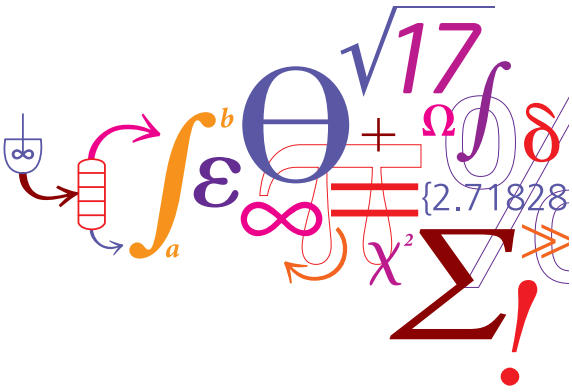
Application of Microorganisms for Enhanced Oil Recovery



Amalia Yunita Halim

Ph.D. Thesis

April 2015

DTU Chemical Engineering
Department of Chemical and Biochemical Engineering

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Preface

This Thesis is submitted as partial fulfillment of the requirement for the PhD degree at Technical University of Denmark (DTU). The work has been carried out at the Department of Chemical and Biochemical Engineering and the Department of System Biology from February 2012 to April 2015 under the supervision of Assoc. Prof. Alexander Shapiro, Senior Researcher Sidsel Marie Nielsen and Assoc. Prof. Anna Eliasson Lantz. The project was funded by Maersk Oil, DONG Energy and The Danish Advanced Technology Foundation as a part of the BioRec project.

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Summary

Microbial Enhanced Oil Recovery (MEOR) is one of the emerging technologies to improve oil production from depleted oil reservoirs. Although the technology is not totally new, very limited information on the application of MEOR in chalk rock has been available to date. This project aims to study the possibility of MEOR application in chalk rock, especially, for the petroleum reservoirs in the Danish sector of the North Sea.

The first challenge of MEOR applications in chalk is the fact that the average sizes of chalk pore throats is comparable to the sizes of microbial cells. In order to investigate microbial penetration into chalk rock, a series of core flooding experiments were conducted with two bacterial strains, *Bacillus licheniformis* 421 (spore-forming) and *Pseudomonas putida* K12 (non-spore-forming). The bacteria were injected into low permeable Stevns Klint outcrop cores with permeability of 2 to 4 mD and pore throat sizes down to 0.5 μm. A significantly higher number of *B. licheniformis* 421 was detected in the effluent as compared to *P. putida* K12. The spore-forming *B. licheniformis* 421 penetrates mainly in the form of spores. It was also demonstrated that both bacteria were capable of plugging the porous rock, as indicated by reduction of the core permeability. A starvation period of 12 days did not allow the permeability to return to initial condition. Based on this finding, *B. licheniformis* 421 was selected for further investigations as the model microorganism.

The second challenge was to identify what mechanisms of additional oil production are the most important for MEOR application in chalk. A number of core flooding experiments were conducted bearing in mind the two objectives. The first objective was to study the selective plugging mechanism. This mechanism was studied by comparative core flooding experiments with homogeneous and heterogeneous cores. The core flooding experiments were conducted using a pressure-tapped core holder that enables pressure monitoring at different points along the core. The second objective was to study the wettability

alteration mechanism by microbial biomass and metabolites. This mechanism was investigated in the core flooding experiments with non-aged and aged cores. It is believed that aging of a core with crude oil will change the pore surface towards more oil-wet. Therefore, if the additional oil production would be different in an aged core, it might indicate that bacteria and/or the metabolites would play a role in the alteration of core wettability; hence, more oil could be produced.

The first study demonstrated that selective plugging plays an important role in improving oil production. It was shown that *B. licheniformis* 421, when injected into homogenous chalk cores after synthetic seawater (SS) flooding, recovered additionally 1.0 to 2.3% original oil in place (OOIP) compared to baseline water flooding. Incremental recovery was significantly higher in heterogeneous chalk cores, where additional 6.9 to 8.8% OOIP was recovered. These results were also supported by the pressure data that showed the increase of the pressure gradient was much higher in the heterogeneous cores. The second study showed that there was no significant difference in the oil production from aged and non-aged cores; the additional oil recovery from the non-aged cores was 1.0-2.3 % OOIP, while from the aged cores it was 3.6-4.3 % OOIP. This might indicate that wettability alteration may not give significant contribution to improve oil production. The reason for this could be that bacteria grew slowly in the presence of hydrocarbons, which was shown in the subsequent experimental study of bacterial growth.

The next challenge was to understand the growth behavior of the microbes, and whether biomass and/or metabolites are produced in sufficient quantities to affect the fluid-fluid and fluid-rock interactions. The growth study was divided into two stages, the aerobic and the anaerobic growth experiments. The aerobic growth experiment was designed as a preliminary study to check whether *B. licheniformis* 421 survives in high salinity environment, and to check the growth behavior with molasses as a nutrient compared to the pure glucose. The anaerobic study aimed to create a more realistic scenario of the bacterial growth under anaerobic conditions and in the presence of n-alkanes to mimic the influence from crude oil. Nitrate was added as electron acceptor. The aerobic study demonstrated that the

bacteria were able to live in the high salinity environment and that they grow better on molasses compared to pure glucose. The anaerobic experiments showed that the bacteria were able to grow on different carbon compounds and to use nitrate as electron acceptor. The bacteria preferred consuming n-alkanes instead of sugar compounds in molasses and produced a lipopetide biosurfactant, lichenysin G. The bacteria grew slowly when n-alkanes were present, however, significant formation of emulsion and interfacial tension (IFT) reduction were still observed, even though almost no bacterial growth occurred. Additionally, it was observed that the bacteria preferred to stay at the interface between the synthetic seawater and n-alkanes; and that they formed aggregates. This showed that the bacterial cells themselves played an important role in production of the emulsions and IFT reduction.

The effect of adding molasses, with and without nitrate, to the changes of indigenous microbial populations was also investigated. Anaerobic incubations using crude oil and brine from a North Sea reservoir were conducted in the laboratory. The results showed that growth of the indigenous microbes was stimulated by addition of molasses. Pyrosequencing showed that specifically *Anaerobaculum*, *Petrotoga* and *Methanothermococcus* were enriched. Addition of nitrate favored the growth of *Petrotoga* over *Anaerobaculum*. Similar phenomena in the *B. licheniformis* 421 growth experiments on fluid-fluid interactions were observed. The growth of the indigenous microbes caused changes in the crude oil-brine system: formation of emulsion and reduction of IFT. Reduction of IFT was associated with microbes being present at the oil-brine interface. These findings suggest that molasses also stimulated the growth of indigenous microbes.

Dansk Resumé

Mikrobiel forbedret olieindvinding (Microbial Enhanced Oil Recovery, MEOR) er en af de fremspirende teknologier, der anvendes til forbedre olieproduktionen i modne oliereservoirer. Selvom teknologien ikke er helt ny, har der omkring anvendelsen af MEOR i kalkreservoirer været begrænset information til rådighed. Dette projekt har til formål at undersøge muligheden for anvendelse af MEOR processen i kalk med fokus på oliereservoirer i den danske del af Nordsøen.

Den første udfordring inden for anvendelse af MEOR i kalk er, at den gennemsnitlige størrelse af porehalsene og selve mikroorganismerne er i samme størrelsesorden. For at undersøge om mikroorganismerne kunne penetrere kalkkernerne, blev der udført en række gennemstrømningsforsøg (core flooding) med to forskellige bakteriestammer. De anvendte bakteriestammer var *Bacillus licheniformis* 421 (sporedannende) og *Pseudomonas putida* K12 (ikke sporedannende). Bakterierne blev injiceret i kerner fra Stevns Klint klippefremspringet med en lav permebilitet på 2-4 mD og med størrelser af porehalsene ned til 0.5 µm. Et markant højere antal *B. licheniformis* 421 blev fundet i udstrømningsvæsken fra kernerne ved sammenligning med *P. putida* K12. Den sporedannende *B. licheniformis* 421 penetrerer hovedsagelig kernen i form af sporer. Det blev også vist, at både bakterier var i stand til delvist at blokere den porøse kerne, hvilket blev indikeret ved, at kernens permeabilitet blev reduceret. En efterfølgende udsultningsperiode på 12 dage bidrog ikke til, at permeabiliteten vendte tilbage til den oprindelige tilstand. *B. licheniformis* 421 blev på basis af disse resultater udvalgt som modelmikroorganismen til de videre forsøg.

Den anden udfordring var at identificere hvilke mekanismer, der er de vigtigste for at forøge olieproduktionen ved anvendelse af MEOR i kalk. Et antal gennemstrømningsforsøg blev udført med to formål. Det første formål var at undersøge den selektive blokeringsmekanisme. Denne mekanisme blev undersøgt ved gennemstrømningsforsøg med homogene og heterogene kerner.

Kerneforsøgene blev udført ved brug af en avanceret kerneholder, der også muliggør trykmåling i flere punkter langs kernen. Det andet formål var at undersøge påvirkningen af mikrobiel biomasse og metabolitter på befugtningsevnen. Dette blev undersøgt ved at udføre gennemstrømningsforsøg med lagrede og ikke-lagrede kerner. Det menes, at lagring af en kerne med råolie vil gøre poreoverfladen mere olievåd. Såfremt den ekstra olieproduktion var anderledes fra en lagret kerne, kunne det indikere, at bakterier og/eller metabolitter spillede en rolle ifm. ændringer af kernens befugtningsevne, og dermed bidrage til at yderligere olie kunne produceres.

Det første studie viste, at den selektive blokeringsmekanisme spiller en vigtig rolle for at forbedre olieproduktionen. Det blev vist, at injektion af *B. licheniformis* 421 i de homogene kalkkerner efter en forudgående gennemstrømning af syntetisk havvand (SS) blev der indvundet 1.0-2.3% ekstra af den oprindelige olie i kernen (OOIP) sammenlignet med referencegennemstrømningsforsøget. Den øgede indvinding var markant højere i de heterogene kalkkerner, hvorfra 6.9-8.8% ekstra OOIP blev indvundet. Disse resultater blev også underbygget af trykdataene, der viste, at trykgradientens forøgelse var større i de heterogene kerner. Det andet studie viste, at der ikke var nogen signifikant forskel på olieproduktionen fra lagrede og ikke-lagrede kerner; den øgede olieindvinding fra de ikke-lagrede kerner var 1.0-2.3% OOIP, mens den fra de lagrede kerner var 3.6-4.3% OOIP. Dette kunne indikere, at ændringer af befugtningsevnen ikke bidrager væsentligt til en forøgelse af olieproduktionen. Årsagen til dette kunne være, at bakterierne voksede langsomt, når der var olie til stede, hvilket blev eftervist i den efterfølgende eksperimentelle undersøgelse af bakterievæksten.

Den næste udfordring handlede om at forstå mikrobernes vækst, og hvorvidt biomasse og metabolitter blev dannet i tilstrækkelige mængder til at påvirke væske-væske og væske-sten interaktionerne. Vækststudiet bestod af to dele; de aerobe vækstforsøg og de anaerobe vækstforsøg. Det anaerobe vækstforsøg var konstrueret som en forundersøgelse for at vurdere, om *B. licheniformis* 421 er

levedygtig i et meget saltholdigt miljø, og hvordan dens vækst påvirkes, når melasse anvendes som næringsstof i sammenligning med ren glukose. Det anaerobe vækstforsøg sigtede mod at skabe et mere realistisk scenario for bakterievæksten under anaerobe forhold og tilsætning af n-alkaner for at efterligne en mulig påvirkning af råolie. Nitrat blev tilsat som elektronacceptor. De aerobe forsøg demonstrerede, at bakterierne kunne overleve i det meget saltholdige miljø, og at bakterierne vokser bedre på melasse i forhold til ren glukose. De anaerobe forsøg viste, at bakterier var i stand til at vokse på forskellige kulstofblandinger og anvende nitrat som elektronacceptor. Bakterierne foretrak at fortære n-alkaner frem for sukkerforbindelserne i melasse og de producerer en lipopetide biosurfactant kaldet lichenysin G. Bakterierne voksede langsomt, når der var n-alkaner til stede. Dog kunne der ses en betydelig dannelse af emulsioner og en reduktion af grænsefladespændingen (IFT) på trods af begrænset bakterievækst. Tillige observeredes der, at bakterierne især dannede aggregater og foretrak at befinde sig på grænsefladen mellem det syntetiske havvand og n-alkanerne. Forsøgene viste, at selve cellerne spillede en vigtig rolle i dannelsen af emulsioner og IFT reduktion.

Effekten af at tilsætte melasse, med og uden nitrat, blev også undersøgt, da det kunne medføre forandringer i de mikrobielle populationer. De anaerobe inkubationsforsøg med råolie og vand indeholdende de oprindelige mikrober fra et reservoir i Nordsøen blev udført i laboratoriet. Resultaterne viste, at væksten af de mikrober, der oprindeligt stammer fra reservoiret, blev stimuleret ved tilsætning af melasse. Pyrosekventering viste, at berigelsen med melasse især var til fordel for *Anaerobaculum*, *Petrotoga* og *Methanothermococcus*. Tilsætning af nitrat betød, at vækstforholdene var mere gunstige for *Petrotoga* end for *Anaerobaculum*. Lignende fænomener for væske-væske interaktionerne blev observeret i vækstforsøgene med *B. licheniformis* 421. Væksten af disse oprindelige mikrober medførte ændringer i olie-vandsystemet: dannelse af emulsioner og reduktion af IFT. Reduktionen af IFT associeredes med mikroberne, der hovedsageligt fandtes i olie-vand grænsefladen. Disse resultater tyder på, at melasse også stimulerede væksten af de oprindelige mikrober.

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CHAPTER 1
Introduction

In the petroleum industry, oil recovery is classified into three phases. The first is primary oil recovery when oil is produced due to the natural drive from the pressure inside the reservoir. However, as the pressure rapidly drops, the primary oil production also decreases [1, 2]. The second phase is secondary oil recovery which is achieved by the injection of water or gas into the reservoir to maintain reservoir pressure and to push oil towards the producing well. Therefore, there is an increase in oil production. The third phase is tertiary oil recovery which includes advanced techniques aimed at increasing microscopic efficiency or sweep efficiency [2]. These include chemical enhanced oil recovery methods such as polymer flooding, surfactant flooding, alkaline flooding, etc. [1].

To date, the primary and secondary oil recovery approaches used in the oil industry can only recover approximately one third of the original-oil-in-place (OOIP), leaving behind a large quantity of residual oil which are trapped in the porous space [3, 4]. The reasons behind this phenomenon are the high viscosity of the trapped oil which result in poor oil mobility [5, 6], the high interfacial tension between oil-brine water which makes the capillary pressure hold the oil in the reservoir rock [5] and the strong bonds between the oil and the surrounding reservoir rock (surface tension) [4, 6] especially in oil wet formations. The present study focuses on injection of microbes as an alternative method to injection of chemicals in the tertiary oil recovery phase.

This chapter begins with an introduction to the microbial world, followed by short history of petroleum microbiology, introduction to microbial enhanced oil recovery (MEOR), the microbial culture used in MEOR, the screening criteria, the objectives of the study and the thesis outline.

1.1 Introduction to microbial world

Based on the phylogenetic relationship, all living organism can be classified into three domains, *Bacteria*, *Archaea* and *Eukarya* (Fig. 1.1) [7, 8]. Microbes can fall into this three domains, they can be either prokaryotes (cells lacking a true nucleus) or eukaryotes (cells with true nucleus) [8]. Microbes are ubiquitous [9], small, free-living organisms (less than about 100 µm) that are generally only visible under the microscope [8]. The smallest known bacteria existing are barely 0.2 µm long, but giant bacteria and protozoa can be up to 1 mm in length or even longer [8].

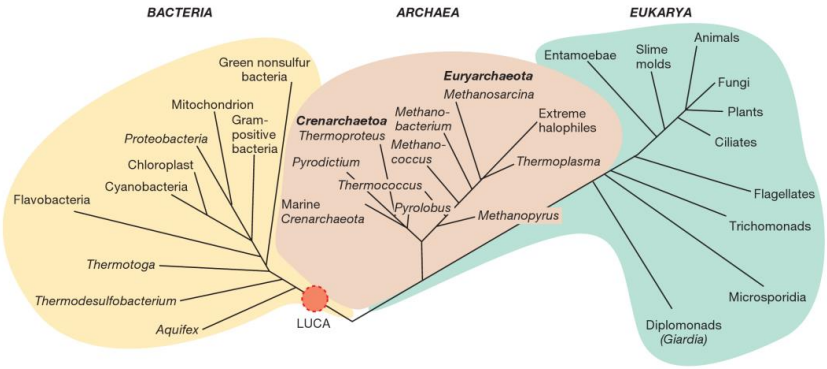

Fig.1.1 The phylogenetic tree of life based on comparative ribosomal RNA homology. The tree shows the three domains of organisms and a few representative groups in each domain. All *Bacteria* and *Archaea* and most *Eukarya* are microscopic organisms. Animals, plants, and fungi are macroscopic *Eukarya*. LUCA, last universal common ancestor. Picture adapted from Madigan *et al.* [7].

The prokaryotic microbes, *Bacteria* and *Archaea*, live in all environments and in many cases are the sole inhabitants of extreme environments. They can be found in deep sea vents to rocks in boreholes 6 km beneath the earth's surface [9] and also in oil reservoirs [10-12].

1.2 History of Petroleum Microbiology

The remarkable history of petroleum microbiology began as early as 1926 when J.W. Beckman finds that microbes are able to release trapped oil in porous rock

formations [3, 10, 11]. Simultaneously in the same year, T.L. Ginzburg-Karagicheva studied microbial formation of hydrogen sulfide in the formation waters of the Aspheron oil fields [10] and Bastin reported that Sulphate Reducing Bacteria (SRB) reside in samples from 67 wells located in California and Illinois [10, 12, 13]. Beckman's discovery developed into new knowledge of enhancement of oil production using a microbial community, or MEOR [1, 3-6, 11]. Meanwhile, Bastin and Ginzburg-Karagicheva's discovery contributed to the knowledge of biodeterioration of materials or corrosion due to the presence and metabolic activities of microorganisms, commonly referred to as Microbiologically Influenced Corrosion (MIC) [14-16]. The extensive research on oil field microorganisms that took place at the Institute of Microbiology, Russian Academy of Sciences by S.I Kuznetsov and his group were summarized in two monographs, *An introduction to Geological Microbiology* in 1962 and *Microflora of Oil Fields* in 1974 [10].

