrient medium with TERIL63 consortium was injected and the system was incubated for 10 days. Subsequently, oil was recovered by displacement at the same temperature with brine. The characteristics of the core, oil and the oil recovery parameters are shown in Table 1.

$$Oil\ recovery\ efficiency\ (ORE)(\%) = (Total\ volume\ of\ oil\ recovery \div original\ oil\ in\ place) \times 100 \quad \textbf{(1)}$$

Where, original oil in place (mL) is the volume of brine displaced by oil saturation;

$$Water\ cut\ \% = (Volume\ of\ water \div Volume\ of\ produced\ liquid) \times 100 \quad \textbf{(2)}$$

Therefore, the enhanced oil recovery (EOR) was estimated by

$$EOR\ (\%) = OREm - OREw \quad \textbf{(3)}$$

Where, OREm is the oil recovery efficiency at the end of the subsequent water flooding and OREw is the oil recovery efficiency at the end of the bacterial injection.

2.8. Pathogenicity of TERIL63

Pathogenicity of TERIL63 was studied under acute oral toxicity/pathogenicity. Forty mice (20 male and 20 female) were assigned to the dose groups: control and test. The test material (TERIL63) was administered (1 mL per mouse) once by gavage to mice. The mice were deprived of feed 3-4 h and 2 h after administration of the test substance. At the end the observation period the surviving experimental animals were sacrificed. Gross necropsy was performed and all animals were carefully examined for the detection of bacterial culture (Results presented in supplementary data). The study was carried out at National Toxicology Centre, Pune with the prior approval from animal ethical committee (Registration no. 40/1999/CPCSEA).

All experiments were performed in compliance with the relevant laws and institutional guidelines.

2.9. Analytical methods

Headspace gas production was quantified by gas chromatograph (model GC-7890A, Agilent Ltd. USA) equipped with a molecular sieve packed stainless steel column (2 m X 2 mm id NUCON, INDIA) and a thermal conductivity detector (TCD) as described by Rathi et al. [20].

The concentrations of C2–C6 VFAs in liquid phase were analysed with GC 7890A Agilent Ltd. USA) equipped with flame ionization detector and DB-WAX etr column (30 m × 530 µm × 1 µm) as described by Singh et al. [21]

Low molecular weight organic acids and solvents were analysed by High Performance Liquid Chromatography (HPLC, Agilent 1100 series, USA) equipped with AminexR HPX-87H, (300 mm × 7.8 mm) column (BIORAD, CA, USA). Sulphuric acid (0.005 M H_2SO_4) was used as mobile phase with the flow rate of 0.6 - 0.9 mL for 21 min in RID detector (optimum temperature 80 °C) as described by Singh et al. [21]. All the mentioned methods were calibrated by injecting standards with the range of concentration. The calibration curve was obtained for all the standards with R^2 approximately 0.998. The methods were found accurate and precise.

Surface tension measurements of culture broth supernatant were performed according to method described by Gudina et al. [3]. A CSC Du Nouy Tensiometer (Cole Parmer India) equipped with platinum ring was used. To increase the accuracy of the surface tension measurements, an average of triplicates was determined. All the measurements were performed at room temperature (25 °C ±1 °C).

3. Results and discussion

The main focus of the present study was to implement MEOR technology in partially depleted oil reservoirs since most of the oil reservoirs have entered into high water cut after initial recovery processes. Improvement in the recovery process is indeed an urgent need. Due to long time exploitation, chemicals, fracturing and acidizing fluids have been injected into the oil reservoir which has been influencing the composition of formation water and also the types and number of microbes in the production wells. Such an oil reservoir is in the Kalol field, which is located 16 km north of Ahmedabad City in the Ahmedabad-Mehsana tectonic block of North Cambay basin, hence was considered in the present study. The oil reservoirs were discovered in June 1961 and was put under production in mid sixties [22.] Development of this field is based on various studies over the past 53 years (REF). Formations from such wells were selected because of their low permeability even after long time exploitation.

Aiming towards successful strategy for effective oil recovery, characteristics of the formation water/oil of the selected oil reservoirs were investigated as tabulated in Table 2. The formation water was found to be slightly alkaline with the presence of fluoride, sulphate, chloride and heavy metals including iron and zinc (Table S2, Supplementary data). Environmental factors together with the composition of formation water in terms of carbon, hydrogen, nitrogen and sulphur (CHNS) plays significant role in the metabolite production of the selected consortium [7]. The viscosity of the selected oil was in the range of 17.80 cP at 70 °C. TPH of the oil was studied gravimetrically with the following composition: 50 % aliphatic, 47 % aromatics and 3% NSO compounds.

3.1. Enrichment of indigenous thermophilic anaerobic microbial consortium for oil recovery

To develop indigenous thermophilic anaerobic microbial consortium for enhanced oil recovery, formation fluids were inoculated into a modified media specific for methanogens and was incubated at the temperature of the respective oil wells. Indigenous microbes with the ability to produce desired bio products for oil recovery, seemed to be the best choice, since they are acclimatized to the reservoir conditions [15]. After three successive enrichments, indigenous consortium TERIL63 showed highest metabolite production (Fig. 2). The selected consortium was named as TERIL63 and was taken up for subsequent experiments.

Highest production of methane (10.10 mmol L^{-1}), carbon dioxide (4.64 mmol L^{-1}) along with VFAs (2037 mg L^{-1}) and solvents (acetone, 1.95 g L^{-1}; butanol, 0.63 g L^{-1}) was observed with TERIL63 in 30 days.

Gudiña et al. [3] showed that microbial production of methane and carbon dioxide is an important activity for MEOR; since these gases can enhance oil recovery by repressurizing the well and reducing the viscosity of oil. Similarly, Kobayashi et al. [4] showed that indigenous microorganisms in the reservoir could be utilized in production of methane for reservoir re-pressurization. MEOR by acid production is one of the mechanisms, which plays an important role in oil recovery, especially in carbonate reservoirs [24, 25]. Solvents alter the rock wettability at the oil–rock interface, releasing the oil from the porous matrix [5.] Indigenous consortium TERIL63 also showed high amount of methane, carbon dioxide, volatile fatty acids and organic solvents which helped in enhanced oil recovery by altering the oil/rock properties at high temperature (70 °C). Moreover, it validates the ability of the consortium TERIL63 towards microbial enhanced oil recovery. Experimental control (uninoculated media bottles) did not show any gas or VFA production.

The biosurfactant producing ability of the consortium TERIL63 was determined by measuring the reduction in surface tension of the medium. The surface tension was reduced from 69 to 35 dynes cm^{-1} in present case, which is significant. Biosurfactants reduces the surface tension between oil water and oil rock interfaces and also alters the wettability of the reservoir rocks [3,5,7, 8, 10] which leads to the mobility of oil towards the well bore. However, characterization of biosurfactant and its efficiency towards enhanced oil recovery will be carried out in future investigations.

3.2. Identification and characterization of TERIL63

Enrichment led to the selection of TERIL63 as it showed huge potential for enhanced oil recovery and was subsequently taken up for phylogenetic analysis. TERIL63 consortium showed the presence of *Thermoanaerobacter* sp. (97 % Bacterial homology) and *Methanothermobacter thermoautotrophicus* (99 % Archaeal homology). The nucleotide sequences obtained in this study were deposited in NCBI Genbank database with the accession numbers as KF683924 to KF683932. Phylogenetic tree was also mapped with closely related matches obtained from BLAST search (Fig. 3a and b). For better understanding, two separate phylogenetic trees were presented in the figures 3a and 3b respectively. Anaerobes have been considered to be dominant in oil reservoirs [26.] Members of the family *Thermoanaerobiaceae* that includes the genera *Thermoanaerobacter* and *Thermoanaerobacterium* were also isolated in low saline reservoirs [26, 27]. In addition, representative of the genera *Methanothermobacter thermoautotrophicus* were also detected in oil fields [18, 28].

Morphological features of TERIL63 were examined by scanning electron microscopy and fluorescent microscopy. Curved rods (0.3 μm in width and 2.2 to 5.9 μm in length) were seen occurring as single or in chains (Fig. 4a). Fluorescence microscopy indicated that these cells had F420 co-enzyme and was consistent throughout the growth period (Fig. 4b). Doddema and Vogels [18] identified F420 as a powerful method for the recognition of methanogens as they emit blue fluorescence at 420 nm. Hence, the presence of methanogens in the consortium TERIL63 was confirmed.

3.3. Effect of reservoir conditions on metabolites production by TERIL63

For effective implementation of MEOR in partially depleted oil reservoirs an attempt was made to evaluate the role of the physiological factors affecting metabolite production. As reported by Cheng et al., [29] environmental factors such as temperature, pH, salinity, and available inorganic and organic nutrients can affect methanogenesis.

The reservoir pressure is not a limiting factor since the growth of most microorganisms is unaffected by pressure up to 300 atm [10]. Several types of microorganisms in a great variety of conditions have been detected in oil fields [8,10,28]. Castorena et al. [8] reported recovery of oil by *Thermoanaerobacter* sp. indigenous to an oil well of pressure 140 atm. Also, Mutai et al. [15] isolated microbes, especially methanogens from the oil reservoir of pressure about 105 atm for enhanced oil recovery. Similarly, in

the present study, the consortium TERIL63 was developed from oil reservoir of pressure 105 atm for enhanced oil recovery. Thus, it is evident that the reservoir pressure is not a limiting factor for growth of microbes for enhanced oil recovery.

The NaCl concentration tolerated by microbes depends on the medium and the environmental conditions such as temperature [30]. In the present study, high salt concentration (NaCl) had detrimental effect on metabolites production (1 mmol L^{-1}methane and 1401.93 mg L^{-1} VFAs at 5 % NaCl) as described in Fig. 5a. Maximum metabolites production (methane, 15-19 mmol L^{-1}; VFAs, 2037-2272 mg L^{-1}) by consortium TERIL63 was observed at 0.5 -1 % salinity, i.e close to the reservoir salinity. Similar to the results in this study, Head et al. [31] also found that the methanogens favour low salinity condition for its growth.

Another important parameter in regulating the anaerobic process is pH as there is a narrow range of pH for every organism. A change in pH of the environment can lead to death of the cells and consequently result in loss of biological activity [32]. Methane production by consortium TERIL63 was seen in pH range of 7.0 to 9.0. Maximum production of methane (10.10 mmol L^{-1}) along with carbon dioxide (4.34 mmol L^{-1}) was observed at pH 7.0 (Fig. 5b) which was close to the pH of the reservoir. Also, maximum VFAs production was also observed (2037 mg L^{-1}) at pH 7.0 (Fig. 5b). Temperature changes have significant effect upon microbial growth and metabolite production. Methanogens have been found at temperatures ranging from 2 to 110 °C [32]. Optimum temperature range for biogenic gas production for methanogens taken from formation water has been reported between 26 and 55 °C [28, 29]. The consortium TERIL63 showed an increasing trend of methane production as the temperature increased from 60 °C to 70 °C, while at 80 °C a sharp decline was observed. The maximum methane (10.10 mmol L^{-1}) and carbon dioxide production (4.64 mmol L^{-1}) along with VFAs (2037 mg L^{-1}) was observed at 70 °C (Fig. 5c). The consortium TERIL63 was found to be an autotroph and a strict anaerobe and had a pH optimum of 7.0 and temperature optimum of 70 °C for growth and metabolite production. In the present study production of methane was due to *Methanothermobacter* sp. in the consortium TERIL63. Besides it methanogenic archaea, syntrophic bacteria, *Thermoanaerobacte*r sp was also identified within the methanogenic consortium TERIL63, which implies the conversion of substrate into hydrogen, carbon-dioxide and VFAs [27, 33], The optimum temperature for *Methanothermobacter* sp. and *Thermoanaerobacter* sp. has been reported in the range of 60 to 70 °C [25, 34]. Since, the fermentation temperature affects substrate utilization, growth

rate, membrane lipid composition and metabolites production. Each microorganism has an optimum temperature range at which maximum growth and metabolite production takes place. This describes the probable reason for the production of VFA and gas at 70 °C. Variation of VFA and gas was observed at 80 °C since change in optimum temperature from 70 to 80 °C had significant effect upon microbial growth and activity. Moreover, the temperature of the reservoir from which the microbe has been isolated also has significant effect. This confirms the fact that the maximum metabolites production occurs at the same temperature from which the microbes has been isolated. The consortium TERIL63 was found to be autotrophic, thermophilic and a strict anaerobe with an optimum temperature of 70 °C for its growth and metabolite production. Thus maximum amount of metabolites were produced at 70 °C, beyond which, growth and activity of microbes were limited.

3.4. Modelling and optimization of nutrient recipe for TERIL63

RSM methodology was employed to study the interactive effect of the culture parameters on the production of metabolites by TERIL63, hence making the process economical and more feasible. Response surface methodology (RSM) is a collection of different statistical techniques, including designing experiments, building models and evaluating the effects of factors to trigger desirable responses [26].

Among the five variables tested, sodium formate had positive effect on the production of metabolites followed by peptone and sodium acetate. Ammonium chloride (X_1) and yeast extract (X_3) expressed strong linear negative effect ($p < 0.05$) (Details are in supplementary data). Significant interactions was noted between sodium formate and peptone followed by ammonium chloride and yeast extract which confirms to the fact that most of the bacterial strains produces metabolites in response to the oil recovery process but nutrient or feed are the major limiting factors [35]. It is evident in Table S3 (Supplementary data) that linear, quadratic and interactive effect of peptone (X_2) was significant than other variables tested. This suggests that the concentration of peptone had a direct relationship with metabolites production in modified MPB medium, i.e. any minor changes in this variable from its zero level values will cause a great positive or negative shift in metabolites production [36]. Analysis of variance (ANOVA) was used to analyse the response under different combinations as defined by the Box Behnken design Table S4 (Supplementary data).

The 2D contour plot is a graphical representation of the regression equation and is plotted to understand the interaction of the variables. From Fig. 6a shows the interactive effect of sodium acetate, yeast extract, ammonium chloride, sodium formate and peptone on gas production. The maximum gas production (> 9 mmol L^{-1}) was observed with yeast extract (0.4 g L^{-1}) and peptone 0.8 g L^{-1} (Fig. 6a). It can be seen that increasing concentration of peptone and yeast extract led to an increase in the gas production (9 mmol L^{-1}). Yeast extract and peptone are the dominant nutrients, whose concentration controls the gas production. However, increasing Sodium acetate concentration did not show any effect on gas production (Fig. 6a). Since the cost of yeast extract is reasonably low, it was considered in the optimized recipe for MEOR application.

From the Fig. 6b (Ammonium chloride versus Peptone) it can be seen that peptone (0-1 g L^{-1}) had approximately negligible effect on VFAs production as shown by the virtually vertical line corresponding to peptone. On the other hand increase in amount of ammonium chloride led to an increase in VFAs production. However, Fig. 6b depicts the interactive effect of yeast extract, ammonium chloride, sodium formate and peptone on VFAs production. Thus, the highest VFAs production was observed when nutrient media containing ammonium chloride, 2 g L^{-1}; peptone, 0.5 g L^{-1}; yeast extract 0.5 g L^{-1}, Sodium formate 1 g L^{-1} (Fig. 6b).

The objective of the present study was to optimize the nutrient recipe, thus to increase the metabolite production rate at minimal concentration of the medium components. Hence, decreases the overall cost of the MEOR process. Therefore, as depicted from Fig. 6, optimized recipe (by RSM) for TERIL63 consisted of ammonium chloride, 2 g L^{-1}; peptone, 0.5 g L^{-1}; yeast extract 0.5 g L^{-1} and sodium formate 1.8 g L^{-1} leading to a maximum metabolite production consisting of 14.74 mmol $L^{-1}$of gases and 2037 mg L^{-1} of VFAs in 10 days instead of 30 days at 70 °C.