1.3 Microbial Enhanced Oil Recovery (MEOR)

1.3.1 A short history and early development

After Beckman's discovery, little was known about MEOR until Zobell and his research group carried out a systematic laboratory study in 1947 [3]. Thereafter, many other researchers made contributions to the knowledge of MEOR [3, 11, 17, 18]. In 1960s and 1970s significant MEOR research took place in European countries. The MEOR research was boosted by the petroleum crisis in the 1970s and later become a substantiated EOR method supported by research projects worldwide [11]. In 1970, a new stage of microbial technology development started in Russia mainly in two laboratories: the Department of Geological Microbiology at the Institute of Microbiology, Academy of Sciences of the USSR, headed by Kuznetsov, and the Laboratory of Microbial Biochemistry and Physiology of Microorganism, Academy of Sciences of the USSR, organized by Ivanov. In 1986, these research groups were united under the basis of the Institute of Microbiology, Russian Academy of Sciences [10]. It is also important to acknowledge the support of U.S. Department of Energy (DOE), who sponsored MEOR basic research and field trials, as well as organizing international meetings

[11]. The decade of the 1990's is noted as a significant year for MEOR development [17]. By the end of the 1990s, MEOR had become a scientific and interdisciplinary method for enhanced oil recovery.

1.3.2 Mechanisms involved in MEOR

To date, MEOR is believed to be one of the advanced technologies able to recover oil in trapped formations inside the reservoirs. This technology is implemented by the addition of nutrients and/or bacteria into oil reservoirs [3-6, 11, 17, 18]. The MEOR technology relies on growth of microbes, either indigenously [10, 11, 19, 20], or exogenously [10, 11, 20-23] to the reservoir, that can produce useful substances [10, 11, 20-25]. These substances, such as gasses, organic acids, solvents, polymers and surfactants, are able to affect the properties of the formation water, oil, and gas mixture(s), as well as to change the properties of the matrix of the reservoir and thus play a significant role in the recovery of residual oil [10]. The advantage of each substance is listed in Table 1.1.

Despite the fact that many substances have been identified and many mechanisms have been proposed, the effectiveness of each substance or mechanism for different reservoir parameters is still debatable [26, 27]. It is most likely that several microbial processes act synergistically to enhance the flow of oil [18, 28]. The widely known mechanisms by which microbes can affect the fluid-fluid and fluid-porous media interaction can be categorized into three major processes: 1) changes in flow behaviour due to bioclogging or selective plugging [26, 27, 29-31], 2) alteration of rock wettability [26, 27, 30-33], 3) reduction of interfacial tension (IFT) [26, 27]. It is also stated by Kaster *et al.* [34] and Jackson *et al.* [35] that formation of emulsions may also be important.

1.3.2.1 Bacteria transport in porous media and selective plugging mechanism

Studies of bacterial penetration are important to understand how far bacteria can travel through the porous formation and plug the water-swept zones of a reservoir, thus altering the flow paths and improving sweep efficiency. Although previous investigations have revealed that bacteria can penetrate deep into porous

formation [36-39], complete understanding of the penetration behavior of bacteria is lacking. In addition, there is a limited amount of publications on microbial penetration/plugging of chalk rocks, as earlier studies were conducted on sandstones [36, 37, 39, 40], micromodels [29, 31], glassbeads [26, 40], or sandpacks [41, 42]. Detailed literature review on microbial transport in porous media is given in the introduction part of Chapter 2.

Table 1. 1 Products and activities of microorganisms potentially useful for enhanced oil recovery*

Product or activity	Example (s)	Example microbes	Application in MEOR
Biomass	Microbial cells	*Bacillus*, *Leuconostoc*, *Xanthomonas*	Selective plugging and wettability alteration
Surfactants	Glycolipids, lipopeptides	*Acinetobacter*, *Arthrobacter*, *Bacillus*, *Pseudomonas*	Emulsification and de-emulsification through reduction of interfacial tension (IFT), wettability alteration
Polymers	Polysaccharides, proteins	*Bacillus*, *Brevibacterium*, *Leuconostoc*, *Xanthomonas*	Viscosity modification of injected water, mobility control, selective plugging
Solvents	Ethanol, butanol, acetone	*Clostridium*, *Zymomonas*, *Klebsiella*	Oil viscosity reduction, wettability alteration, dissolve and remove heavy-long chain hydrocarbons from pore throat (increase effective permeability)
Acids	Acetic, butyric, lactic	*Clostridium*, *Enterobacter*, mixed *acidogens*	Improve effective permeability by dissolving carbonate precipitate from pore throat, emulsification, increase porosity (in carbonate reservoir)
Gases	CO_2 and CH_4	*Clostridium*, *Enterobacter*, *Methanobacterium*	Increased pressure, oil swelling, IFT and viscosity reduction, permeability increase due to dissolution of carbonate rock in reaction with CO_2
Emulsifiers	Heteropolysaccharides, proteins	*Acinetobacter*, themophilic *methanogens*	Oil emulsification, wax and paraffin control
Hydrocarbon metabolism	Oil degradation	Aerobic: *Pseudomonas*, *Rhodococcus*, *Geobacillus*, *Bacillus*, Anaerobic: denitrifying bacteria, sulfate-reducing bacteria	Paraffin control, viscosity alteration, CH_4 production

* Adapted from McInerney *et al.* [18], Sen [4], Lazar [11] and Belyaev *et al*.[10]

During secondary oil recovery, when water is injected into the reservoir, the water preferentially flows into high-permeable channels (water thief channels), thus bypassing the low-permeable parts of the reservoirs filled by oil [43, 44]. Injection of nutrients/and or bacteria into these preferential pathways and stimulating the bacterial growth will block the water thief channels, either with bacterial cells or polymer produced in-situ by the growing bacteria. Blocking of the channels will alter the water pathways to the previously unswept pore space [43, 45-48]. This concept is known as microbial selective plugging, as illustrated in Fig.1.2. Selective plugging of the more permeable zones may result in increasing oil production, correcting microscopic and volumetric sweep efficiency and redirecting water flow to the low permeable, oil-bearing zones [49]. A detailed literature review on the selective plugging mechanism can be found in the introduction section of Chapter 3.

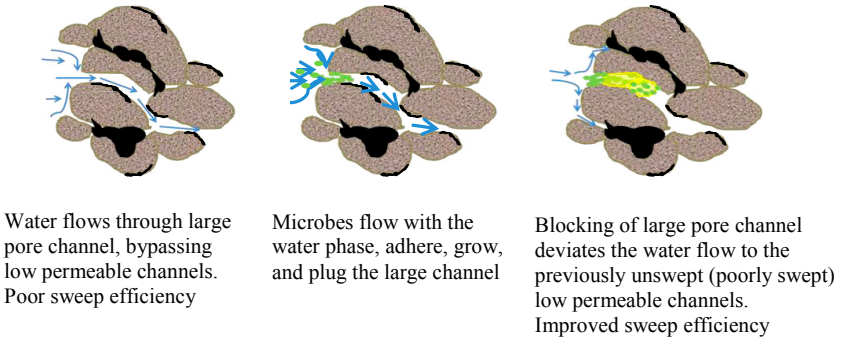

Fig.1. 2 Illustration of selective plugging mechanism.

1.3.2.2 Alteration of rock wettability

In a rock/oil/brine system, wettability can be defined as a measure of the preference that the rock has either for the oil or for the water [50] . Wettability of the rock/fluid system is of great importance because it controls the location, flow, and distribution of fluids in porous media [50].

Several possible mechanisms have been proposed to explain wettability alteration by microbes, such as metabolite (biosurfactant) production, bacteria attachment and/or biofilm formation on the rock surface, brine chemistry of the bacterial

solution [27, 29, 30, 33, 51]. However, to date, the published literature on wettability alteration by bacteria mostly conducted on sandstones and the minerals that compose sandstone rock [30, 32, 33, 51]. It is also important to mention that the change in wetting properties is dependent on the initial wetting conditions [27, 33], surface properties, types of microorganisms and the metabolites [33]. Bacterial growth in an initially oil-wet system can result in a more water-wet condition [51], while an initially water-wet system can change wettability towards a less wet condition [27, 32].

Karimi *et al*.[33], investigated *Enterobacter cloacae*, a facultative, anaerobic Gram-negative bacterium on a silica glass slide using atomic force microscopy (AFM). The glass slides were aged for 7 and 21 days in crude oil to mimic the rock surfaces in a mature reservoir with a tendency towards greater oil wetness. It was found that *E. cloacae* is able to adhere to the desired surfaces and retains their attachment even after 15 days of starvation without nutrient. The authors concluded that wettability alteration takes place due to absorption of biosurfactant and other products on the rock, bacteria attachment and biofilm formation. The biofilm formation is considered to be the key player for wettability alteration. Bacteria are able to use organic compounds adsorbed on the glass surfaces during aging process and are able to partly remove the thin adsorbed oil film, and thus, there is a shift of the wettability towards more water wet.

Polson *et al*.[32] investigated the wettability alteration of quartz after exposure to microbial consortium isolated from core handling facilities using environmental scanning microscopy (ESEM). Twenty quartz chip samples exhibiting initial hydrophilic (water wet) behavior were used in the investigation. After 2 days of exposure to microbial consortium, the quartz chips showed a change in wettability from hydrophilic to hydrophobic. The authors proposed that this phenomenon is likely to happen due to the presence of microbial consortium cellular or extracellular compounds (e.g., protein). However, the authors also mentioned that not all biofilms produce a hydrophobic surface; and referred to the study by Schaumann *et al.* [52], where it was shown that biofilms may develop hydrophilic

microenvironments. Therefore, it is important to keep in mind that the possible effect on wettability of each biofilm or microbial consortium may be different, and thus has to be investigated individually.

Afrapoli *et al*. [30] conducted the Amott tests on Berea sandstone cores to study macroscopic wettability index changes. The investigation involved spontaneous uptake of fluids and forced displacement steps. Two types of *Rhodococcus* sp.094 were used, the first type is a surfactant-producing bacterium, while the second type is non-surfactant-producing. The two different metabolisms are obtained by manipulating the carbon source of the media. The bacterium produces surfactant when grown on media supplemented with dodecane and does not produce surfactant when it is grown on media supplemented with acetate. Different modifications of wettability are noticed. The surfactant producing bacterium changes the wettability to a less water wet condition, while the non-surfactant-producing bacterium changes the wettability to a more water wet condition. It is also observed that wettability changes occur mostly when the bacteria biomass attaches to the grain surfaces and by the adsorption of bacterial metabolites on the rock surface.

Kowalewski *et al*. [27] studied the oil/brine/rock wettability and IFT changes in the presence of bacteria and phosphate ions. Full Amott-Harvey tests were performed on Beitheimer sandstone cores using different types of hydrocarbons (crude oil and n-decane) and water compositions (with and without phosphate ions). A mixture of aerobic mesophilic bacteria isolated from seawater and backflooded water injection wells was used in this study. Several conclusions are drawn from the study: 1) crude oil with higher viscosity and lower IFT compared to n-decane makes the imbibition curves more “S-shaped”; 2) there is no change in the Amott-Harvey index, but the dynamic behaviour of the spontaneous imbibition shows a possible change in wettability towards less water-wet system in the cores treated with microbes; 3) phosphate increases interfacial tension (IFT) but does not affect the shape of the spontaneous imbibition curve; 4) reduction in IFT, although not significant, may be an important factor for the enhanced oil

recovery process. The reduction of IFT is most likely to take place because the bacteria produce biosurfactant in order to get access to the crude oil. The reduction in the oil-water IFT will change the oil-rock contact and thus alters the rock wettability.

1.3.2.3 Reduction of interfacial tension (IFT)

The residual oil in a porous medium exists as disconnected and dispersed globules. Under reservoir conditions, there is insufficient pressure gradient to move these globules through the surrounding pore throats. Mobilization of the globules is dependent on viscosity and capillary forces. A useful correlating parameter is the capillary number (N_{ca}), which represents the ratio of viscous to capillary forces between the two immiscible fluids, as given by Eq. (1) [53, 54]:

$$N_{ca} = \frac{\upsilon \times \mu}{\sigma_{ow}} \qquad (1)$$

where υ is the velocity of the fluid around a globule (m/s), μ is the viscosity (Pa·s), and σ_{ow} is the oil-water interfacial tension (N/m) [53, 54]. Reduction of IFT will increase the capillary number; thus, capillary forces become less dominant and the likelihood of oil mobilization increases [53]. Usually, the IFT needs to be reduced at least by two orders of magnitude to mobilize the oil significantly [53].

Microbes are known to produce diverse biosurfactants that can reduce the IFT between the water and hydrocarbons [5, 55]. Microbial surfactants are structurally diverse groups of compounds containing both hydrophilic and hydrophobic domains within the same molecule. This structure make the surfactants preferentially stay at the interface between fluid phases of the different degrees of polarity and hydrogen bonding (such as oil/water or air/water interfaces) [55]. Biosurfactants include a wide variety of chemical structures, such as glycolipids, lipopeptides, polysaccharides-protein complexes, phospholipids, fatty acids and neutral lipids [55]. The biosurfactants produced by microbes may be cell-bound or

free [27]. However, Kowalewski stated that there is also a strong indication that the presence of bacteria cells themselves is important in the reduction of IFT [27].

The limiting concentration of surfactant in solution above which micelles formed is known as critical micelle concentration (CMC). The interfacial tension between the aqueous phase and the oleic phase changes very little above the CMC. At the concentrations above the CMC, amphiphilic molecules associate readily to form supramolecular structures such as micelles, bilayers and vesicles. The mixture of oil, brine and surfactant exhibits complicated phase behaviour above CMC. The formation of micelles can result in solubilisation of oil or water in another phase, giving rise to microemulsions [55].

1.3.2.4 Emulsions

Formation of emulsions is considered to be an important factor that can affect fluid flow through porous media [34]. It is mentioned by Kaster *et al.* [34] that emulsions can be useful in MEOR as they may prevent or reduce re-trapping of mobilized oil. Microbes have been shown to form emulsions, either by production of surfactant [56, 57] or by attaching and surrounding hydrocarbon globules [58]. Due to the former reason, the words bioemulsifiers and biosurfactants are sometimes interchangeable.

The low-molecular-mass bioemulsiers reduce the surface and interfacial tensions, whereas the higher molecular-mass bioemulsiers are more effective at stabilizing oil-in-water emulsions [59]. Rosenberg and Zon [59], proposed three natural roles of bioemulsifiers: (i) increasing the surface area of hydrophobic water-insoluble growth substrates; (ii) increasing the bioavailability of hydrophobic substrates by increasing their apparent solubility or desorbing them from surfaces; (iii) regulating the attachment and detachment of microorganisms to and from surfaces.

Kaster *et al.* [34] mentioned about their studies that emulsification is related to the presence of biomass itself and that the degree of emulsification is propositional to

the amount of biomass. In addition, studies by Rosenberg and Rosenberg [60] revealed that some microorganisms can form emulsions even without cell growth or uptake of hydrocarbons.

1.3.3 Microbes used in MEOR and reservoir screening criteria

Table 1. 2 Screening criteria of reservoirs for MEOR application

Permeability	> 50 to 100 mD [35] > 20mD [66] 0.1 – 600 mD [67] > 100 mD [68]
Temperature	< 60–70 ºC [35], < 132ºC [66], < 55ºC [69], 38-104 ºC [67] < 71ºC [68]
Salinity	< 6% TDS [35] < 10% [66, 68] 100-150 g/l [69] < 120 g/l [67]
pH	5-9 (6-8 ideal) [35] 7-8 [67]
Oil viscosity	< 500 cP [35] 5-50 cP [66, 69]
Down hole pressure	< 3000 psi [35] < 13500 psi, with pressure gradient at least 0.25 psi/ft [67]
Porosity	>10% [66]
Reservoir depth	1000-1500 m [69] < 3000 m [68]
Pore throats	0.3-2.1 microns [67]
Residual oil saturation	> 20% [67] > 25-30% [68]

> = should be greater than, < = should be lower than

In general, oil reservoirs have low redox potential and hence harbor mainly anaerobic and facultative anaerobic microorganisms. Availability of electron donors and acceptors, temperature and salinity are also important environmental factors that shape the microbial community that can survive in the oil reservoirs [25]. It is strongly recommended that the microorganisms used for MEOR should be able to live under anaerobic conditions under restricted electron acceptor [35]. Bacteria from genera *Bacillus*, *Pseudomonas* and *Clostridium* have been repetitively reported being used in field application with positive outcomes in many different countries [11]. To date, *B. licheniformis* is one of the bacillus genera that has been widely investigated for MEOR [44, 48, 61-65]. A more detailed literature study on this bacterium can be found in Chapter 5.

Other reservoir conditions that should be taken into considerations, when choosing the microorganisms for MEOR, have been collected from various reviews and are listed in Table 1.2. It should be remarked that these conditions are not absolute and depends on specific conditions in a particular reservoir.

1.4 Research Objectives:

This study was performed to understand some of the challenges for MEOR applications in chalk reservoirs, as listed below:

1. To study microbial penetration/transport in chalk rock. This considers being important as the microbial cell sizes are almost comparable to the sizes of the chalk pore throats.
2. To study whether bacteria injection improves oil production compared with water flooding
3. To investigate whether bacterial selective plugging is one of the possible mechanisms for enhanced oil recovery in chalk.
4. To investigate whether wettability alteration is one of the mechanisms for enhanced oil recovery in chalk.
5. To study bacterial growth with different carbon nutrients (glucose vs. molasses) and to test the bacterial survival in a high salinity environment.

6. To investigate bacterial growth and metabolites production under anaerobic conditions using the chosen carbon nutrient and hydrocarbons. In addition, the effects of the bacterial biomass and/or metabolites to the fluid-fluid interaction were also addressed.
7. To study the effects on the dynamic changes of the indigenous microbial populations and the fluid-fluid interactions by adding the carbon nutrient chosen and nitrate.
8. To identify similar phenomena that may have taken place in batch culture experiments of the model study microbes and the indigenous microbes; and the proposed possible mechanisms and recommendations for MEOR applications in chalk reservoirs based on similar phenomena.