Varying concentration of media components were used to optimize the nutrient recipe for MEOR application. The objective was to maximize the metabolites production and also to reduce the cost. The authors have done a detailed evaluation of the media concentration before and after optimization. Also, the cost of each component has been taken into account. Before optimization, recipe contains ammonium chloride, 1 g L^{-1}; peptone, 1 g L^{-1}; yeast extracts 1 g L^{-1}, and Sodium formate 2 g L^{-1}. Whereas in the optimized recipe where sodium acetate was eliminated and the concentration of peptone was reduced to 0.5g L-1 which led to an approximately 43% reduction in the cost of medium. Along with nutrient cost, growth period of microbial consortium was also reduced from 30 days to 10

days. Thus, due to this nutrient optimization higher metabolites production rate in lesser time was achieved. At present, our main focus for nutrient optimization is to maximize growth of the consortium and its metabolites in minimum duration so that the MEOR process should be economical and feasible. Thus, the optimization study lead to an overall reduction of cost by optimizing concentrations of expensive components, making it a viable and cost effective option for successful MEOR application.

In previous studies it was reported that significant increase in methane production was observed after adding the nutrient and other materials [35, 36]. As per to the author's knowledge this is the first report where RSM has been used for designing an economical media for enhanced metabolite production for use in MEOR by thermophilic methanogenic consortium.

3.5. Enhanced oil recovery

In the core-flooding assay, oil recovery was evaluated at each stage. Firstly, core was saturated with 3.2 pore volume (PV) of brine (Linch#63 formation water). Then, 2.4 PV Linch#63 oil was flooded at 15mL h^{-1} rate, which lead to 43.2% (10.8 mL) irreducible water saturation obtained with an oil saturation of 56.8% (14.2 mL). After 2 days, 1.9 PV of brine was flooded at 15 mL h^{-1} rate, which lead to 9 mL i.e. 63.4% of oil displacement. At the MEOR stage, the optimized nutrient solution with consortium TERIL63 at their exponential growth phase (10^5 cells mL^{-1}) of 1 PV was injected into the core at a rate of 5mL h^{-1} at 70 °C. After 10 days incubation, 1 PV of brine with 15 mL h^{-1} rate was flooded which lead to 2.2 mL of oil recovery corresponding to an oil displacement efficiency of 15.49 % over original oil in place (OOIP). Also, 10^2 cells mL^{-1} of the indigenous bacterial consortium TERIL63 was observed at the outlet after 10 days of incubation at 70 °C, when flooded with brine. In this experiment, overall 78.89 % oil was recovered. Out of which, 15.49% of incremental oil was recovered at 70 °C due to microbial action after 10 days of incubation. However in the control set no incremental oil recovery was seen and also no metabolites were produced. This can be well attributed to the fact that microbes were not present in the control.

The results demonstrated that the inoculated systems containing TERIL63 cells were effective in releasing oil.

Earlier, Xu and Lu [38] demonstrate 17-25 % oil recovery when genetically engineered bacteria produced 11.44 mmol L^{-1} CO_2 at 45 °C. Oil recovery of 5 to10% has been reported due to the presence of organic acids (135-217 mg L^{-1}) in microbial core flood experiments[37, 38]. Similarly, TERIL63 showed enhanced organic acid (VFAs) production (2037 mg L^{-1}) at 70 °C by using sodium formate as carbon source helping in increased reservoir permeability and porosity.

Previous report have shown 5.6 % increase in oil recovery after 7 days at 70 °C in an oil displacement system employing the injection of bacteria [39]. Recently Castorena et al. [8] also demonstrated a mixed culture of *Thermoanaerobacter* sp. having ability to produce gases, VFA (693 mg L^{-1}) and solvent (as ethanol 173 mg L^{-1}) showing 12% of heavy oil recovery in 10 days. Similarly, the metabolites produced by TERIL63 with optimized nutrient recipe lead to 15.49 % residual oil recovery after 10 days at 70 °C. Therefore, Consortium TERIL63 has immense potential in MEOR application as it produces organic acid, solvents as well as gases leading to repressurization of oil reservoirs, dissolution of carbonates. Thus, increasing porosity and permeability together with enhanced oil migration from wells in lesser time.

Earlier, microbial enhanced oil recovery was demonstrated mainly due to the production of surfactant, solvents, acids and carbon dioxide [8, 9, 11]. However, in the present study significant amount of biogenic methane generation was reported first time for enhanced oil recovery at high temperature.

In the core flood study, TERIL63 also displayed reduction in surface tension value (35 from 69 dynes cm^{-1}). This reduction in surface tension confirms the ability of TERIL63 to produce surface-active agents, which can reduce interfacial/surface tension followed by alteration in the wettability of rocks thus enhancing the oil mobility. Biosurfactant are biological products used for oil recovery processes. The biosurfactant synthesised by *Pseudomonas* MR01 increased the 15 % recovery of residual oil by reducing the surface tension by surface-active agent [3, 11, 40]. Detailed biosurfactant characterization studies with TERIL63 need to be undertaken to provide further insights into the MEOR process. In addition, a pathogenicity test for TERIL63 was also performed. Acute oral toxicity in mice showed no presence of live bacterial culture in any of the organs thereby rendering the culture non-toxic, non-virulent and safe for use in the field (Supplementary data).

4. Conclusions

The primary objective of the present study was to investigate the potential of the selected indigenous consortium in MEOR. The enrichment culture favoured the growth of fermentative, methanogenic, anaerobic and halo tolerant microbes. Among the five oil wells investigated, consortium TERIL63 of Linch oil reservoir was selected due to its ability to produce highest amount of metabolites in comparison to other indigenous consortia with the potential for MEOR application. Moreover TERIL63 had an added advantage of decreasing the surface tension. The microorganism in the consortium TERIL63 were identified and showed similarity to *M. thermoautotrophicus* and *Thermoanaerobacter* sp. The consortium has immense potential for enhanced oil recovery in different oil reservoirs by producing metabolites at wide range of temperature, salinity and pH. A cost effective nutrient recipe was designed which increased the production of metabolites favouring quick recovery of oil from partially depleted oil wells. Consortium TERIL63 presented the ability of improved oil recovery by production of high rate of metabolites in just ten days making the overall process a very cost-effective strategy.

In the core flood study, the produced metabolites interacted with the oil entrapped in the porous medium and overall oil mobility 15.49%.

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Figure Captions

Fig. 1 Schematic of Core flood apparatus.

Fig. 2 Production of hydrogen, methane carbon dioxide and VFAs by microbial consortia (K#529, K#243, K#152, L#63 and N#60) isolated from oil fields.

Fig. 3 Phylogenetic tree based on 16S rRNA sequences phylotypes retrieved from methanogenic culture TERIL63 and closely related sequences from Genbank database

(a) Bacterial primers (16S rRNA)

(b) archeal primers

Alignments to related sequences were performed with MEGA5 software with the neighbor-joining method. Bootstrap values (n=1000 replicates) of > 75 % are reported. Based on 1000 replication are represented on each node. Scale bar = nucleotide change per site

Fig. 4 Morphology of the microbial consortium TERIL63.

a. Scanning electron micrograph; bar 2 µm (Fig. 4a).

b. Fluorescence micrograph of cells with UV excitation filter (380-420 nm) (Fig. 4b).

Fig. 5 Effect of reservoirs conditions on production of methane carbon dioxide and VFAs by TERIL#63 in MPB medium with nitrogen as headspace

Data recorded after 30 days of incubation.

a. At various salts concentrations (0.5, 1, 2.5, 5 %).

b. At various pH values (5, 7, 9).

c. At different temperatures (60 °C 70 °C, 80 °C, 90 °C, 100 °C).

Fig. 6 RSM contour plots of interactive effects of yeast extract, sodium acetate, sodium formate, ammonium chlorides and peptone.

(a) Headspace gas analysis.

(b) VFA production at zero levels of other variables.