1.5 Thesis outline

The work presented in this thesis is organized into seven chapters, where the chapters are mainly based on articles that are either published, accepted or under preparation. The summary of each chapter is described below:

Chapter 1 provides short introduction to microbial world, history of petroleum microbiology, general introduction of MEOR and organization of the thesis.

Chapter 2 is based on a published article to answer the first research objective. This chapter demonstrates the penetration behaviour of two bacteria strains, *Bacillus licheniformis* 421, a spore-forming bacterium, and *Pseudomonas Putida* K12, a non-spore forming bacterium, into chalk rock. The penetration study was carried out by injecting microbial cultures into water saturated Stevns Klint chalk cores and analysing the microbial cells in the effluents. It was shown that bacteria were able to penetrate the chalk rocks even though the permeability was as low as below 4 mD and the pore sizes were comparable to bacterial sizes.

Chapter 3 is based on a submitted article and answers the third research objective. This chapter shows the performance of *B. licheniformis* 421 in recovering the residual oil when injected into homogeneous and heterogeneous

reservoir chalk cores. The core flooding experiments were carried out in crude oil saturated cores. It was demonstrated that the main mechanism of the MEOR action was selective plugging on the small-scale level.

Chapter 4 shows the answers for the second and the fourth research objectives. This chapter aims to investigate whether injection of *B. licheniformis* 421 increases oil production and whether wettability alteration plays a role in increasing oil production or not. It is believed that injection of bacteria change the wettability towards less oil-wet system and hence increases oil production. Several core flooding experiments were conducted in homogeneous chalk cores. The results demonstrated that injection of bacteria increased oil production and wettability alteration did not give a significant effect on the additional oil production.

Chapter 5 is divided into two parts to answer research objectives 5 and 6. The first part of the chapter considers aerobic growth, where investigations of bacterial growth on different carbohydrate nutrients in a high salinity environment were carried out. It was shown that *B. licheniformis* 421 grows better with molasses compared with glucose and it grows well in a high salinity environment. Molasses was hereafter chosen as carbohydrate nutrient. The second part of the chapter deals with *B. licheniformis* 421 anaerobic growth on molasses and n-alkanes and production of metabolites. It was shown that *B. licheniformis* 421 was able to use different carbon sources under anaerobic conditions and produced amongst others a lipopeptide, lichenysin G.

Chapter 6 is based on a published article that answers the 7th research objective. In this chapter, the effects of adding molasses to the indigenous microbial population sampled from the Dan field were investigated. The microbial population was monitored by qPCR and pyrosequencing. In addition, the effects on the fluid-fluid interactions such as oil-brine interfacial tension (IFT) and emulsions were also investigated. It was shown that molasses stimulated and altered the indigenous microbial populations. Furthermore, the microbial growth

caused changes in the crude oil-brine system: reduction of IFT and formation of oil emulsions.

Chapter 7 summarizes the findings and gives recommendations for future work.

The joint author statements and copy right clearance are attached in Appendix 4.

1.6 List of Research Papers and Conference Contributions

The following is a list of relevant papers to the thesis which has been written by the author of this thesis:

1. **Halim, A.**, A. Shapiro, A.E. Lantz, S.M. Nielsen. 2014. Experimental Study of Bacterial Penetration into Chalk Rock: Mechanisms and Effect on Permeability. Transport in Porous Media, 101 (1): 1-15.
2. **Halim, A.Y.**, D.S. Pedersen, S.M. Nielsen, A.E. Lantz. 2015. Profiling of Indigenous Microbial Community Dynamics and Metabolic Activity During Enrichment in Molasses-Supplemented Crude Oil-Brine Mixtures for Improved Understanding of Microbial Enhanced Oil Recovery. Applied Biochemistry and Biotechnology, April 2015,
   DOI: 10.1007/s12010-015-1626-y
3. **Halim, A. Y.**, S.M. Nielsen, A.E. Lantz, V.S. Suicmez, N. Lindeloff, A. Shapiro. 2015. Investigation of Spore Forming Bacteria Flooding for Enhanced Oil Recovery in North Sea Chalk Reservoir. Journal of Petroleum Science and Engineering (accepted article in Journal of Petroleum Science and Engineering)

The following is a list of relevant conference contributions which have been performed during the PhD study:

1. **Halim, A.Y**, A. Shapiro, S.M. Nielsen, A.E. Lantz. The effect of bacteria penetration on chalk permeability. 75th EAGE Conference & Exhibition incorporating SPE EUROPEC 2013, 10th-13th June, 2013, London, United Kingdom. (Oral)

2. **Halim, A.Y.**, D.S. Pedersen, A.E. Lantz, S.M. Nielsen, A. Shapiro. Molasses injection as a MEOR strategy: Enrichment incubations of brine/oil from North Sea Oil Field. 4th International Symposium on Applied Microbiology and Molecular Biology in Oil Systems (ISMOS), 25th-28th August, 2013, Rio de Janeiro, Brazil. (Poster)
3. **Halim, A. Y.**, S.M. Nielsen, A.E. Lantz, A. Shapiro. Optimization of Spore Forming Bacteria Flooding for Enhanced Oil Recovery in North Sea Chalk Reservoir.20th Reservoir Microbiology Forum (RMF), 18th-19th November, 2014, London, United Kingdom. (Oral)
4. Katika K., **A. Y. Halim**, A. Shapiro, I. L. Fabricius. Quantification of the recovered oil and water fractions during water flooding laboratory experiments with the use of low field NMR and UV/visible spectroscopy, 77th EAGE Conference & Exhibition, 1-4 June, 2015 (accepted for oral presentation, Appendix 2).

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Paper 1

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CHAPTER 2
Experimental Study of Bacterial Penetration into Chalk Rock: Mechanisms and Effect on Permeability

Abstract

Bacterial selective plugging is one of the mechanisms through which microorganisms can be applied for enhanced oil recovery, as bacteria can plug the water-swept zones of a reservoir, thus altering the flow paths and improving sweep efficiency. However, complete understanding of the penetration behavior of bacteria is lacking, especially in chalk formations where characteristic pore throat sizes are comparable with the sizes of bacterial cells. In this study, two bacterial strains, *Bacillus licheniformis* 421 (spore-forming) and *Pseudomonas putida* K12 (non-spore forming) were used to investigate the penetration of bacteria into chalk and its effect on permeability reduction. The core plugs were produced from Stevns Klint outcrop with low permeability (2 -4 mD) and with pore sizes comparable to bacterial sizes. Both types of bacteria were able to penetrate and to be transported through the cores to some extent. A significantly higher number of *B. licheniformis* 421 was detected in the effluents as compared to *P. putida* K12. It was demonstrated that the spore-forming *B. licheniformis* 421 penetrates in the form of spores. *P. putida* K12 is found to penetrate the core, however, in smaller numbers compared to *B. licheniformis*. It was shown that both bacteria, under different injection concentrations, were capable of plugging the porous rock, as indicated by reduction of the core permeability. An incubation period of 12 days did not allow the permeability to return to initial condition. Based on the results it can be concluded that, when injected into chalk, spore forming bacteria have higher chance to survive and penetrate into deeper formation; and both types of bacteria may cause permeability reduction.

2.1 Introduction

Numerous mechanisms have been proposed in the literature through which microorganisms can be used for enhanced oil recovery. One of them is the change in flow behavior due to bioclogging or selective plugging [1-3]. Injection of

bacterial populations may cause plugging of water-swept zones and attaining more uniform displacement of oil from heterogeneous rocks [4]. Studies of bacterial penetration are important to understand how deep/far bacteria can travel through the porous formation and plug the water-swept zones of a reservoir, thus altering the flow paths and improving sweep efficiency. Although previous investigations have revealed that bacteria can penetrate deep into porous formation [5-8], a complete understanding of the penetration behavior of bacteria is lacking. Furthermore, there is a limited amount of publications on microbial penetration/plugging of chalk rocks, as earlier studies were conducted on sandstones [5-7, 9], micromodels [2, 3], glassbeads [1, 9], or sandpacks [10, 11].

Experiments on Berea sandstone have revealed that the permeability of 100 mD was a limiting value for instantaneous bacteria penetration [6]. However, permeability is not always a good indicator, since pore throat size and tortuosity may be more crucial for bacteria penetration [6]. In agreement with this, other researcher [5] has stated that the probability of retaining a cell as it travels with the suspending media is related to the structure of the porous rock. Motility (that is, an ability to "swim" or to move spontaneously and actively through water-based environments by using the flagella in the outer cell) has also been shown to be an important factor for bacteria penetration in porous rocks as motile bacteria penetrated the rock at a rate three to eight times faster than the nonmotile bacteria [6]. Furthermore, it has been demonstrated that flow rate, flow pattern, specific cellular attachment mechanisms and chemotaxis influence cells and/or spores transportability in porous media [5].

Core flooding experiments conducted on Berea sandstone using injection of three different bacteria populations: *Pseudomonas putida*, *Bacillus subtilis* and *Clostridium acetobutylicum* led to the recommendation that injection should be performed with low to intermediate (~ 10^6/ml) bacterial cell suspension concentrations [5]. Thereby, plugging of the inlet surfaces can be avoided and further bacterial growth can be enhanced by injection of nutrients. In addition, bacteria with self-aggregating tendencies are not recommended for the microbial

enhanced oil recovery (MEOR) process, since plugging in the inlet due to the bacterial aggregates is likely to occur. The authors also have concluded that injection of spores is recommended for MEOR processes due to their relatively small size and rigidity that makes it easier to push through porous rocks [5].

Afrapoli *et al.* [2] and Crescente *et al.* [3] have conducted investigations on pore scale plugging mechanisms using a glass micro-model. Two different growth variants of *Rhodococcus* sp.094 were used in their investigation, the surfactant producing bacteria (SPB) and non-surfactant producing bacteria (NSPB). The SPB cells are hydrophobic, therefore tend to form aggregates, while the NSPB cells are hydrophilic and travel as loose particles. The SPB travel as large pieces, precipitate and block the pores faster than NSPB. The NSPB have higher mobility and deeper penetration. It should be noticed that most mechanism described at this work (like the aggregates or biofilm formation) are probably irrelevant to propagation of bacteria in chalk rock where pores can hardly accommodate many bacteria.

MacLeod et al. [7] has studied the plugging mechanisms by flowing starved cells of *Klebsiella pneumonia* though artificial rock cores. The starved cells reduced the core permeability less than the vegetative cells, and the starved cells were distributed more uniformly throughout a core. This was due to the fact that the starved cells decreased in size and were able to penetrate further. In addition, the starved cells did not produce glycocalyx, responsible for irreversible adhesion of bacteria to surfaces.

A recent publication reported core flooding experiments on bacteria penetration and plugging mechanisms in low permeability Liege Chalk cores (0.28 – 0.6 mD). The enrichment bacteria consortium from Draugen field, North Sea, was used in the experiments. The bacteria were found to be able to penetrate, to be transported and to grow at different core sections, without causing any permeability reduction or plugging. However, no data were presented on the bacterial population in the influent (inlet) and effluent (outlet). The authors related the core flooding results to the fermentative enrichment tests [12].

In summary, there are many factors that influence the transport behavior of bacteria in porous media. These include (but are probably not limited to) i) bacteria characteristics such as sizes and shapes [8, 10], hydrophobicity [2, 10], motility [6, 10], tendency to form aggregates [8]; ii) electrostatic or surface charge [10, 13]; iii) rock permeability [6]; iv) pore throat sizes [14], and v) tortuosity [6]. In the practical applications of the deep bed filtration, it is generally believed that the 1/3-1/7 rule can be applied: 1) Particles larger than 1/3 of the pore diameter will form a bridge at the entrances of formation face and form an external filter cake; 2) Particles smaller than 1/3 but larger than 1/7 the pore diameter will invade the formation and are trapped, forming an internal filter cake; 3) Particles smaller than 1/7 the pore diameter will cause no formation impairment because they will be carried through the formation [15]. The data on bacterial penetration seem to contradict to this rule: while the bacterial sizes are comparable to the pore sizes, so that the ratio of "particle" to pore diameter is around 1, bacteria are still able to penetrate deeply into the porous medium. Therefore, analysis of the size exclusion mechanisms for this case (why they still allow the bacteria to penetrate) is of the utmost importance.

This investigation was conducted to understand the penetration and plugging behavior of freshly grown bacteria in chalk by comparing penetration behavior of spore forming and non-spore forming bacteria. We flooded the cores of the outcrop chalk rock (Stevns Klint) with the brine containing bacteria and nutrients, in the different sequences. The chosen bacteria, *Bacillus licheniformis* 421 and *Pseudomonas putida* K12 were isolated from a high temperature oil field in China [16]. Dynamics of the permeability difference around the cores was analyzed. The amounts of bacteria in the effluent were quantified, and analyzed under microscope. The resulting data enables us to conclude that spore formation and change of the cell shapes are the two main mechanisms behind bacterial penetration through low permeable rocks.

2.2 Description of material/equipment and experimental sequence

2.2.1 Materials

Outcrop rock samples from Stevns Klint about 40 km south of Copenhagen were used as porous media. They have an unimodal pore throat size distribution, with the pore sizes in the range of 0.004-6.1 μm with mean value of about 0.5 μm [17]. The core plugs with dimension of 1.5 inch in diameter and 3 inch in length were produced. The cores were homogeneous (as was indicated by preliminary X-ray computer tomography scanning), and had a white color and a fine grained texture.

Two bacterial strains, *Bacillus licheniformis* 421 and *Pseudomonas putida* K12, were used in this study. Both cultures were obtained from Professor T. Nazina (Winogradsky Institute of Microbiology Russian Academy of Sciences) and were originally isolated from a high temperature oil field in China [16]. *B. licheniformis* 421 is a rod shaped, motile (having petrichous flagella arrangement), Gram positive bacterium. *P. putida* K12 is a rod shaped, motile (having lophotrichous flagella arrangement), Gram negative bacterium. The classification onto Gram positive and Gram negative bacteria is based on the structure of their cell wall – see more detailed explanation in Sharma *et al.* [13]. The vegetative *B. licheniformis* 421 has an approximate cell size 0.5 μm in diameter and 2-4 μm in length, while the vegetative *P. putida* K12 has an approximate cell size 0.5 μm in diameter and 1-2 μm in length, which is comparable to the pore sizes in chalk.

Enrichment medium was used for bacteria inoculum preparation and for the determination of bacteria enumeration. The medium consists of bacto™ tryptone (0.5 g/l), bacto™ yeast extract (2.5 g/l), glucose (1 g/l), NaCl (2 g/l) and bacto™ agar (15 g/l). The liquid used for the core flooding experiment was synthetic seawater (SS) supplemented with 5 mM $NaNO_3$ and 4 % molasses (Table 2. 1), hereafter called SS medium. The molasses was kindly supplied by Nordic Sugar, containing 59% of sucrose. The SS medium was sterilized by filtering through 0.2 μm mixed ester cellulose membrane filter (Advantec®).

Table 2. 1 Synthetic seawater (SS) composition

Chemical	Amount (g/l)
NaCl	18.00
$NaHCO_3$	0.17
KCl	0.74
$MgCl_2.6H_2O$	9.15
$CaCl_2.2H_2O$	1.91
Na_2SO_4	3.41

2.3 Experimental procedure

Schematic drawing of the experimental sequence is presented in Fig. 2.1 and detailed explanations of the procedure are given in sections 2.3.1 to 2.3.5.

2.3.1 Core preparations

Prior to flooding experiments, all the cores were scanned using a fourth generation Siemens SOMATOM scanner. The X-ray computer tomography (CT) scanning was conducted in lateral and longitudinal directions to ensure no fractures in the cores used for the experiments. Each core was then cleaned by flooding with toluene and absolute (99%) ethanol inside the Hassler core holder until a clear solution was observed in the effluent. A 260D-ISCO pump was used to control a constant injection rate of 1 ml/minute. After cleaning, a core was dried in the oven at 80°C to constant weight. The dry weight and wet weight were measured in an analytical balance to calculate the core porosity.

2.3.2 Cleaning and sterilization process

The Hassler core holder, the injection cylinders, the injection lines and the effluent glass collectors were sterilized by autoclave at 121°C, 15 psi, for 20 minutes. The dry core was inserted into the sterile Hassler core holder. Approximately seven pore volumes injected (PVI) of 75 % ethanol were applied in order to sterilize the core plug. The saturated 75 % ethanol core was left overnight inside the core holder. During this process, an overburden pressure of 20 bars was applied using a 260D-ISCO pump.

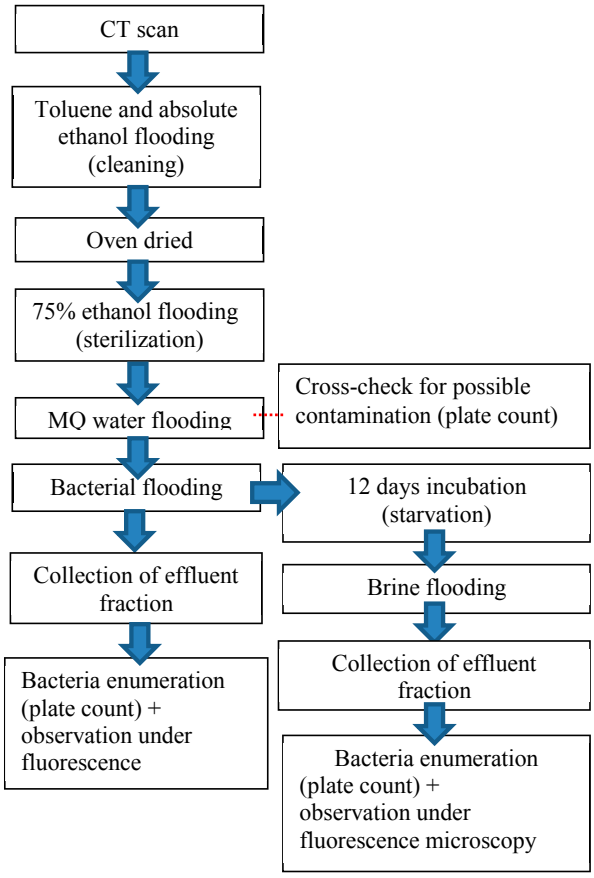

Fig.2.1 Schematic drawing of experimental sequence

2.3.3 Core flooding with bacteria suspensions

Prior to bacteria injection, the 75 % ethanol saturated core was flooded with approximately 7 PVI sterile MQ water to displace the ethanol. MQ water is ultrapure water that has been purified using an ion exchange cartridge, having a resistivity of 18.2 MΩ.cm at 25 °C. The purity of MQ water was monitored by measuring the conductivity and further filtration through a 0.22 μm membrane filter. The effluent was collected every 1 PVI to cross-check for possible contamination. The overburden pressure was fixed at 20 bars and temperature was raised to 50°C by a heating jacket. The injection pressure and the pressure

difference around a core were monitored throughout this process. The recorded pressure difference during MQ flooding was used to calculate the average core permeability according to the Darcy Law. The core was then flooded with bacteria inoculum in SS medium. Various inoculum concentrations were tested to verify the plugging effect of the bacteria. The effluent was collected every 1 PVI for bacterial enumeration. Some of the cores were incubated inside the core holder for 12 days for the study of bacterial development under incubation without any additional nutrient. Post-flushing of these cores with SS was conducted.

2.3.4 Inoculum preparation and bacteria enumeration

For inoculum preparation, bacteria were grown in the enrichment medium for 24 hours and afterwards diluted with 0.85% NaCl. The optical density (OD) of the bacterial solution was adjusted to a desired OD value at the wave length of 600 nm using a spectrophotometer (the OD of ca. 0.05 corresponding to ~10^6 cells/ml). Approximately 10% (v/v) bacterial solution was inoculated into SS medium for core flooding experiments. To obtain a homogeneous solution, the mixture was vortexed for 3 minutes. Bacteria enumeration was conducted by the serial dilution plate method using enrichment medium. The plates were incubated for 24 hours at 50°C before counting the cells. The number of bacteria cells growing on the agar (viable cells) is defined as colony forming unit (cfu), i.e. one visible colony on the plate has grown from one initial cell plated. The unit used for the bacteria cell counting data in this paper is expressed in cfu/ml.

2.3.5 Observation of bacterial cell morphology under fluorescence microscopy

The potential changes of bacterial cell shapes were monitored by use of a fluorescence microscope. The bacterial inoculum suspension and the collected effluents after injection were fixed by 2% (v/v) formaldehyde for 30 minutes and then 0.5% (v/v) 4',6-diamidino-2-phenylindole (DAPI) staining was added. After DAPI addition, the liquid was incubated for 20 minutes in darkness and filtered through a black polycarbonate membrane filter. Subsequently, the filter was fixed on a glass slide and observed under Nikon Eclipse E1000 fluorescence

microscope, equipped with a Retiga Ex*i* camera. The image was processed with Image ProPlus 5.1 software.

2.3.6 Permeabilities

The effective core permeabilities were calculated from the recorded pressure difference by application of the Darcy Law. The brine viscosity was measured at 50°C, with a result of 0.55 cP. It was verified that addition of the filtered molasses and bacteria did not significantly modify the viscosity. The results are presented here in terms of the permeability ratios defined as an average permeability at a given time (*k*) over the initial permeability (*ki*). The plugging curve is the *k/ki* ratio plotted against porous volumes injected (PVI). The permeability reduction that will further be analyzed is defined as (*ki-k)/ki*. Under the conditions of the experiment, the pressure differences around the cores may be used instead of permeabilities in these calculations.

2.4 Results

A total of eight core flooding experiments, four each strains, were conducted (for experimental detail see section 2.3) to study bacterial penetration into chalk rock, the mechanism of the penetration and its effect on permeability. A summary of the bacterial inoculum concentrations used for the experiments, pore volume, the initial permeability of the cores, permeability ratios after bacteria injection (k/ki during the 1st PVI and total k/ki), average bacteria breakthrough concentration (B_{out}/B_{in}) and total number of bacteria retained inside the cores is presented in Table 2.2.

2.4.1 Bacteria cells found in the effluent

To evaluate to what extent bacteria can penetrate chalk rock, the total number of bacterial cells passing through the cores (bacteria breakthrough concentration) was determined. An initial *B. licheniformis* 421 concentration of 3.8×10^7 cfu/ml in the injected incoculum resulted in concentration in the order of 10^1 cfu/ml in the effluent (Fig 2.2a, core 18 and Fig. 2.2b, core 10). These were much lower than the concentrations in the order of 10^4-10^6 cfu/ml obtained in the effluents,

when an inoculum with bacterial cell concentration of 7.0-8.8x0^{7} cfu/ml was injected (Fig. 2.2a, core 1 and Fig. 2.2b, core 2). The relative average amount of bacteria at breakthrough (B_{out}/B_{in}) of *B. licheniformis* 421 increased at a higher value of inoculum (Table 2.2). It was approximately 0.32-1.09 % for inoculum concentration of 7.0-8.8 x 10^{7} cfu/ml and 10^{-4}-10^{-5} % for inoculum concentration of 3.8 x 10^{7} cfu/ml. The total number of retained bacteria cells increased at a higher inoculum concentration (Table 2.2). After 12 days of incubation without extra addition of nutrients, an increase in cell numbers in the effluents compared to before incubation was detected (Fig 2.2b, core 2 and core 10). This increase was particular significant for the experiment where low inoculum concentration was used (Fig. 2.2b, core 10). The pH of the collected effluents slightly decreased for the four replicates during bacteria injection (Fig. 2.2a, core 1 and core 18; Fig. 2.2b, core 2 and core 10). After incubation, the pH of the collected effluent increased from 6.6 to 8.3 (Fig 2.2b, core 2) and from 7.0 to 8.3 (Fig. 2.2b, core 10).

Table 2. 2 Summary of the bacteria inoculum concentrations, the initial conditions and permeability reduction of the cores

Core number	Bacteria	Pore volume (ml)	ki (mD)	Bacteria inoculum (cfu/ml)	1st PVI k/ki	Total k/ki	k/ki after 12 days incubation	Bacteria breakthrough B_{out}/B_{in} (%)	Total bacteria retained inside (cfu/ml)
1	*B. licheniformis* 421	36.98	4.25	7.0×10^{7}	0.44	0.13	-	0.32	1.5×10^{10}
2	*B. licheniformis* 421	36.78	1.82	8.8×10^{7}	0.59	0.21	-	1.09	2.0×10^{10}
10	*B. licheniformis* 421	37.35	4.47	3.8×10^{7}	0.70	0.15	0.51	1.2×10^{-4}	1.0×10^{10}
18	*B .licheniformis* 421	36.02	2.90	3.8×10^{7}	0.64	0.17	0.53	8.5×10^{-5}	9.8×10^{9}
5	*P. putida* K12	38.49	4.56	3.5×10^{6}	0.82	0.41	0.58	2.4×10^{-4}	8.1×10^{8}
7	*P. putida* K12	38.05	3.76	3.7×10^{6}	0.83	0.55	-	3.6×10^{-4}	8.4×10^{8}
9	*P. putida* K12	37.15	4.20	2.1×10^{8}	0.80	0.54	0.42	6.3×10^{-6}	5.6×10^{10}
17	*P. putida* K12	36.70	4.07	4.0×10^{9}	0.73	0.10	-	2.6×10^{-7}	1.0×10^{12}

k: permeability at a given pore volume, ki: initial permeability, B_{out}: bacteria in the effluent, B_{in}: bacteria in the influent (inoculum)

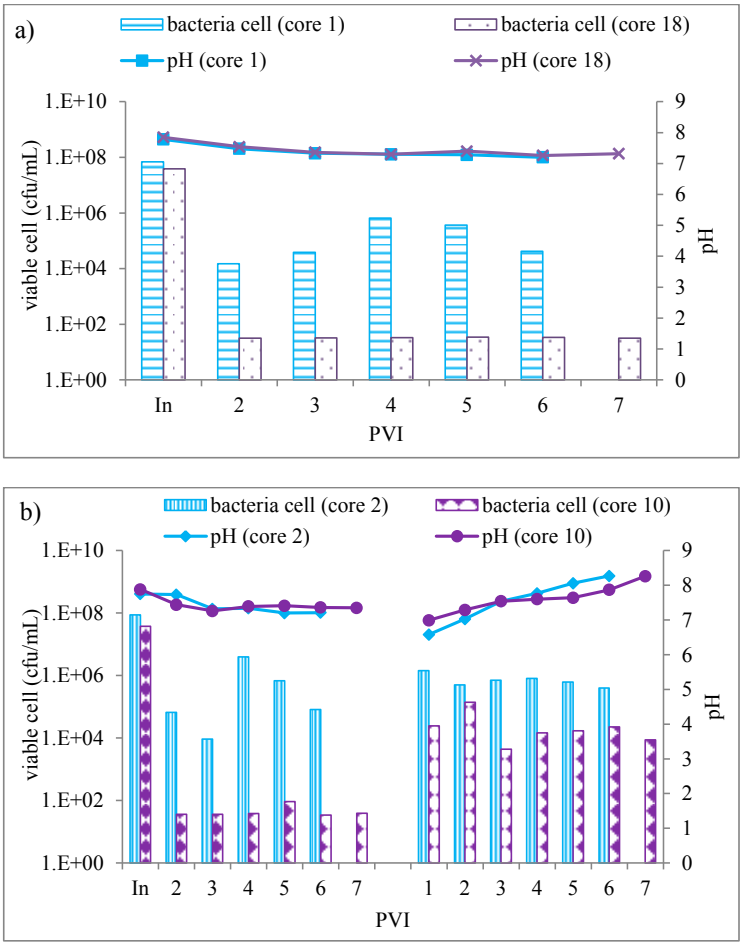

Fig.2.2 Viable *B. licheniformis* 421 cells detected in the effluent (a) during injection of bacteria suspension in core 1 and core 18, (b) during injection of bacteria suspension (left) and after 12 days (right) of incubation of the core 2 and core 10. The injection and incubation were conducted at 50°C. (In : concentration of injected bacteria/inoculum)

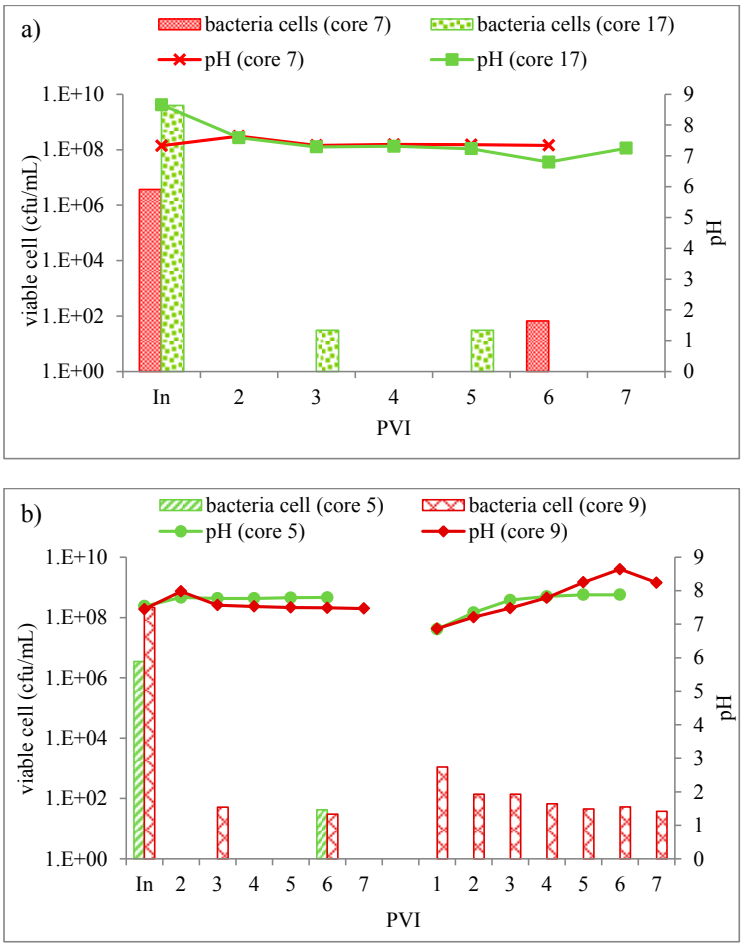

Fig.2.3 Viable *P. putida* K12 cells detected in the effluent (a) during injection of bacteria suspension in core 7 and core 17, (b) during injection of bacteria suspension (left) and after 12 days of incubation (right) of the core 5 and core 9. The injection and incubation were conducted at 50°C. (In : concentration of injected bacteria/inoculum)

Penetration behavior of the *P. putida* K12 was a function of the inoculum concentration with a higher amount of cells detected in the effluent when higher inoculum concentrations were used (Fig 2.3). When the inoculum concentrations were as low as 3.5–3.7 x 10^6 cfu/ml, bacterial cells were only detected in one of the effluent fractions collected (Fig 2.3a, core 7 and Fig. 2.3b, core 5). On the

other hand, when higher inoculum concentrations were used, bacteria were detected in more than one effluent fraction meaning improved penetration of the bacteria (Fig 2.3a, core 17 and Fig. 2.3b, core 9). However, the relative average amount of bacteria at breakthrough (B_{out}/B_{in}) decreased at a higher value of inoculum (Table 2.2). It was approximately 10^{-6} down to 10^{-7} % for inoculum concentration above ~ 10^{6} cfu/ml and 10^{-4} % for inoculum concentration of 10^{6} cfu/ml. For both low and high inoculum concentrations, the number of bacteria in the effluents was around 10^{1} cfu/ml, which is remarkably lower than the corresponding results for *B. licheniformis* 421. The total number of retained bacteria cells increased at a higher inoculum concentration (Table 2.2). After 12 days of incubation without extra addition of nutrients, a significant increase in number of the bacterial cells in the effluent was seen for the experiment with high inoculum concentration (Fig. 2.3b, core 9). However, no bacterial cells were detected in the effluent after 12 days incubation when a low inoculum concentration was used (Fig 2.3b, core 5). In general, pH of the effluents slightly decreased for the four replicates during bacteria injection (Fig. 2.3a, core 7 and core 17; Fig. 2.3b, core 5 and core 9). After incubation, the pH increased from 6.9 to 7.9 (Fig. 2.3b, core 5) and from 6.9 to 8.2 (Fig. 2.3b, core 9).

2.4.2 Bacterial morphology before and after flooding

Photos of the bacterial cells are shown in growth media and in the effluent in Fig. 2.4. It can be seen from the sizes and the rod shaped that the bacteria in injected into the core were in form of vegetative cells for both *B. licheniformis* 421 and *P. putida* K12. The vegetative *B. licheniformis* 421 had an approximate cell size of 0.5 µm in diameter and 2-4 µm in length (Fig. 2.4a). In the collected effluent after injection, no vegetative cells were seen when samples from *B. licheniformis* 421 experiments were investigated in microscope. Instead, small round particles with a size of approximately 0.5 µm were found, which are likely to be spores (Fig. 2.4b). The vegetative *P. putida* K12 in the growth media had an approximate cell size 0.5 µm in diameter and 1-2 µm in length (Fig. 2.4c). In the effluent after injection, exposure to high salinity and high pressure, the *P. putida* K12 became slender (Fig. 2.4d).

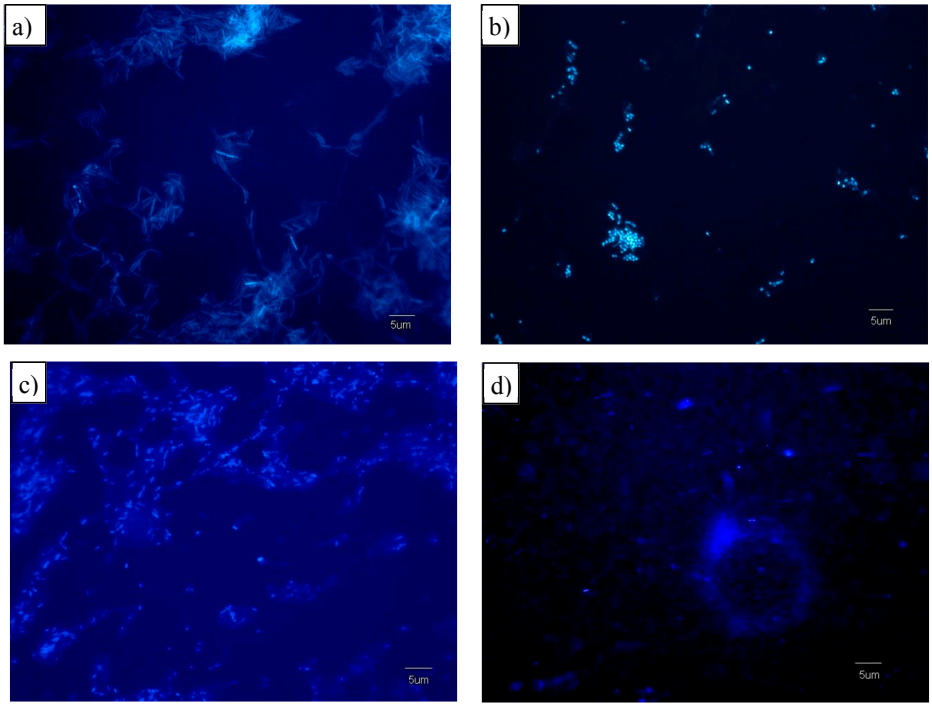

Fig.2.4. *B. licheniformis* 421 cells under DAPI staining (a) in growth media, (b) in the effluent from a chalk core plug. *P. putida* K12 cells under DAPI staining (c) in growth media, (d) in the effluent from a chalk core plug.