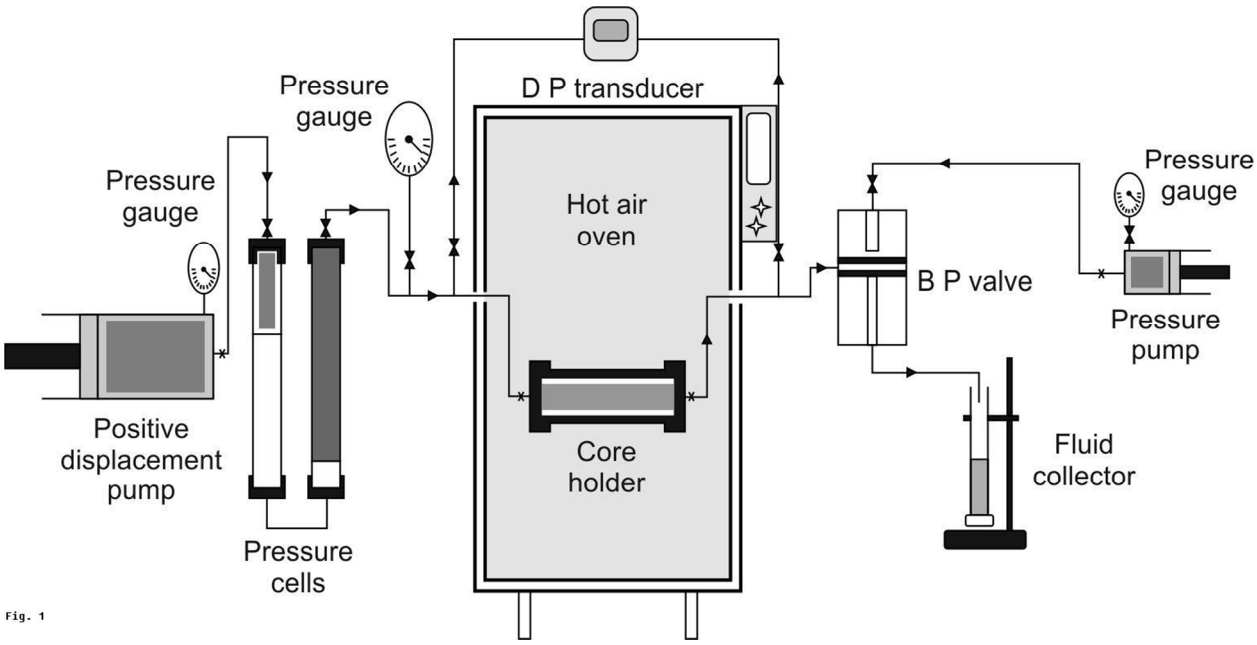
Pressure gauge
D P transducer
Pressure gauge
Hot air oven
B P valve
Pressure gauge
Pressure pump
Positive displacement pump
Core holder
Fluid collector
Pressure cells
Fig. 1

Figure 1

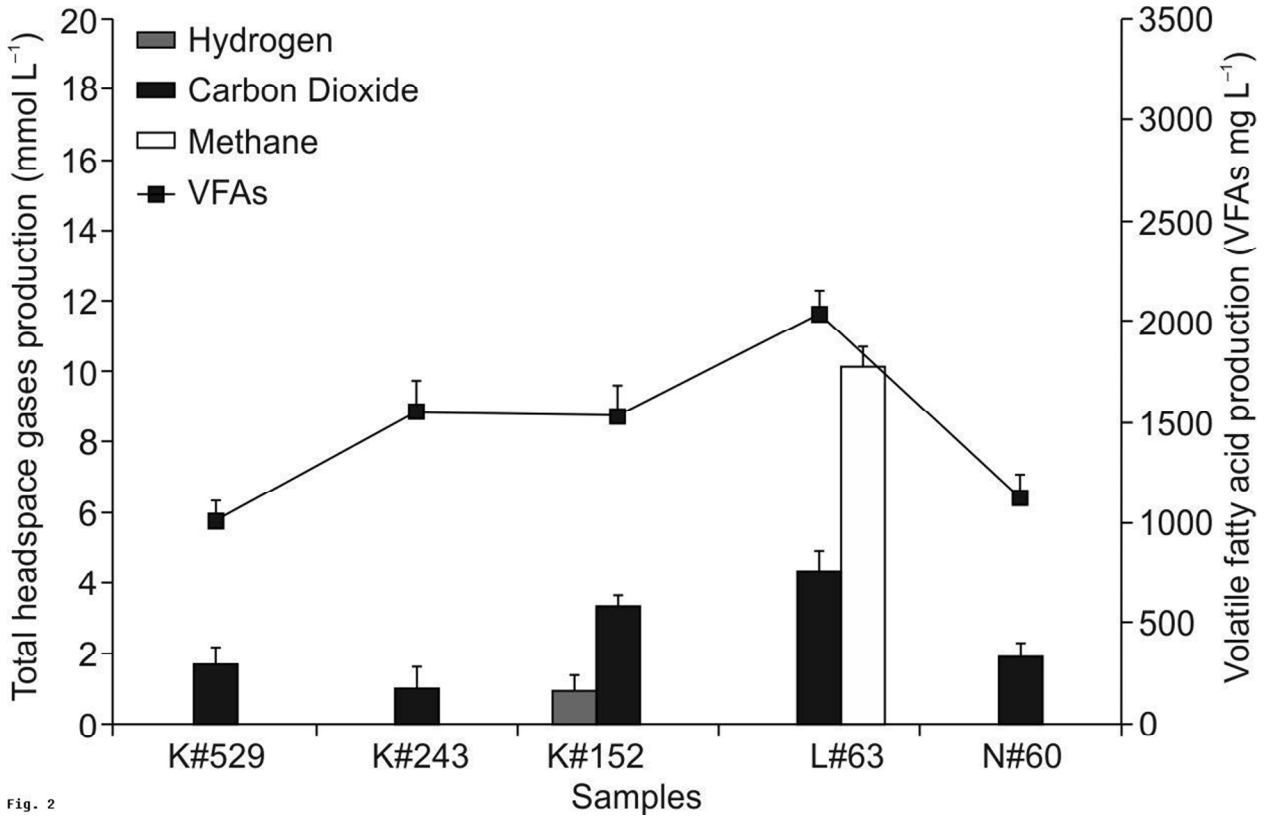

Fig. 2

Figure 2

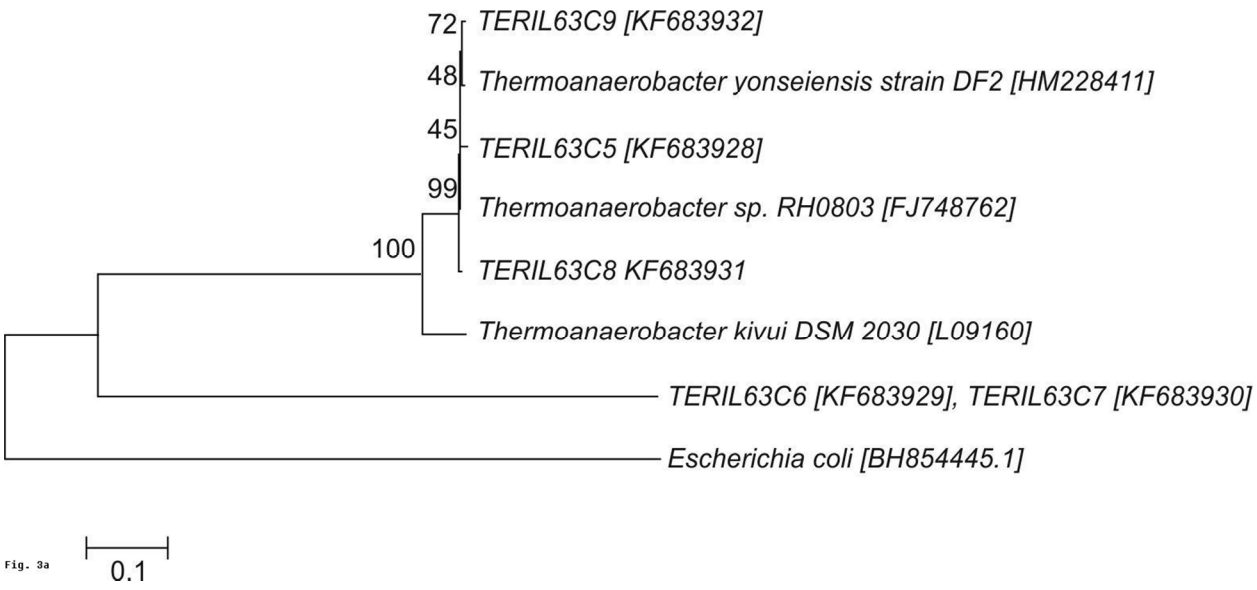
72
TERIL63C9 [KF683932]
48
Thermoanaerobacter yonseiensis strain DF2 [HM228411]
45
TERIL63C5 [KF683928]
99
Thermoanaerobacter sp. RH0803 [FJ748762]
100
TERIL63C8 KF683931
Thermoanaerobacter kivui DSM 2030 [L09160]
TERIL63C6 [KF683929], TERIL63C7 [KF683930]
Escherichia coli [BH854445.1]
Fig. 3a
0.1