2.4.3 The effect of bacteria penetration on core permeabilities

Fig. 2.5a and Fig. 2.5b show the effect of *B. licheniformis* 421 injection on the chalk permeability. The injection rate was kept constant, and injection pressure increased, as plugging occurred. For all four core flooding experiments the core permeability was reduced continuously in the course of injection; and permeability reduction was influenced by the bacterial inoculum concentration (Fig 2.5, Table 2.2). The reduction in permeability occurred much faster at the beginning (the first PVI), as shown by the low *k/ki* value obtained after 1 PVI (Fig. 2.5, Table 2.2). The higher the inoculum concentration, the faster was permeability reduction (Fig. 2.5a, core 1 and Fig. 2.5b, core 2). Experiments with replicate cores having an initial permeability below 4 mD, exhibited similar dependencies that resemble effective permeability profiles appearing under filter cake formation (Fig. 2.5a, core 18 and Fig. 2.5b, core 2). Overall, the ratio *k/ki*

dropped to 0.13–0.20 after 6-7 PVI. Post-flushing with SS after 12 days incubation without extra addition of nutrients increased the permeability compared to before incubation and it stabilized at a level around half the *ki* for cores 2 and 10 (Fig. 2.5b).

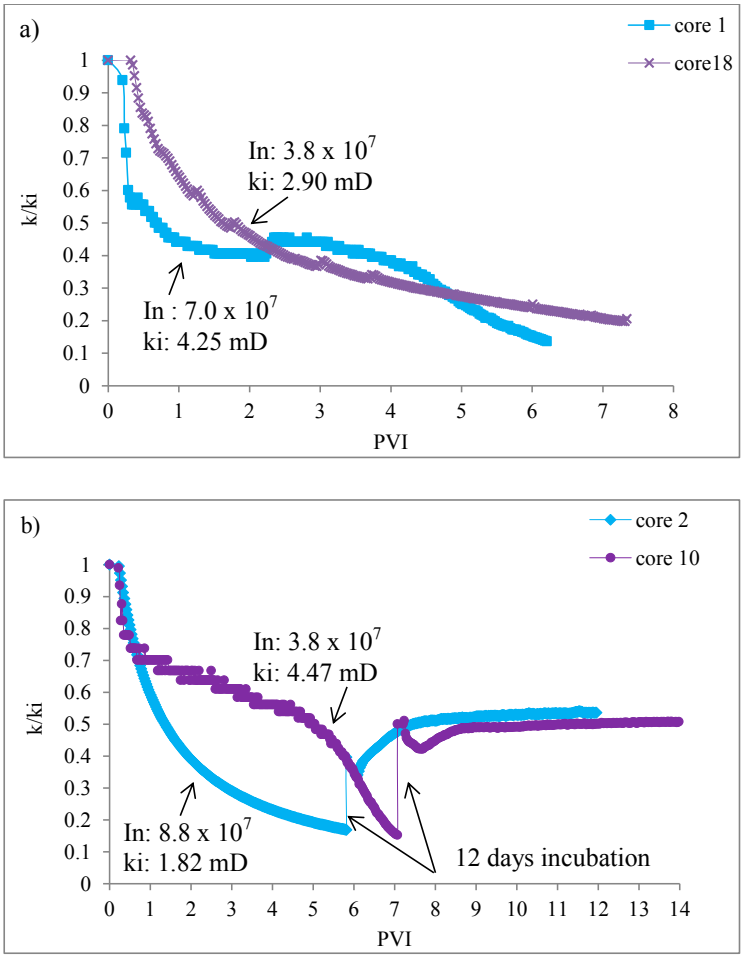

Fig.2.5 Permeability changes by *B. licheniformis* 421 (a) during injection of bacteria suspension in core 1 and core 18, (b) during injection of bacteria suspension and after 12 days of incubation of the core 2 and core 10. The injection and incubation were conducted at 50°C. (In: bacteria inoculum in cfu/ml; ki : initial permeability).

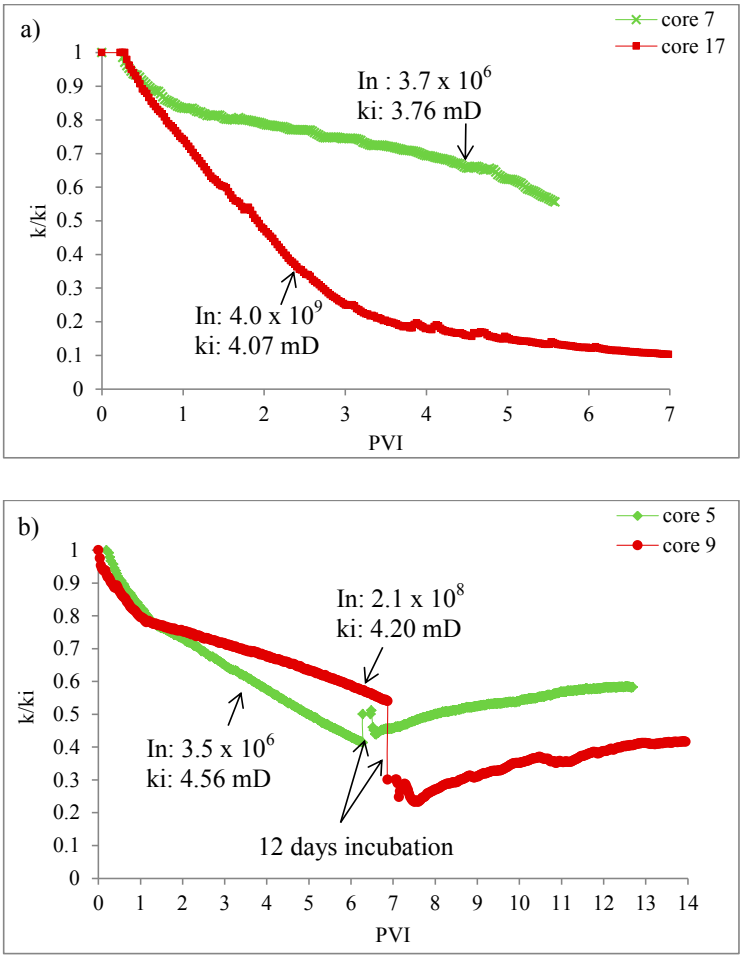

Fig.2.6 Permeability changes by *P.putida* K12 (a) during injection of bacteria suspension in core 7 and core 17, (b) during injection of bacteria suspension and after 12 days of incubation of the core 5 and core 9. The injection and incubation were conducted at 50°C. (In: bacteria inoculum in cfu/ml; ki : initial permeability)

Fig. 2.6a and Fig. 2.6b show the effect of *P. putida* K12 injection on the chalk permeability. For these four core flooding experiments the core permeability was also reduced continuously during injection. In contrast to permeability damage by *B. licheniformis* 421, the permeability reduction occurred at a much slower rate and plugging curve had a different shape. However, also for *P. putida* K12 injections a rapid permeability reduction happened during the first PVI, but for the

high bacterial inoculum injection, where the rapid reduction continued until 3 PVI (Fig 2.6a, core 17). The total permeability reduction was higher at high inoculum concentrations (Table 2.2, Fig. 2.6), where permeability reductions curve resembled that characteristic of the filter cake formation (Fig 2.6a, core 17). The ratios *k/ki* after 6-7 PVI for the low and medium bacteria inoculum concentrations were 0.41 to 0.55, resulting in lower reduction in permeability than for *B. licheniformis* 421 injection. Nevertheless, for the high bacteria inoculum concentration after 6-7 PVI the ratio *k/ki* decreased down to 0.10. Similar to *B. licheniformis* 421 incubation experiments, during further SS flooding after incubation of 12 days the permeability stabilized at a constant level (0.58 of the *ki* for the low bacteria concentration and 0.41 of the *ki* for the high bacteria concentration).

2.5 Discussion

One of the major concerns for MEOR application in chalk formations is whether the bacteria can penetrate deeply enough into formations as the pore throat sizes are small and, hence, similar to the sizes of bacterial vegetative cells. In our study we focused on the penetration of freshly grown cells of *B. licheniformis* 421 and *P. putida* K12 into chalk. Both strains are common rod shaped bacteria that have been applied for microbial enhanced oil recovery (MEOR). *B. licheniformis* 421 is a "typical" spore forming bacteria, while *P. putida* K12 is a usual non-spore forming bacteria. We were aiming at evaluating how far these two bacteria could penetrate into the chalk rock, and the subsequent effect on permeability reduction. The molasses and nitrate used in the experiment were mainly to mimic the injection liquid used for MEOR. The time required for injection process in each core flooding experiment was approximately 3.5- 4 hours. Therefore the bacteria growth during injection was negligible.

Our results showed that both bacteria types were able to penetrate through the cores, even when the core permeability was below 4 mD and the mean values of the pore throat sizes were approximately 0.5 μm [17], which is comparable to the sizes of the specific bacterial strains used in this study. This means that the 1/3 –

1/7 rule commonly used for straining [15] is not applicable for bacteria penetration, since bacteria are not rigid as particles. Our results are in agreement with an earlier report [12] that although the permeability was below 1 mD the injected bacteria were still able to penetrate the cores. Our results demonstrated that *B. licheniformis* 421 penetrated in the form of spores, which are much smaller than the vegetative cells. Stressful conditions such as high salt concentrations and high pressure might have induced *B. licheniformis* 421 to form spores that then enabled a deeper penetration than possible with vegetative cells. *P. putida* K12 was able to penetrate in form of vegetative cells, which were detected in the effluent and the cells were thus small enough to pass through some pore throats. It is known that bacteria can change shapes due to environmental stresses, such as the force to flow through geological strata, entrapment in geological strata, nutrient limitation, changes in osmotic pressure and predatory by other microorganisms [11]. In our experiment, *P. putida* K12 growing in the saline SS medium most likely have shrunk due to changes in osmotic pressure. However, the injected concentration of *P. putida* K12 needed to be above a certain threshold value before the cells could be detected in the effluent.

The number of the viable cells found in the effluent (bacteria breakthrough concentration) was significantly higher for *B. licheniformis* 421 as compared with *P. putida* K12. In the case of *P. putida* K12, it can be seen clearly that the number of cells being able to penetrate the core depended on the bacteria inoculum concentration. Although more cells were detected when high inoculum concentration was injected, the bacteria at breakthrough concentration (B_{out}/B_{in}, Table 2) decrease with increasing inoculum. This could happen because higher values of inoculum block the pore more rapidly. For *B. licheniformis* 421, there seems to be "a penetration threshold" between the inoculum concentration of $3.8x10^7$ cfu/ml and higher inoculum concentrations ($7.7\text{-}8.0 \times 10^7$ cfu/ml). This is still to be explained in further study.

Davis and Updegraff [14] stated that the pore throat sizes of the rock should be at least twice the cell sizes to allow passage of microbes through the rock without

substantial plugging. In most of our experiments, plugging occurred relatively rapidly at the beginning of injection, which indicates that a large fraction of the bacteria did not penetrate deeply into the cores but were caught at the beginning. The plugging effect (permeability reduction) was more pronounced under injection of *B. licheniformis* 421, as the sizes of *B. licheniformis* 421 are larger than the sizes of *P. putida* K12. We also sliced the cores and made microscopic pictures of the slices (not shown here). The results indicated that there were some bacteria stuck in the core. However, these pictures did not allow for more certain conclusions. In summary, although the cells of *B. licheniformis* 421 are considerably larger than *P. putida* K12, and they plug the porous media stronger, considerably larger amounts of *B. licheniformis* 421 penetrate deeper than of *P . putida* K12. This may only be attributed to the spore formation.

During the first PVI, similar trends with a rapid decrease of permeability were seen for all bacterial concentrations. However, after 6-7 PVI the permeabilities became different. For low injected concentrations the curve flattened out, whereas there was a continuous decrease in *k/ki* when a high bacterial concentration was injected. The measured values of *k/ki* in the core flooding experiments of *B.licheniformis* 421 clearly showed that the initial permeability of the cores influenced the plugging curves, as only the permeabilities below 4 mD exhibited curves that resemble the filter cake formation [11]. An unclear point in the experiments with *B.licheniformis* 421 was, why the inoculum concentration of 7.0 $x10^7$ cfu/ml (Fig 2.5a, core 1) gave lower ratios of *k/ki* as compared to the inoculum concentration of 8.8 $x10^7$ cfu/ml (Fig 2.5b, core 2). In the experiments with *P. putida* K12, we also observed that injection of $2.1x10^8$ cfu/ml (Fig. 2.6b, core 9) gave lower ratios of *k/ki* as compared to the inoculum concentration of $3.5x10^6$ cfu/ml (Fig. 2.6, core 7 and core 5). For both cases, it can only be speculated that this observation may be attributed to specific properties of the core porous spaces. It is important to mention that complete (100 %) permeability reduction was never reached, which is in coherence with the previous studies of bacterial plugging of the porous rocks [5, 6, 18].

Since the pore sizes in the cores are comparable to the bacterial sizes, formations of large bacterial colonies in the porous space are unlikely. However, small aggregates still can be formed inside relatively large cavities. In experiments on Berea sandstone, it was observed that *P. putida* when injected at a higher concentration of $5x10^7$cells/ml formed a filter cake outside the inlet surface, whereas for injection at lower concentration of 10^6 cells/ml no filter cake was found [5]. This happened because *P. putida* at high concentrations tends to form aggregates, thus easily plugging the inlet surface and preventing further bacterial penetration. In accordance with previous findings [5], the present study also demonstrates the difference in the permeability behavior under injection of low or high concentrations of bacteria.

Further SS flooding after 12 days of incubation did not allow permeability to return to the initial value for any of the bacterial strains. This means that bacteria remained trapped inside the core and/or bacterial metabolites might precipitate and cause plugging in some pore channels. In the case of *P. putida* K12 injection, permeability restoration after incubation was lower as the bacterial inoculum concentration increased (Fig. 2.6b, core 5 and core 9). This might be due to the fact that *P. putida* at high concentrations may form stable aggregates [5].

The initial decrease of pH during bacterial injection might indicate production of organic acids and/or CO_2 by the bacteria. Further increase of pH after injection was most probably due to release of Ca^{2+} ions from the rock as indicated by previous publication [19]. The authors observed a linear correlation between pH and the release of the Ca^{2+} ions. The increase in pH of the liquid with time is indicative of carbonate mineral dissolution that leads to formation of water soluble bicarbonates. As the concentration of HCO_3^{2-} increases, the liquid become less acidic and pH increases [19].

2.6 Conclusion

In this work we have shown that bacteria were able to penetrate and to be transported through the chalk porous rocks even though the permeability is as low

as below 4 mD and the pore sizes are comparable to bacterial sizes. The spore-forming *B. licheniformis* 421 predominantly penetrates in form of spores while the *P. putida* K12 penetrate as vegetative cells, however, smaller in size than during normal growth conditions due to the high salinity conditions. The number of cells being able to penetrate the core depended on the bacteria inoculum concentration. The higher the injected inoculum, the higher the number of cells was found in the effluent. Retention of bacteria and, probably, of produced metabolites in the cores caused irreversible permeability damage, which could not be repaired by further brine flooding after a 12-day waiting (no addition of nutrients). We were not able to take further conclusion on the distribution of the bacteria inside the cores by analyzing the cores slices, although the analysis gave clear indications that bacteria were retained.

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Paper 2

Halim, A.Y, S. M. Nielsen, A. E. Lantz, V. S. Suicmez, N. Lindeloff and A. Shapiro. Investigation of Spore Forming Bacteria Flooding for Enhanced Oil Recovery in North Sea Chalk Reservoir. (Accepted version in Journal of Petroleum Science and Engineering)

CHAPTER 3

Investigation of Spore Forming Bacteria Flooding for Enhanced Oil Recovery in North Sea Chalk Reservoir

Abstract

Little has been done to study microbial enhanced oil recovery (MEOR) in chalk reservoirs. The present study focused on core flooding experiments to see microbial plugging and its effect on oil recovery. A pressure tapped core holder was used for this purpose. *Bacillus licheniformis* 421 was used as it was shown to be a good candidate in a previous study. Bacterial spore can penetrate deeper into the chalk rock, squeezing through the pore throats. Our results showed that injection of *B. licheniformis* 421 as a tertiary oil recovery method, in the residual oil saturation state, was able to produce additionally 1.0 to 2.3 % original oil in place (OOIP) in homogeneous cores and 6.9 to 8.8 % OOIP in heterogeneous cores. In addition, the pressure gradient was much higher in the heterogeneous cores and thus confirms that bacterial selective plugging plays an important role in higher oil production from the heterogeneous chalk cores. In all cases, an incubation period ('shut-in') after the bacterial and/or nutrient injection was needed to give sufficient time for the bacteria to grow inside the core and to produce more oil. Our findings show potential application of bacteria as a plugging agent in heterogeneous chalk cores to improve oil production.

Key words: microbial enhanced oil recovery, microbial selective plugging, spore-forming bacteria, low permeable chalk reservoir

Abbreviation

MEOR, Microbial Enhanced Oil Recovery; OOIP, original oil in place; cfu, colony forming unit; SS, synthetic seawater; $S_{or,1st\ SS}$, residual oil saturation after 1^{st} synthetic seawater flooding.

Unit conversion to SI

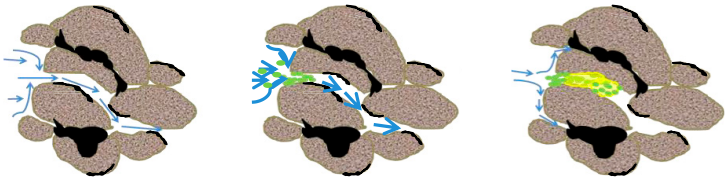
1°C = 273.15 K

1 psi = 6.894,76 Pa
1 bar = 10^5 Pa
1 D = 10^{-12} m^2
1 ml = 10^{-6} m^3

3.1 Introduction

To date, conventional oil recovery technologies used in the oil industry can only recover about one third to one half of the original-oil-in-place (OOIP), leaving behind a large amount of residual oil being targeted for enhanced oil recovery (EOR) [1-4]. Microbial Enhanced Oil Recovery (MEOR) is believed to be one of the advanced technologies enabling recovery of the residual oil due to the ability of bacteria to produce biosurfactants, biopolymers, bioacids, biomass, biosolvents, gases, and enzymes. This technology is implemented by addition of nutrients and/or bacteria to the injected fluid [2-5].