Figure 3a

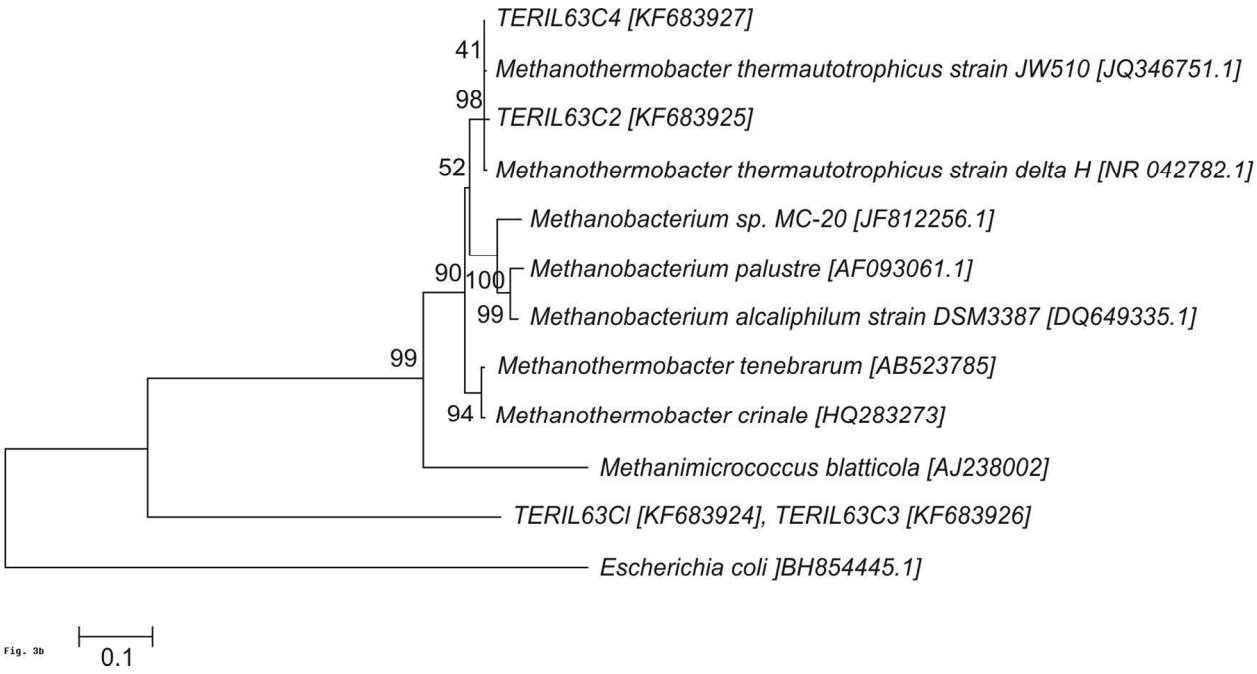
TERIL63C4 [KF683927]
41
Methanothermobacter thermautotrophicus strain JW510 [JQ346751.1]
98
TERIL63C2 [KF683925]
52
Methanothermobacter thermautotrophicus strain delta H [NR 042782.1]
Methanobacterium sp. MC-20 [JF812256.1]
90
100
Methanobacterium palustre [AF093061.1]
99
Methanobacterium alcaliphilum strain DSM3387 [DQ649335.1]
99
Methanothermobacter tenebrarum [AB523785]
94
Methanothermobacter crinale [HQ283273]
Methanimicrococcus blatticola [AJ238002]
TERIL63Cl [KF683924], TERIL63C3 [KF683926]
Escherichia coli]BH854445.1]
Fig. 3b
0.1

Figure 3b

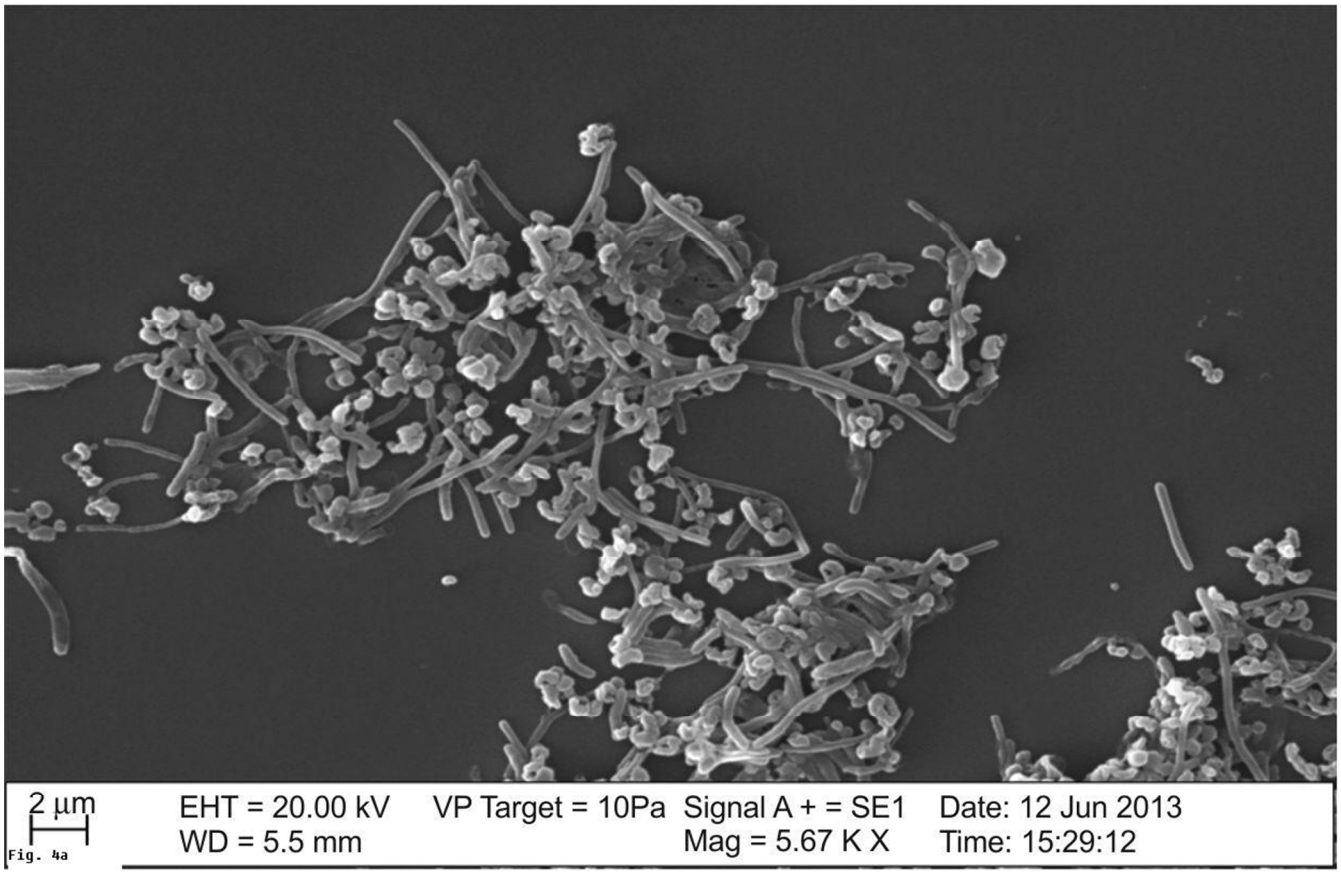
2 µm
EHT = 20.00 kV
WD = 5.5 mm
VP Target = 10Pa
Signal A + = SE1
Mag = 5.67 K X
Date: 12 Jun 2013
Time: 15:29:12
Fig. 4a