A major challenge of EOR technologies in North Sea chalk reservoirs is finding ways to mobilize the residual oil in low permeability reservoirs. During secondary oil recovery, when water is injected into a reservoir, on the microscopic level, it preferentially flows into high permeable channels, thus bypassing the low permeable, oil-filled pores parts of the reservoirs [6, 7]. Injection of nutrients/and or bacteria into these preferential pathways and stimulation of the bacterial growth in situ will block the water thief channels, either with bacterial cells or polymers produced in situ by the growing bacteria. Blocking of these channels will alter the water pathways to the previously unswept pore space [6, 8-11]. This concept is known as microbial selective plugging as illustrated in Fig. 3.1. Selective plugging of the more permeable zones may result in increasing oil production, correcting microscopic and volumetric sweep efficiency and redirecting water flow to the low permeable, oil-bearing zones [1]. Even though studies of microbial plugging have been conducted by many researchers, to the best of our knowledge none has been performed on chalk. Earlier studies have been conducted in packed glass beads [12], sandstones [1, 7, 8, 11, 13, 14], sand packs [1, 15] outcrop limestones [14, 16, 17] and outcrop carbonates [18, 19].



Water flows through large pore channel, bypassing low permeable channels. Poor sweep efficiency

Microbes flow with the water phase, adhere, grow, and plug the large channel

Blocking of large pore channel deviates the water flow to the previously unswept (poorly swept) low permeable channels. Improved sweep efficiency

Fig.3. 1 Illustration of selective plugging mechanism.

The phenomenon of microbial plugging has been investigated in a number of previous works where bacteria were injected into the different porous media in the water phase, without oil present. Flooding was carried out by injecting microbial culture and/or nutrient into various porous media and observing (or not observing) microbial plugging and fluid flow diversion. Shaw et al. [12] used a sintered glass bead core to simulate the spaces and surfaces of reservoir rock and *Pseudomonas* sp. to study the bacterial plugging phenomenon. The authors observed that *Pseudomonas* sp. blocked the inlet face of a core and decreased its permeability. A dense 'bacterial mat' at the inlet face was observed using scanning electron microscopy (SEM). Raiders et al. [13], investigated microbial plugging using Berea sandstone cores with a pressure tapped core holder along the length of the core. Indigenous microbes from the cores and *Bacillus* strain 47 were used in this study. Continuous injection of sucrose mineral salt medium resulted in a large permeability reduction (70-98%) by indigenous microbes from the core. Injection of *Bacillus* strain 47 prior to nutrient injection resulted in a more rapid plugging. Analysis of the pressure gradient along the core showed that plugging was localized at the inlet and outlet faces of the cores. The authors further tested the selectivity of the microbial plugging process by using a dual core system to create a contrast permeability layer. Two cores with different permeabilities (240 and 760 mD) were connected using a crossflow. After the system was injected with *Bacillus* strain 47 and nutrient, the flow pattern changed and about 90% of the

total injected fluid was diverted to the low permeability core. Raleigh and Flock [14], conducted core flooding using four distinct core types: Berea sandstone (homogeneous type), Cardium sandstone (less uniform sand grains compared to Berea, but a clean, well-sorted rock type), Devonian rock (a completely heterogeneous type), Indian limestone (outcrop rock, composed of oolitic porosity with secondary, small, vuggy and pinpoint porosity). *Bacillus subtilis* was used and it was observed that the majority of plugging observed in clean, homogeneous, uniformly grained, consolidated sandstone was located near the inlet face of a core. The heterogeneous carbonate behaves irregularly and plugging was observed along the entire length of the core. The authors concluded that depth of plugging within porous rock is a function of pore geometry.

The effect of microbial plugging on oil recovery was studied by injecting microbial culture into crude oil saturated core plugs. Crescente et al. [8], conducted a series of core flooding in Berea sandstone using two types of *Rhodococcus* sp. 094. The first type was a surfactant-producing bacterium, while the second type was a non-surfactant-producing bacterium. The authors used dodecane as hydrocarbon, instead of crude oil, to saturate the cores. Different injection scenarios were studied. The bacteria were injected directly before and after water flooding. The injection of bacteria after water flooding resulted in an additional recovery of 3.1-3.4% OOIP for surfactant-producing and 5.1-9.7% OOIP for non-surfactant-producing bacteria. However, when the surfactant-producing bacteria were injected directly before water flooding, the process resulted in a higher oil recovery (67-84% of OOIP) as compared with the non-surfactant-producing bacterial injection (60-80% of OOIP). The authors concluded that when the cores were flooded with the non-surfactant-producing bacteria, the recovery was faster initially, but the recovery curves of the non-surfactant-producing bacteria and surfactant-producing bacteria would intersect and in the long run the surfactant-producing bacteria would achieve a higher recovery. A possible reason for this behavior could be that the biosurfactant produced by bacteria plays a role in addition to the selective plugging process [8]. Suthar et al. [15] investigated the selective plugging strategy based MEOR using

sandpack column and *B. licheniformis* TT33, a facultative anaerobic, halotolerant, thermotolerant, and biofilm forming microorganism. Injection of a 0.6 PVI (pore volumes injected) bacterial culture at residual oil saturation, followed by a 20-day incubation period at 50°C, resulted in an additional oil recovery of 13.0-17.8% OOIP. The bacteria produced biopolymer that increased the thickness of the biofilm. The biofilm diverted the flow to low permeability zones (a previously unflooded area) and released oil from this area. The presence of biofilm in the sandpack was confirmed by the Environmental Scanning Electron Microscope (ESEM). Al-Hattali et al. [16], conducted core flooding experiments with Indiana limestone cores (permeability 200 mD and porosity 13%). The cores were sliced along the core using a thin blade to introduce fractures in the cores. *Bacillus licheniformis* W16, isolated from the Omani oil field, was used in this study. The bacteria were injected in residual oil saturation condition and the cores were incubated with the bacterial culture for 18-24 hours. Two different carbohydrate sources, sucrose and molasses, were tested to enhance the bacterial growth. The results showed a quite significant additional oil recovery, of 16.5-21.3% OOIP and 6.0-14.0% OOIP for sucrose and molasses, respectively. The authors added that lower oil recovery in the experiment with molasses could be due to slower bacterial growth in molasses as compared to sucrose. The presence of bacterial cells at the inlet, middle, and outlet of the cores was visualized by SEM at the end of the experiment. A recent publication by Kaster et al. [17], reported core flooding experiments on bacterial plugging in low permeability Liege limestone cores (0.28 – 0.6 mD) and its effect on oil recovery. The enrichment bacteria consortium from the Draugen field, the North Sea, was used in the experiments. The bacteria were found to be able to penetrate, to be transported, and to grow at different core sections, without causing any permeability reduction or plugging. The core flooding experiments showed an additional oil recovery of 1.6-3.9% OOIP when the Draugen field microbial culture and nutrient were injected, followed by 'shut-in' periods for incubation. Salehizadeh and Mohammadizad [19] conducted core flooding experiments with low permeable carbonate cores and *Alcaligenes faecalis*. The bacteria were able to produce additionally 9.2%-10.7% OOIP.

Field application of microbial selective plugging also showed promising results both in sandstone [6] and carbonaceous fields [20]. Injection of microbe strain CJF-02, a biopolymer producer microbe, significantly decreased the water cut and increased cumulative oil production during six months after microbial treatment in the Fuyu Oilfield, China [6]. A microbial profile modification process using sequential injection of *Bacillus licheniformis* spores and nutrient was able to plug the thief zone in a low permeable carbonate reservoir in Lea County, Mexico [20].

This study was conducted in order to investigate the mechanism of microbial selective plugging by means of core flooding experiments in low permeability chalk rock cores from the reservoirs of the Danish part of the North Sea. The chalk formations are high porous, but low permeable. For our study it is important that the pore sizes in such formations vary in wide ranges, and the sizes especially of the pore throats are comparable to the sizes of bacteria. The investigation was conducted using the model study organism *B. licheniformis* 421 that can penetrate deeply into chalk rock [21]. The microbial plugging mechanism was investigated by comparison of the additional oil recovery produced after bacterial injection into homogeneous and heterogeneous chalk cores.

3.2 Materials

3.2.1 Crude oil, reservoir rock, and bacteria samples

Crude oil and reservoir rock samples from the Danish North Sea reservoir were used. Maersk Oil provided the core material used for the experiment. A total of five reservoir cores was used, three were homogeneous (Ho 1, Ho 2, and Ho 3), and the other two were heterogeneous with micro fractures (He 1 and He 2, respectively). The porosity of the core samples was varied from 25.3% to 42.9% and the permeability from 0.5 mD to 6.4 mD. The homogeneous and heterogeneous cores were taken from different fields and hence from different formations.

The bacterium used in this study was *Bacillus licheniformis* 421, obtained by courtesy of Professor T. Nazina (the Winogradsky Institute of Microbiology, the Russian Academy of Sciences). This is a spore forming, facultative anaerobic bacterium, isolated from a high temperature oil field in China. It has previously been reported as having a potential to penetrate deeply into a chalk formation [21].

3.2.2 Chemical and growth media

Synthetic seawater (SS) was used as injection liquid throughout the experiment (the composition is given in Table 3.1). The bacterial inoculum was prepared in the SS nutrient medium (SSN), a medium which has a basic composition of SS, supplemented with nutrients (see Table 3.1). The SS and SSN were sterilized by filtering through 0.2 µm mixed ester cellulose membrane filters (Advantec®). Enrichment medium (EM) was used for quantification of viable bacterial cells. The medium consists of bacto™ tryptone (0.5 g/l), bacto™ yeast extract (2.5 g/l), glucose (1 g/l), NaCl (2 g/l), and bacto™ agar (15 g/l).

3.2.3 Core flooding

Fig. 3.2 illustrates the core flooding setup. Injection pressure, pressure along the flooded core plug, differential pressure (dPi) and sleeve pressure were monitored throughout the experiment by means of the Agilent data logger connected to a computer. All the pressure transducers (Vegabar 17, Germany) and the differential pressure (dPi) transducer (Valydine, USA) were calibrated using a dead weight tester (Desgranges et Huot, France). Microbial plugging can be observed by increase of the pressure at the different sections along the core length as illustrated in Fig. 3.3 A duplicate setup was used in order to run experiments in parallel, but only one setup contained a pressure tapped core holder, needed to measure the intermediate pressure values. The pressure tapped core holder (DCHH-1.5) was manufactured by Core Laboratories L.P., USA., while the Hassler type core holder used in the second setup and injection cylinders were made in-house. All experiments were carried out in the pressure tapped core holder, except for the core Ho 3 flooding, which was performed with the ordinary Hassler-type core

holder. A biological fraction collector (BioRad, USA) was used to collect the effluent of the experiments. The fraction collector was placed inside a fridge to prevent bacterial growing and contamination from the air. A camera was placed at the transparent outlet tube from the core holder. The camera was controlled by YamCam software to take pictures every 30 seconds, in order to visualize the oil production throughout the experiment.

Table 3.1. Synthetic seawater (SS) composition

Chemical	Amount (g/l)		
	SS	SSN (for growth)	SSN (for injection)
NaCl	18.00	18.00	18.00
$NaHCO_3$	0.17	0.17	0.17
KCl	0.74	0.74	0.74
$MgCl_2.6H_2O$	9.15	9.15	9.15
$CaCl_2.2H_2O$	1.91	1.91	1.91
Na_2SO_4	3.41	3.41	3.41
$NaNO_3$		2.0	2.0
KH_2PO_4		0.1	0.1
Molasses (59% sucrose)		16.9	16.9
Vitamin [22]		1% (v/v)	1% (v/v)
Trace elements [23]		1% (v/v)	1% (v/v)
MOPS (buffer)		20	

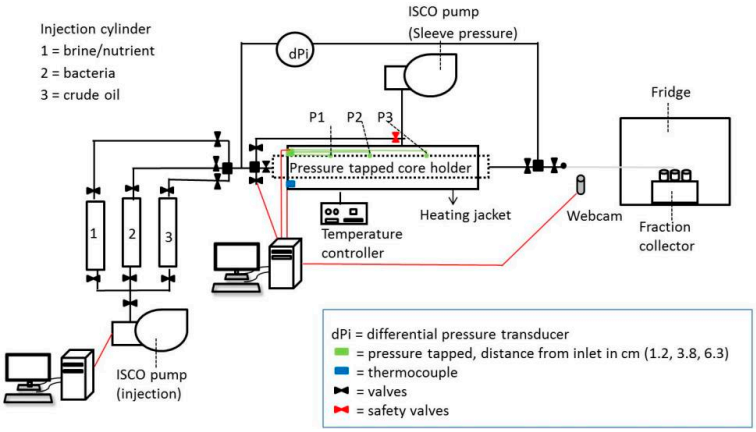


Fig.3.2 Core flooding experimental setup.

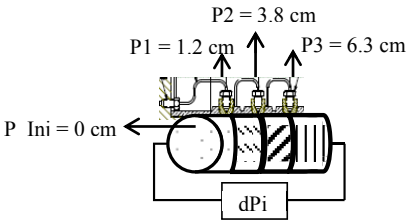


Fig.3.3 Illustration of different sections of the core plug based on the pressure transducer position along the core.

3.3 Methods

The experimental sequence is presented in Fig. 3.4 and detailed explanation of the procedure is given in the section below.

3.1 Bacterial inoculum preparation and enumeration

The bacterial inoculum was grown in the SSN medium for 18 hours (corresponding to a late logarithmic phase). The cells were then harvested by centrifugation (10.000xg, 10 min, 4°C) and washed by re-suspension of the pellet in 0.85% NaCl followed by another centrifugation. This process was repeated three times and the bacteria were then re-suspended into SSN medium without 3-(N-morpholino) propansulfonic acid (MOPS). The volume was adjusted to

provide an optical density of the bacterial SSN solution of OD_{600}=0.5. This solution was then homogenized by a vortex for three minutes before being used in the core flooding experiments. Bacterial enumeration was conducted by the serial dilution plate method using EM.

3.2 Cell-to-spore ratio measurement

Cell-to-spore ratios were verified to confirm that bacterial suspensions used for injection mainly consisted of growing cells. Approximately, 1 ml of a bacterial suspension was taken and diluted 10-fold with the 0.85% NaCl solution, washed three times using the same solution and harvested by centrifugation at 10.000xg for 10 minutes. The resulting pellet after the third washing was re-suspended in 5ml 0.85% NaCl solution and fixed with 2% (v/v) formaldehyde for 30 minutes. The cells (and potential spores) were then washed and 0.5% (v/v) 4',6-diamidino-2-phenylindole (DAPI) staining was added. After the DAPI addition, the liquid was incubated for 20 minutes in darkness and filtered through a black polycarbonate membrane filter. Subsequently, the filter was fixed on a glass slide and observed under a Nikon Eclipse E1000 fluorescence microscope, equipped with a Retiga Ex*i* camera. Approximately, 20-30 random spots of the filter were observed. The image was processed with Image ProPlus 5.1 software.

3.3 Sterilization process

The Hassler type core holder, the pressure tapped core holder, the injection cylinders, the injection lines and the effluent glass collectors were sterilized in the autoclave at 121°C, 15 psi, for 20 minutes. The desiccator used for vacuum saturation was sterilized by soaking in disinfectant (Pefectant^{TB}) overnight.

3.4 Core cleaning and basic properties measurement

Prior to flooding experiments, all the cores were scanned using a fourth generation Siemens SOMATOM scanner. The X-ray computer tomography (CT) scanning was conducted in lateral and longitudinal directions to check the homogeneity of the cores. Each individual core was then cleaned by cyclic flooding of toluene and methanol inside the Hassler type core holder to remove any organic material

inside the core. After cleaning, the core was dried in the oven at 100°C overnight. The core porosity and permeability were measured by a steady state gas permeameter (Poroperm, Vinci Technologies) before and after the experiment. An overburden pressure of 350 psi was applied during the measurement. The software has a capability to calculate the Klinkenberg correction, in order to compute the liquid permeability based on the gas permeability data.

3.5 Core saturation preparations

Each core was saturated stepwise with SS under vacuum for 24 hours, followed by further saturation for 48 hours with vacuumed SS at high pressure (100 bar) inside a stainless steel cylinder. The saturated core was inserted in a core holder. The overburden pressure was fixed at 50 bar using a 260D ISCO pump, and the temperature was fixed to 50°C by a heating jacket.

3.6 Core flooding experiments

A 260D ISCO pump was used to keep control of a constant injection rate of 0.1 ml/min throughout the experiment. After the temperature inside the core holder was stabilized, the core was subjected to 3-5 PVI of sterile SS. This was followed by injection of crude oil until no more water was produced, in order to create irreducible water saturation (S_{wi}). The oil saturation under this condition was considered to be original oil in place (OOIP). The core was then flooded with SS (1^{st} SS flooding) until irreducible oil saturation (S_{or}), where no more oil was produced. Then 1 PV of bacteria was injected, and the core was incubated with bacteria for three days by closing both inlet and outlet valves. The core was again flooded with SS (2^{nd} SS flooding) until no more oil was produced. In the experiments with heterogeneous cores (He 1 and He 2), after no more oil was produced in the 2^{nd} SS flooding, 1 PV of nutrient was injected, followed by three days of incubation. The core was then flooded again with SS (3^{rd} SS flooding) until no more oil was produced.