Figure 4a

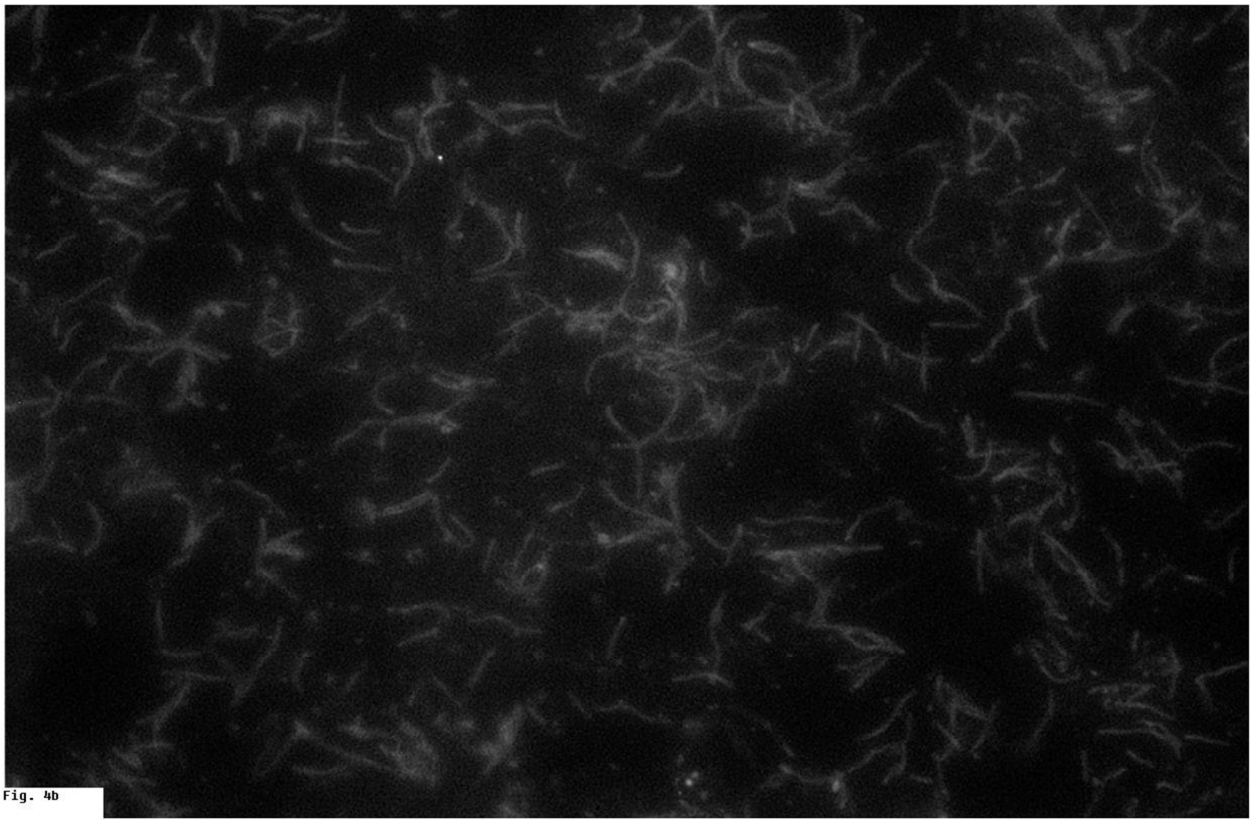

Figure 4b

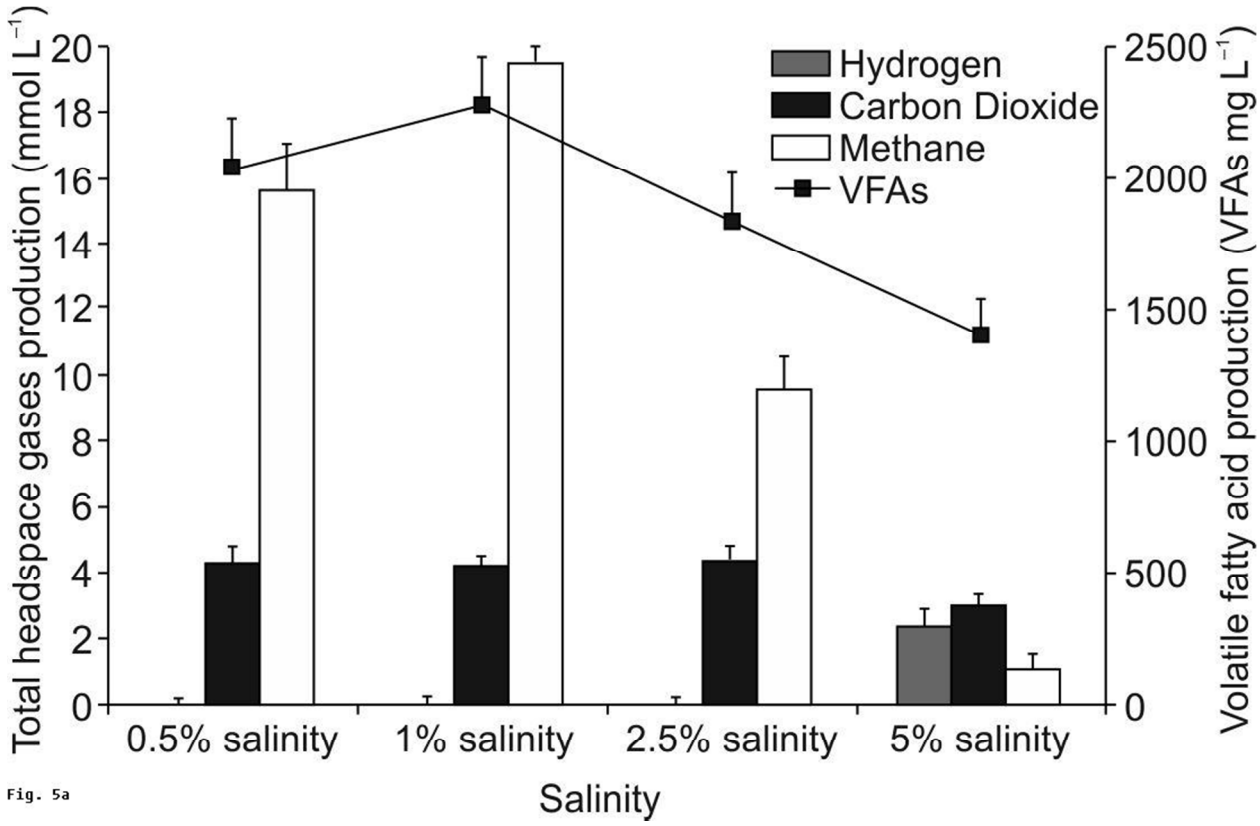
Total headspace gases production (mmol L⁻¹)
Volatile fatty acid production (VFAs mg L⁻¹)
Hydrogen
Carbon Dioxide
Methane
VFAs
0.5% salinity
1% salinity
2.5% salinity
5% salinity
Salinity
Fig. 5a

Figure 5a

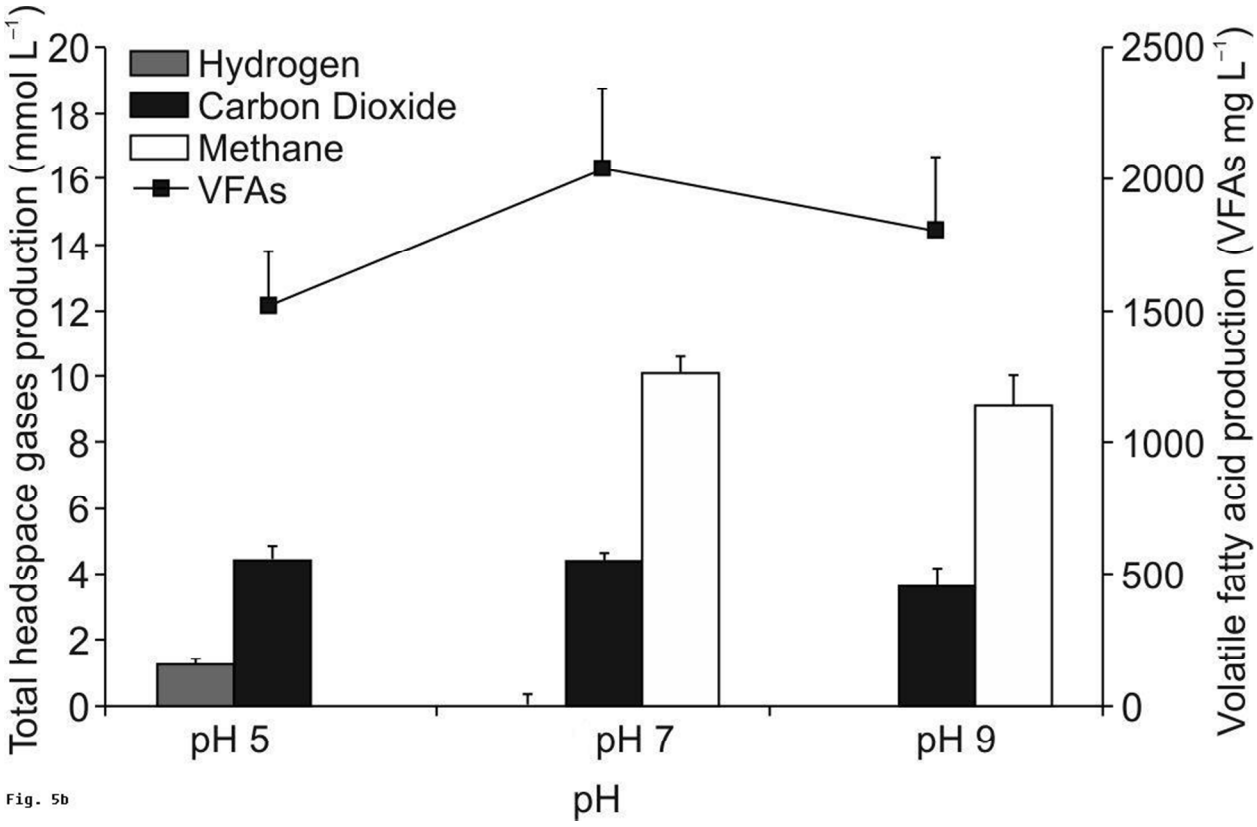
Hydrogen
Carbon Dioxide
Methane
VFAs
Total headspace gases production (mmol L⁻¹)
Volatile fatty acid production (VFAs mg L⁻¹)
0
2
4
6
8
10
12
14
16
18
20
0
500
1000
1500
2000
2500
pH 5
pH 7
pH 9
pH
Fig. 5b