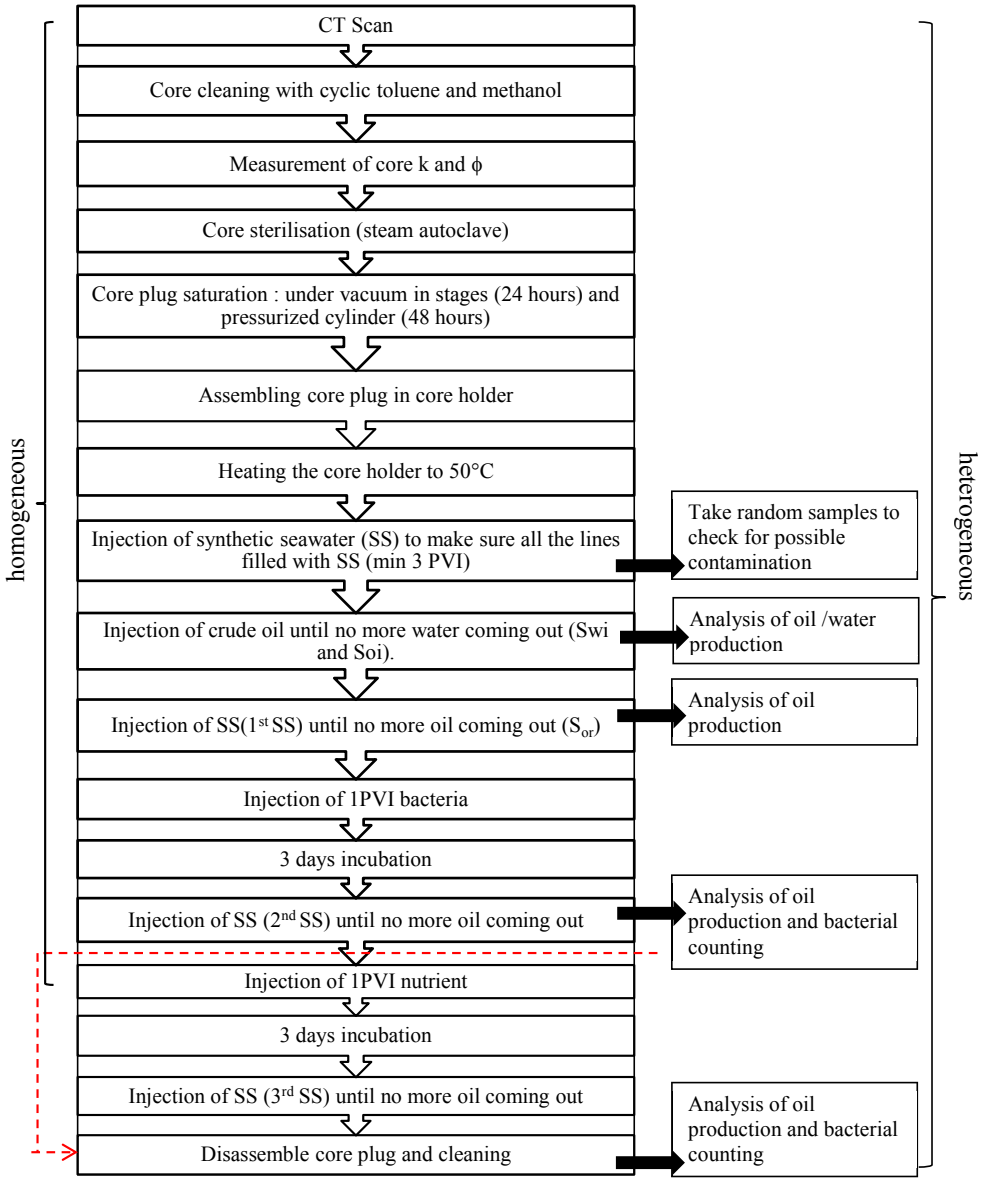


Fig.3.4 Schematic drawing of experimental sequences. In the experiment with homogenous cores, the nutrient injection was not performed, the setup was dissembled and cores were cleaned after 2nd SS flooding.

3.7 Effluent analysis

The collected effluents were analyzed for oil production, bacterial enumeration and pH. The viable bacterial cells were enumerated by the plate count method. The amounts of oil produced were found visually, by comparison of the vials containing oil and water with a standard set of samples prepared beforehand with known amounts of liquids. When an oil amount was below 1 ml, it was measured using the UV-vis method [24]. It has been verified that such measurements provide stable and reliable results [25].

3.4 Results

A total of five core flooding experiments, three with homogeneous cores (Ho 1, Ho 2 and Ho 3) and two with heterogeneous cores (He 1 and He 2), was conducted to study the microbial selective plugging mechanism and its effect on oil recovery. A summary of the core properties before and after the experiment is presented in Table 3.2. Furthermore, a summary of the oil production history with different flooding schemes is presented in Table 3.3.

3.4.1 Core properties

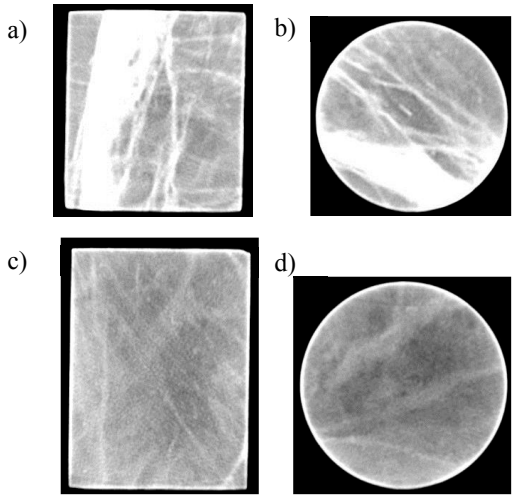


Fig.3.5 CT scan pictures of heterogeneous cores He 1 at lateral section (a) and longitudinal section (b); He 2 at lateral section (c) and longitudinal section (d).

Core permeability and porosity were measured under dry conditions by the steady state gas permeameter. There was no significant change in the core properties of both porosity and permeability of the homogeneous cores before and after the experiment (Table 3.2). The results of CT scanning show that cores Ho 1, Ho 2, and Ho 3 are relatively homogeneous, while cores He 1 and He 2 are heterogeneous. Core He 1 has a significant dense area in the direction of the flow. This area is seen in the CT scan by the white lines along the lateral section of the core (Fig. 3.5a) and also on its cross-section (Fig. 3.5b). Core He 2 has a more evenly distributed high density area compared to core He 1 (Fig. 3.5c and 3.5d). However, the direction of the high density area is the same as that of core He 1. No open or partially open fractures were detected in the cores and it was not possible to see the micro fractures clearly because the practical limit of the CT scan resolution is approximately 1 mm. The variation of the CT number was much higher in the heterogeneous cores compared to the homogeneous cores, and the pictures indicate a specific structure of porosity distribution inside the cores. This might indicate that the fractures are also oriented and aligned along the directions of the core heterogeneity. The permeability and the porosity of the heterogeneous samples were not measured after the experiment, because their variations could not be interpreted in terms of bacterial activity. These samples are rather fragile and are affected by experimental stresses.

3.4.2 Oil production history

The initial water saturation (S_{wi}) was approximately 0.25 and 0.31 in the homogeneous, and the heterogeneous cores, respectively. The cores were flooded with SS (1st SS flooding) until no more oil was produced to establish the residual oil saturation (S_{or}). The 1st SS flooding of homogeneous cores Ho 1, Ho 2, Ho 3 recovered 56 to 62 % OOIP, while from heterogeneous cores He 1 and He 2 it recovered 65 to 70 % OOIP. Although lower waterflood recoveries may initially be expected in a heterogeneous core plug due to relatively earlier water breakthrough, our observation is not unexpected for a chalk system. Due to the water wet characteristics of the chalk, more efficient spontaneous imbibition via the micro fractures into the chalk matrix which often boost the recovery from such

resources may be observed. By application of MEOR as a tertiary oil recovery method, bacterial injection after the 1st SS flooding was able to produce additionally 1.0 to 2.3 % OOIP in homogeneous cores and 6.9 to 8.8 % OOIP in heterogeneous cores.

Table 3.2. Summary of core plug properties

Core number	Texture	Diameter (cm)	Length (cm)	Pore volume (ml)	Ki (mD)	Ka	ϕi	ϕa
Ho 1	Homogenous	3.8	7.5	27.4	3.2	3.1	31.1	30.7
Ho 2	Homogenous	3.8	7.5	37.1	6.4	6.4	42.9	43.1
Ho 3	Homogenous	3.7	5.2	13.9	0.5	0.5	25.3	25.2
He 1	Heterogeneous, micro fractures	3.8	4.1	17.4	3.4	n/a	37.3	n/a
He 2	Heterogeneous, micro fractures	3.8	4.9	19.4	2.9	n/a	35.2	n/a

ki = permeability before experiment, ka = permeability after experiment, ϕi = porosity before experiment, ϕa = porosity after experiment

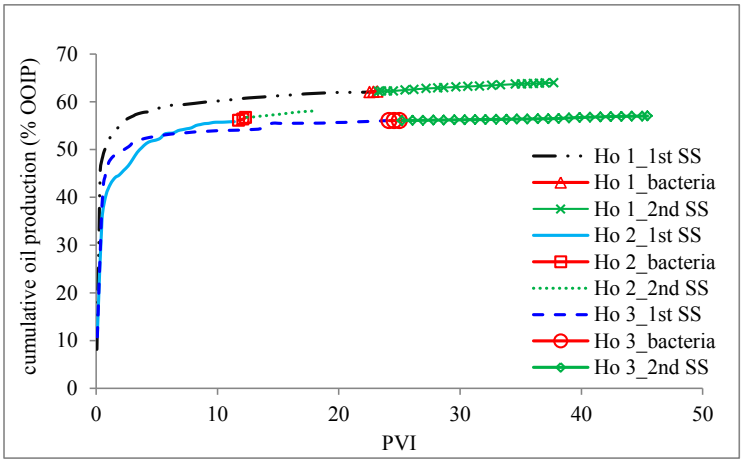


Fig.3.6 Cumulative oil production vs. pore volumes injection for core Ho 1, Ho 2 and Ho 3.

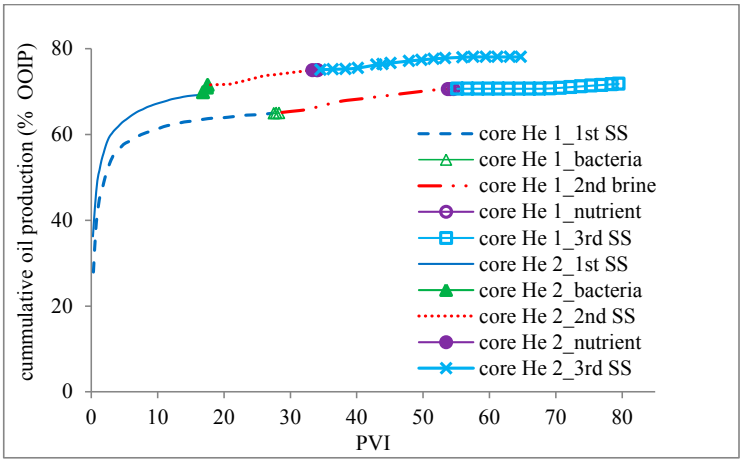


Fig.3.7 Cumulative oil production vs. pore volumes injection for core He 1 and He 2.

In the experiment with core Ho 1, the oil production at the 1st SS flooding stopped after 11.5 PVI. Afterwards, 1 PV of bacteria was injected, resulting in an additional oil recovery of 0.9 % OOIP. After the three-day incubation period, 1.4% OOIP was additionally produced during the 2nd SS flooding (Table 3.3, Fig. 3.6). In core Ho 2, the oil production during the 1st SS flooding stopped at 22.2 PVI. Afterwards 1 PV of bacteria was injected, resulting in an additional oil recovery of 0.1 % OOIP. Additional oil amounting to 1.9% OOIP was produced during the 2nd SS flooding (Table 3.3, Fig. 3.6). In core Ho 3, the oil production stopped at 24.1 PVI of the 1st SS flooding. Subsequent injection of 1 PV of bacteria gave no additional oil. However, after the three-day incubation period, additional oil of 1.0 % OOIP was produced during the 2nd SS flooding (Table 3.3, Fig. 3.6). Table 3.3 summarizes the recoveries achieved at each stage.

In core He 1, the oil production during the 1st SS flooding stopped at 27.2 PVI, after which 1 PV of bacteria was injected, resulting in a very small production of additional oil, as low as 0.1 % OOIP. However, after the three-day incubation period, an additional recovery of 5.6 % OOIP was produced during the 2nd SS flooding (Table 3.3, Fig. 3.7). Finally, during the 3rd SS flooding, 1.2 % OOIP was additionally produced after supplementary nutrient incubation. The

experiment with another heterogeneous core plug (core He 2) showed a slightly different result, with a higher total oil recovery. In core He 2, the oil production stopped at 16.6 PVI of the 1st SS flooding, and injection of 1 PV of bacteria produced additionally 2.2 % OOIP. During the 2nd SS flooding, a relatively significant recovery of 3.5 % OOIP was observed. Further nutrient injection and the three-day incubation period produced additionally 3.1 % OOIP (Table 3.3, Fig. 3.7).

The webcam at the outlet of the core holder confirmed that when the cumulative oil production curve flattened, no more oil was produced. The camera also captured the moments where small oil fractions were produced at late stages of recovery (a video from the experiment with core He 2 is available as a supplementary file).

Table 3.3. Summary of oil production history with different flooding schemes*

Core number	S_{wi}	S_{oi}	$S_{or,1st\ SS}$ (%OOIP)	1st SS (%OOIP)	Bacteria (%OOIP)	2nd SS (%OOIP)	Nutrient (%OOIP)	3rd SS (%OOIP)	Total additional recovery (%OOIP)	Bacterial inoculum (cfu/ml)
Ho 1	0.25	0.75	44.2	55.8	0.9	1.4	n/a	n/a	2.3	2.9×10^7
Ho 2	0.26	0.74	38.0	62.0	0.1	1.9	n/a	n/a	2.0	2.6×10^8
Ho 3	0.24	0.76	43.9	56.1	0	1.0	n/a	n/a	1.0	7.2×10^8
He 1	0.31	0.69	35.1	64.9	0.1	5.6	0	1.2	6.9	2.4×10^8
He 2	0.31	0.69	30.7	69.3	2.2	3.5	0	3.1	8.8	3.3×10^8

* The experiments were conducted at overburden pressure 50 bar and temperature at 50°C. Oil density used is 854.2 kg/m^3 @20°C

S_{wi} = initial water saturation, S_{oi} = initial oil saturation, $S_{or,1st\ SS}$ = residual oil saturation after 1st SS flooding, 1st SS = 1st SS flooding, Bacteria = bacteria flooding, 2nd SS = 2nd SS flooding, Nutrient = nutrient flooding, 3rd SS = 3rd SS flooding, OOIP = original oil in place, cfu = colony forming unit

3.4.3 Microbial plugging

Plugging occurred when the bacteria were injected, as indicated by the pressure increase in all experiments (Fig. 3.8 - Fig. 3.12). The pressure tapped core holder was able to monitor bacterial plugging and migration by pressure increase/decrease at different sections of the core. It is important to mention that for the pressure data, only pressure data after the 1st SS flooding (at the start of the bacterial injection) are plotted.

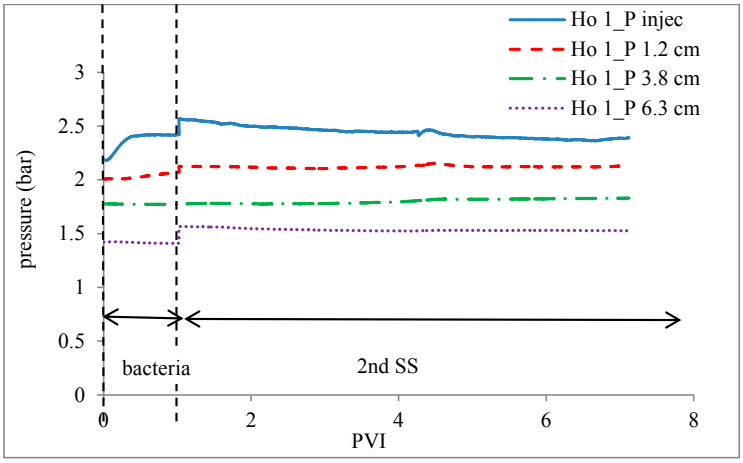


Fig.3.8 Pressure at different positions of core Ho 1 during different injection schemes. Data was plotted as PVI after 1st SS flooding, start from bacteria injection onwards. There was a 3 days incubation period after bacteria injection before 2nd SS flooding.

There was a noticeable increase of pressure at the injection point, P_injec, for cores Ho 1 (Fig. 3.8), Ho 3 (Fig. 3.10) and He 1 (Fig. 3.11) during the bacterial injection and only a slight pressure increase for cores Ho 2 (Fig. 3.9) and He 2 (Fig. 3.12). In addition, P_1.2 cm also increased slightly during the bacterial injection into core Ho 1. The pressure data of core Ho 1 showed that after the three-day incubation, the pressure increased at P_injec and P_6.3 cm. Later on, during the 2nd SS flooding, P_injec and P_1.2 cm decreased, while P_ 3.8 cm increased slightly (Fig. 3.8). Core Ho 2 showed that the pressure was more uniformly distributed at the different sections of the core (Fig. 3.9). The pressure was only slightly increasing when bacteria were injected. However, a significant

pressure increase was observed after the core was incubated with the bacteria, and this pressure decreased rapidly during the 2nd SS flooding. Core Ho 3 was run in a Hassler type core holder. Therefore only data from P_injec is presented (Fig. 3.10). In general, the pressure data for core Ho 3 have the same tendency, the pressure increased during bacterial injection and decreased during the 2nd SS injection.

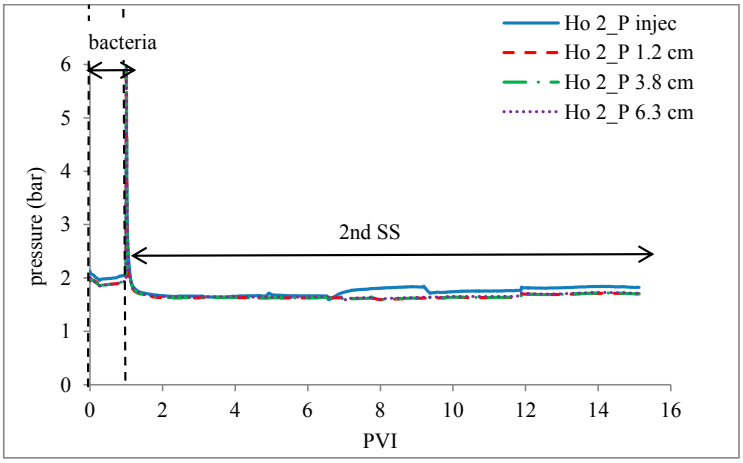


Fig.3.9 Pressure at different positions of core Ho 2 during different injection schemes. Data was plotted as PVI after 1st SS flooding, start from bacteria injection onwards. There was a 3 days incubation period after bacteria injection before 2nd SS flooding.