Figure 5b

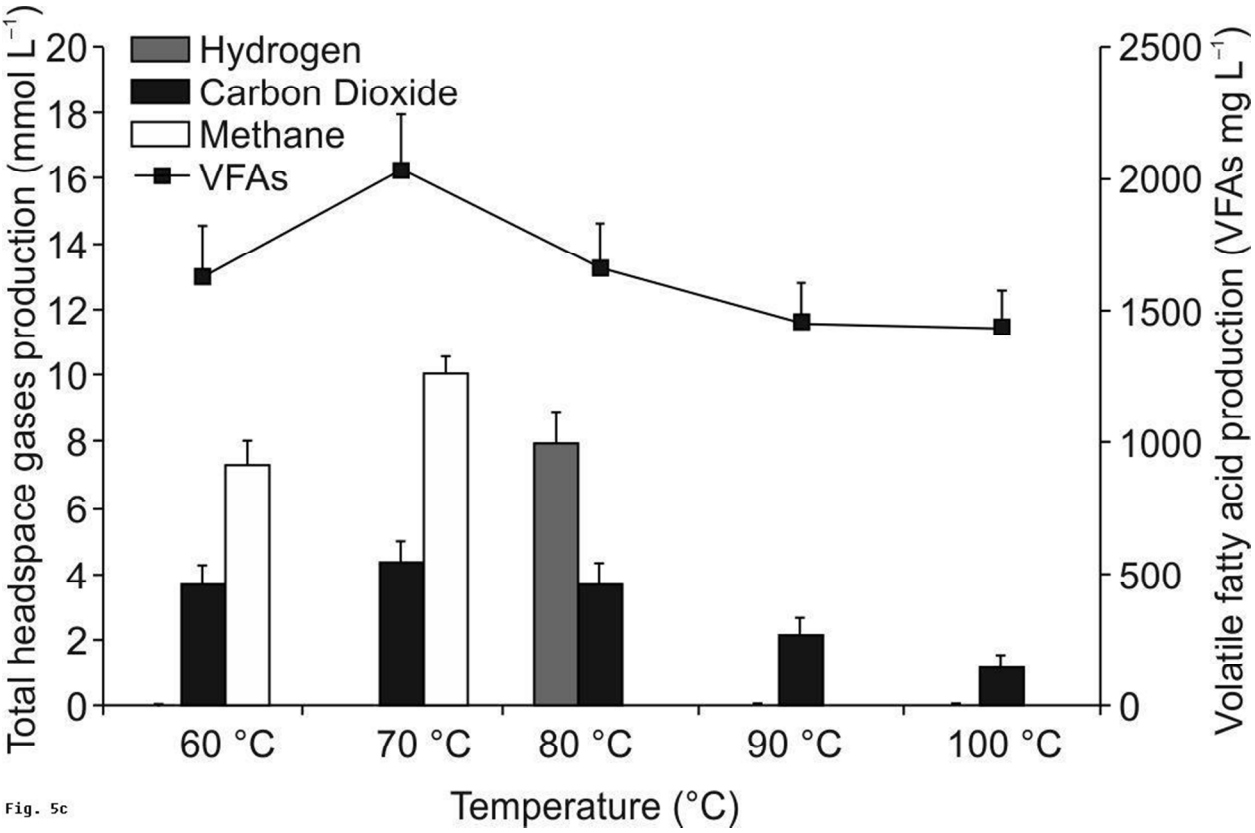

Fig. 5c

Figure 5c

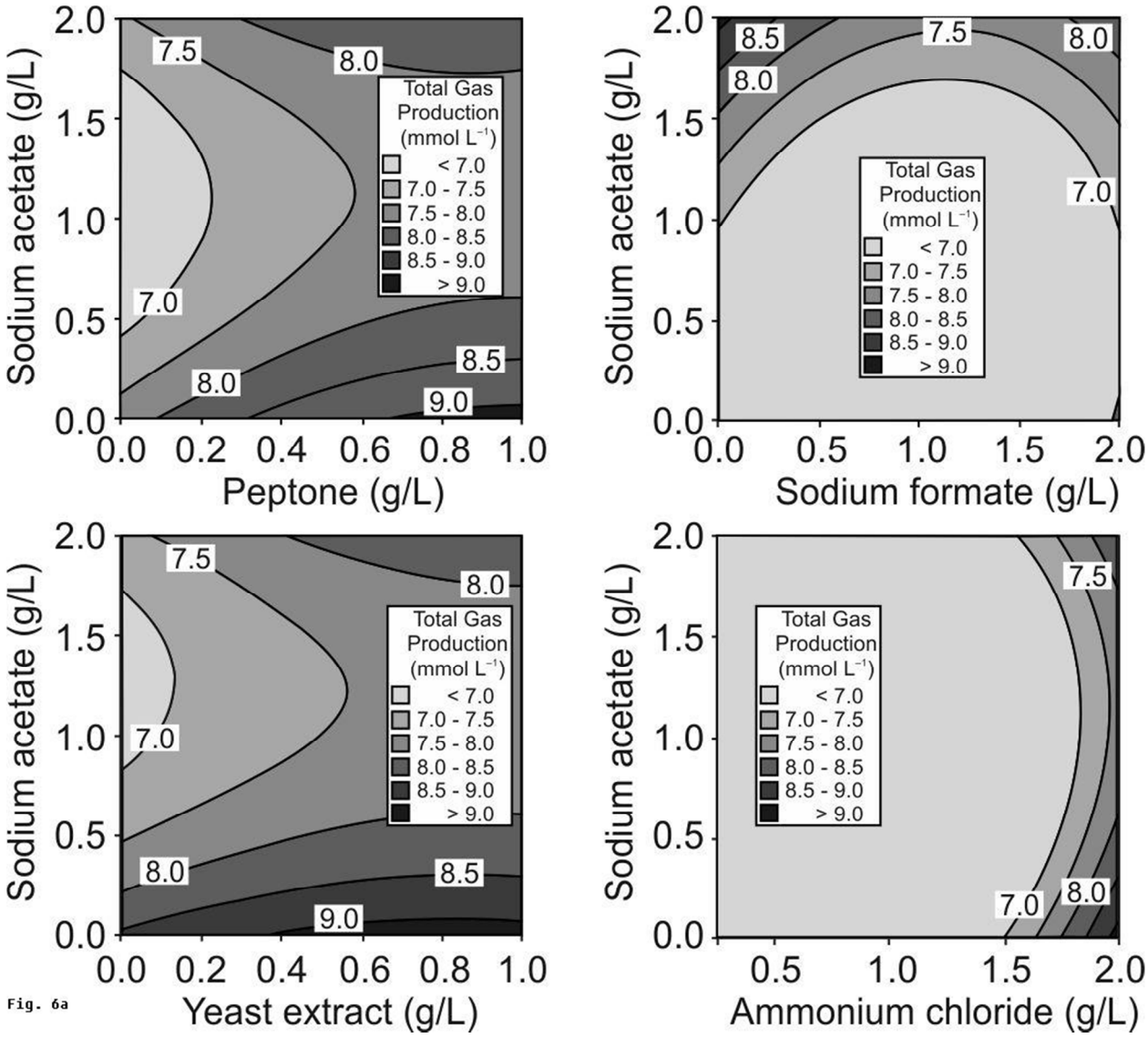

Fig. 6a

Figure 6a

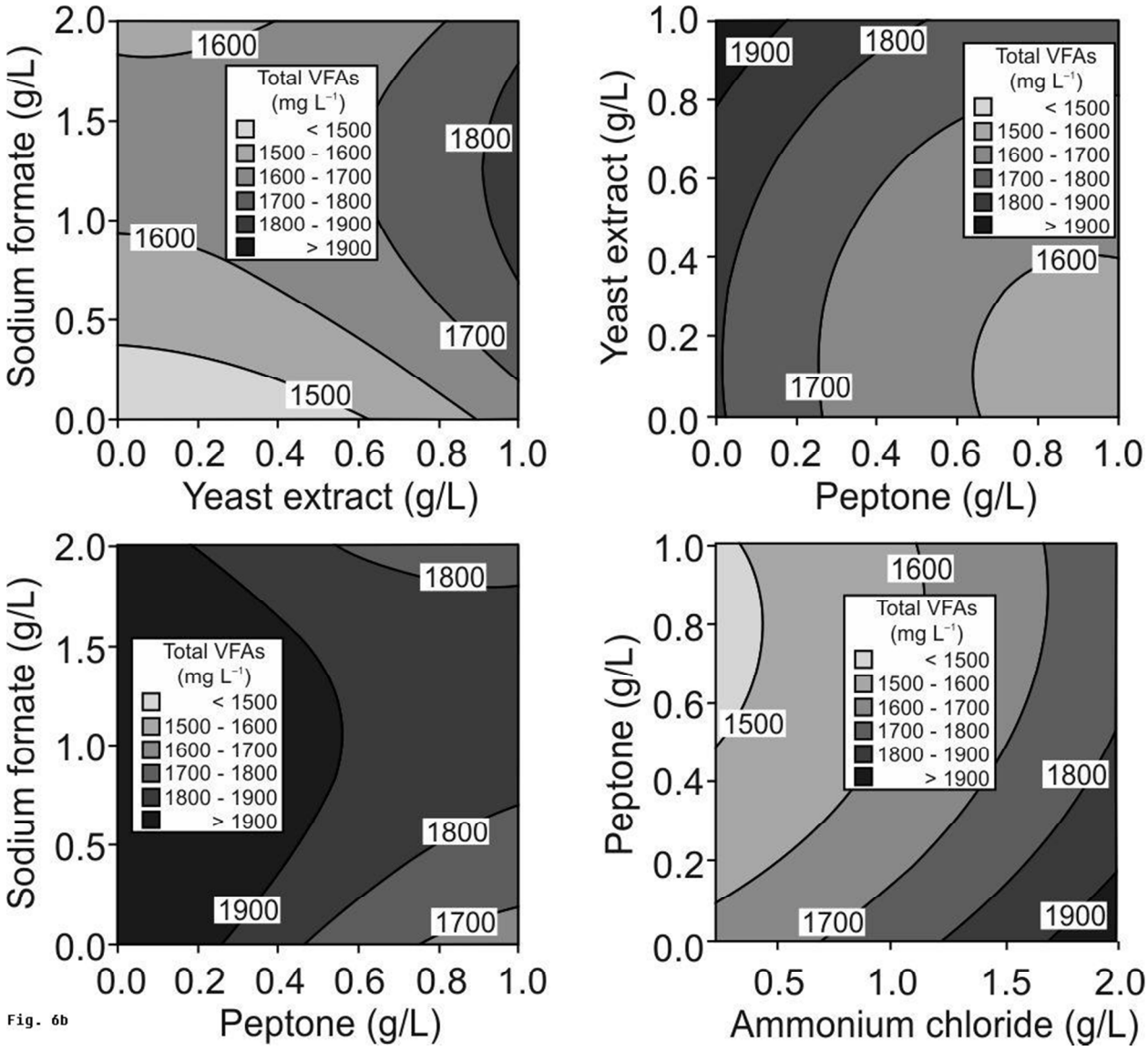

Fig. 6b

Figure 6b

Table 1 Cumulative oil recovery in core flood study

Characteristic/conditions	Value
Crude oil	
Viscosity (cP S) at 70 °C	17.80
Saturated (%)	64
Aromatic (%)	25
Core flood study	
Type of rock Sandstone	Linch
Length (cm)	7.3
Diameter (cm)	3.6
Area (cm^2)	10.2
Pore volume (mL)	25.0
Porosity (%)	33.7
Permeability (mD)	29.01
Oil saturation, SO (%)	56.8
Residual water saturation, SWR (%)	43.2
Original oil in place (OIIP), (mL)	14.2
Residual oil saturation (ROS), (%)	52.8
Oil recovery over ROS, (%)	15.49
Operation conditions	
Microbial culture	TERIL63
Inoculum concentration (%)	10
Brine, NaCl ($g\ L^{-1}$)	10.40
Temperature (°C)	70
Pressure (MPa)	Atmospheric

Flow (mL h^{-1})	10

Table 2 Characteristics of formation fluids/ oil of Linch, Kalol and Nandasan oil fields

Parameter	Method	Oil wells				
		L#63	K#529	K#152	K#253	N#60
Temperature (° C)		70	90	84	84	100
pH		6.74	7.99	7.10	8.0	8.38
Salinity (g L^{-1})		10.40	4.29	9.68	9.98	5.44
Conductivity(mho cm^{-3})		17.65	7.79	16.32	16.99	9.72
TDS (g L^{-1})		10.83	4.90	10.52	10.20	5.85
API gravity (°)		39.0	38.0	37.0	38.0	35.0