The increase of the pressure gradient was much higher in the heterogeneous cores compared to the homogeneous cores. This indicates that the effect of microbial plugging is higher in the heterogeneous cores. Pressure data for He 1 and He 2 are slightly different. In core He 1 the inlet pressure increased during bacterial and nutrient injection (Fig. 3.11). It was also observed that in core He 1, P_3.8 cm increased during nutrient injection. Meanwhile, core He 2 (Fig. 3.12) showed no inlet pressure changes during bacterial and nutrient injection. The pressure data during the 2nd and the 3rd SS flooding showed the same tendency for both cores. Among the three different ports (P_injec, P_1.2 cm and P_3.8 cm), the pressure increased at P_injec and decreased at P_1.2 cm and P_3.8 cm.

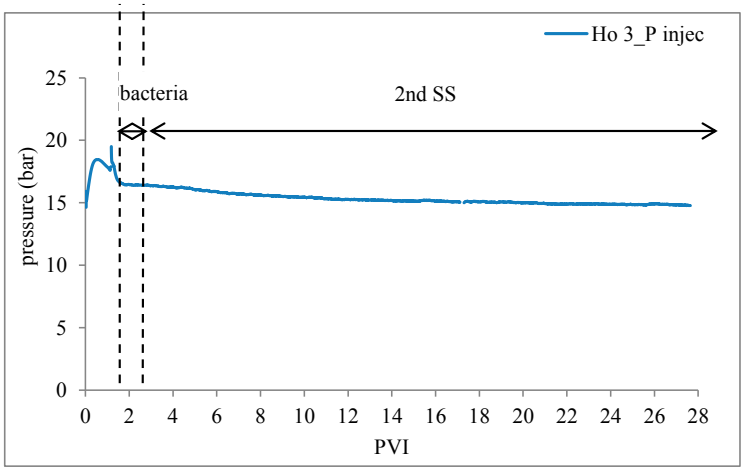


Fig.3.10 Injection pressure of core Ho 3 during different injection schemes. Data was plotted as PVI after 1st SS flooding. There was a 3 days incubation period after bacteria injection before 2nd SS flooding.

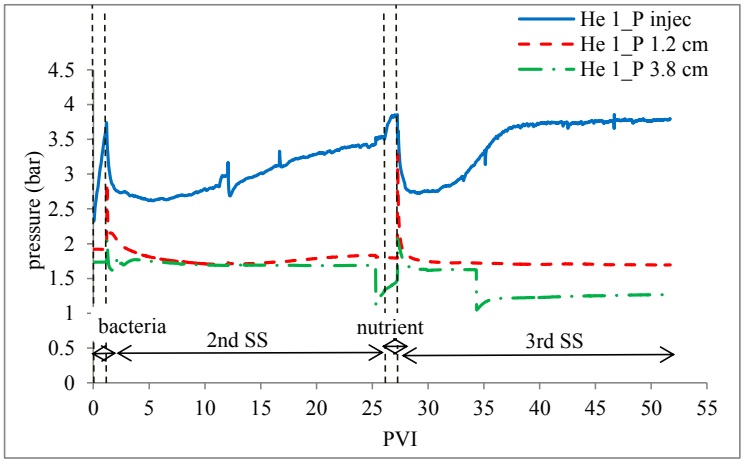


Fig.3.11 Pressure at different positions of core He 1 during different injection schemes. Data was plotted as PVI after 1st SS flooding, start from bacteria injection onwards. There was a 3 days incubation period after bacteria and nutrient injection before 2nd SS flooding and 3rd SS flooding.

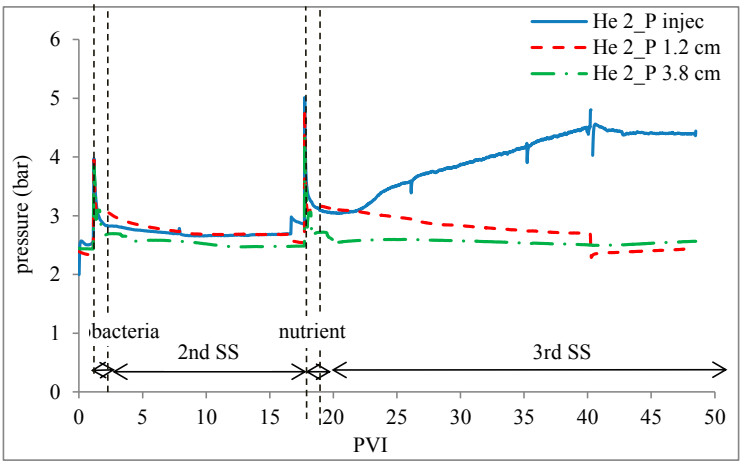


Fig.3.12 Pressure at different positions of core He 2 during different injection schemes. Data was plotted as PVI after 1st SS flooding, start from bacteria injection onwards. There was a 3 days incubation period after bacteria and nutrient injection before 2nd SS flooding and 3rd SS flooding.

3.4.4 Cell-to-spore ratio and bacterial enumeration

DAPI staining and image analysis confirmed that the injected bacterial suspension contained above 98% growing cells and hence that the propagation methodology was satisfactory (data not shown). Quantification of bacterial cells in the effluents gave different results for the experiments with all the five different cores (Figs. 3.13a-3.13c). In Ho 1 (Fig. 3.13a), viable bacterial cells were observed in all the representative produced fractions. In cores Ho 2 and Ho 3, no viable cells were found in the effluent (data not shown). The results of cell quantification in the effluents for core He 1 (Fig.3.13b) and He 2 (Fig.3.13c) showed that viable cells were only detected randomly in some fractions. However, in general, only less than 1% of the injected bacterial cells was found in the effluents of the five different cores. Hence, most likely, the majority of the cells were retained inside the cores. It was not possible to detect bacterial spores in the effluents by the current method. This was due to the traces of oil dissolved in the collected effluents giving autofluorescence when the filter was observed under the microscope.

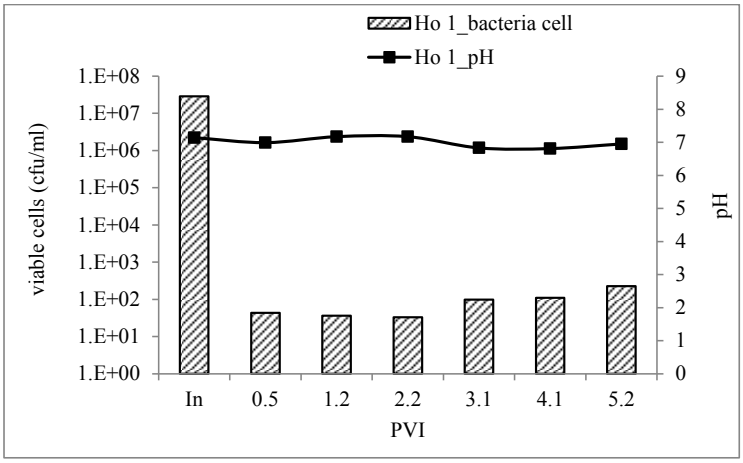


Fig.3.13a Viable *B. licheniformis* 421 cells detected in the effluent of the 2nd SS flooding, after 3 days incubation in the Ho 1 at 50°C (In = concentration of injected bacteria/inoculum).

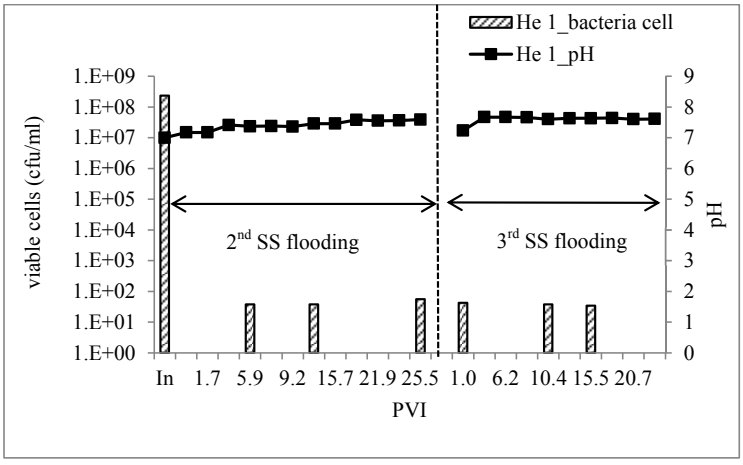


Fig. 3.13b Viable *B. licheniformis* 421 cells detected in the effluent of core He 1 during the 2nd SS flooding (left) and during the 3rd SS flooding (right). The injection and incubation were conducted at 50°C (In = concentration of injected bacteria/inoculum).

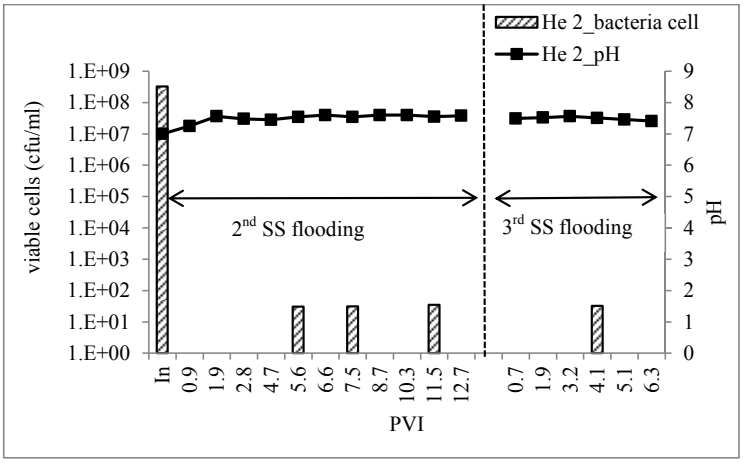


Fig.3.13c Viable *B. licheniformis* 421 cells detected in the effluent of core He 2 during the 2nd SS flooding (left) and during the 3rd SS flooding (right). The injection and incubation were conducted at 50°C (In = concentration of injected bacteria/inoculum).

3.5 Discussion

Our results showed that injection of bacteria (*B. licheniformis* 421) into low permeablity chalk cores gave an additional oil recovery of 1.0-8.8% OOIP. In all cases, it was necessary to have an incubation (shut-in) period after injection of bacteria and/or nutrients to produce more oil. This incubation period allows the bacteria to grow inside the core, to plug more, and to produce some useful substances that may help releasing the trapped oil [1, 11, 15-17]. To the best of our knowledge, no core flooding study has been conducted in low permeable chalk. The closest similar study was conducted by Kaster et al. [17] where it was shown that injection of Draugen indigeneous microbial culture followed by incubation periods was able to recover additional oil of 1.6-3.9% of OOIP. This is within the range of our results. However, there is no information in Kaster et al. [17] regarding the heterogeneity of the cores used in the experiments and, hence, no further conclusions can be drawn to compare our results with Kaster et al. [17].

Our study clearly shows that application of MEOR in heterogeneous cores produced a much higher oil recovery (6.9% - 8.8% OOIP) compared with the

homogeneous cores (1.0% -2.3% OOIP). This indicates that selective plugging may play an important role in MEOR application in chalk. In addition, the pressure data also supports this hypothesis as the pressure gradient increase is higher in heterogeneous cores.

In our experiment, each core was cleaned with organic solvents to remove all the organic materials and salts after each experiment before measuring the permeability in the gas permeameter. The degree of plugging cannot be observed during such measurements, since, if plugging occurs, it will disappear when the bacterial cells are removed. This is in accordance with the observations of Shaw et al. [12], who investigated bacterial plugging in the packed glass beads. It is mentioned in Shaw et al. [12] that bacterial plugging was removed when the bacterial biofilm/cells were removed.

In our study, the bacterial plugging was monitored by pressure increase in the pressure tapped core holder during different injection schemes. The pressure increase during bacterial injection into all the five different cores indicates that bacteria accumulate and plug the porous medium near the injection point. This result is expected and is similar to observations by other investigators for the different porous media [12-14]. The pressure data clearly show that effective permeability decreases more significantly in the presence of bacteria in heterogeneous cores. It is not clear whether the decrease of porosity is proportional. Bacteria may accumulate in specific places (e.g. microfractures) where they significantly change permeability without much affecting the overall porosity.

In core Ho 1, after the three days of incubation, a noticeable pressure increase at the inlet and outlet side of the core was observed. This might indicate bacterial plugging. This phenomenon is similar to the observations by Raiders et al. [13] who found that bacterial plugging was higher at the inlet and the outlet of a core, compared with its middle sections. The authors stated that bacterial plugging at the inlet took place due to biomass accumulation, while at the outlet it was caused

by the biogas production. Plugging at the outlet could also be attributed to the capillary end effect, since the gas dissolved in the injected fluid comes out of the solution as the pressure drops to the atmospheric level at the outlet end of the core. It was also stated in Raiders et al. [13] that dissolution of the gas present in the injected fluid might have contributed to the increase of the pressure at the outlet end.

During the 2nd SS injection into core He 1, the pressure decreased in P_injec and P_1.2 cm while the pressure at P_3.8 cm increased. This might indicate bacterial migration from the first section (P_injec – P_1.2 cm) to the second section of the core (P_1.2 cm – P_3.8 cm). Core Ho 2 showed a different pressure behavior, as the pressure was more uniformly distributed and increased significantly only after bacterial incubation. The different behavior of the pressure data in cores Ho 1 and Ho 2 might be due to the different permeability, pore geometry and tortuosity.

In the heterogeneous cores with micro fractures (He 1 and He 2), the pressure increased in all the sections of the cores after bacterial/nutrient injection followed by an incubation period. This could happen because bacteria were able to penetrate deeper into the cores with micro fractures, or because the bacterial cell had more space to grow and produced more gas. Possibly, in the heterogeneous cores the interconnectivity of the porous space was higher (due to the matrix micro fracture interface) and the gas produced by bacteria was distributed more evenly along the core.

We are not able to give a detailed explanation for the results of the bacterial cells enumeration in the effluents. In core Ho 3 no viable cells were found in the collected effluents, which could be due to the low permeability of the core matrix. However, core Ho 2, which has a higher permeability, shows the same results. This might indicate that tortuosity, instead of permeability, plays an important role in bacterial penetration [26].

3.6 Conclusions

In this work, it was shown that bacteria (*B. licheniformis* 421) may be used for MEOR in the North Sea low permeable heterogeneous chalk rocks. It was demonstrated that the main mechanism of the MEOR action is selective plugging on a small-scale level. The bacteria can selectively block some permeable channels of the reservoir chalk, alter the water paths and thus improve oil production. Injection of bacteria after synthetic seawater flooding in homogeneous chalk cores resulted in an additional oil recovery of 1.0-2.3 % OOIP. In the heterogeneous cores, we observed a noticeably higher recovery range of 6.9-8.8% OOIP.

The pressure tapped core holder was used to monitor the bacterial plugging by pressure difference increase around the different sections of the cores. Pressure data supported the oil production data, as the increase of the pressure gradient was much higher in the heterogeneous cores.

In all cases, injection of bacteria and/or nutrient needed to be followed by an incubation period in order to allow bacterial growth inside the cores.

Less than 1% of the injected cells was found in the effluents. This indicates that most of the bacteria were retained inside the cores.

Acknowledgments

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core flooding with core He 3, which was a part of her master thesis. Nordic Sugar is acknowledged for providing the molasses for the experiment.

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CHAPTER 4
Bacteria Flooding vs. Water Flooding and Wettability Alteration Study

4.1 Introduction

This chapter covers the investigation whether wettability alteration by bacterial biomass and/or metabolites play a role in increasing oil production and how much oil is produced when bacteria is injected into homogeneous chalk cores. Three different core flooding schemes were conducted using the same core plug for these purposes. The core was re-cleaned after each experiment and re-used for the next experiment. The first core flooding was injection of synthetic seawater (SS) without bacteria. This was performed as a baseline study to check whether oil was produced in SS flooding without bacteria. The second was core flooding using non-aged core and 1 pore volume injection (PVI) of bacteria. This was performed as a confirmation whether bacteria injection could recover the residual oil in the core. The third was core flooding using aged core and 1 PVI of bacteria. This was performed to check whether more oil was produced in the aged core as compared to the non-aged core. It is believed that aging of the core in crude oil will change the core towards more oil-wet system. If the oil production is higher in the aged core, it may indicate that bacteria and/or the metabolites play a role in the alteration of core wettability towards less water wet system hence more oil is produced. Detailed literature review on wettability alteration by bacterial biomass and/or metabolite can be found in Chapter 1, section 1.3.2.2.

Wettability alteration of the core towards more oil-wet system was carried out by aging the core in crude oil. There are many factors that influence the aging process of a core plug. These include (but are probably not limited to): acid numbers of the crude oil [1], asphaltene concentration [2, 3], aging time, and temperature [3-5]. The approach to age the core in crude oil has several shortcomings, such as it is very difficult to get a uniform wettability alteration along a core and no established way to check its uniformity [6]. In addition, the strength of the wettability alteration cannot be controlled, especially when the experiment is conducted at high temperature. Under this condition, organic

molecules of crude oil might be detached from the rock surface, resulting in returning of wettability to the original water-wet state [6]. In this study, we simplified the aging process by aging the core for 3 weeks, at 50°C (the temperature of which the experiment was conducted).

4.2 Materials and methods

Materials used in the experiments (crude oil, reservoir rocks, bacteria samples, chemicals, growth media and core flooding apparatus) are similar to the ones used in Chapter 3. Three homogeneous cores were used in this study (Ho 1, Ho 2 and Ho 3). Further in the text, a core will be referred as core ID followed by type of experiment, e.g.: core Ho 1_baseline, refers to baseline experiment with core Ho 1 (no bacteria injection), core Ho 2_aged core, refers to experiment with core Ho 2 using aging, etc.

Each core was subjected to three different core flooding schemes: base line – no bacteria, injection of bacteria at residual oil state in non-aged core and injection of bacteria at residual oil state in aged core. All experiments were carried out in the pressure-tapped core holder, except the baseline of core Ho 1 flooding and the experiments with core Ho 3, which were performed with the ordinary Hassler-type core holder. Schematic drawing of the experimental set up can be seen in Chapter 3, Fig. 3.2.

The experimental sequence is presented in Fig. 4.1 and detailed explanations of the procedure are similar to the description in Chapter 3. The part that is different from the descriptions in Chapter 3 is the experimental sequence which will be described below.

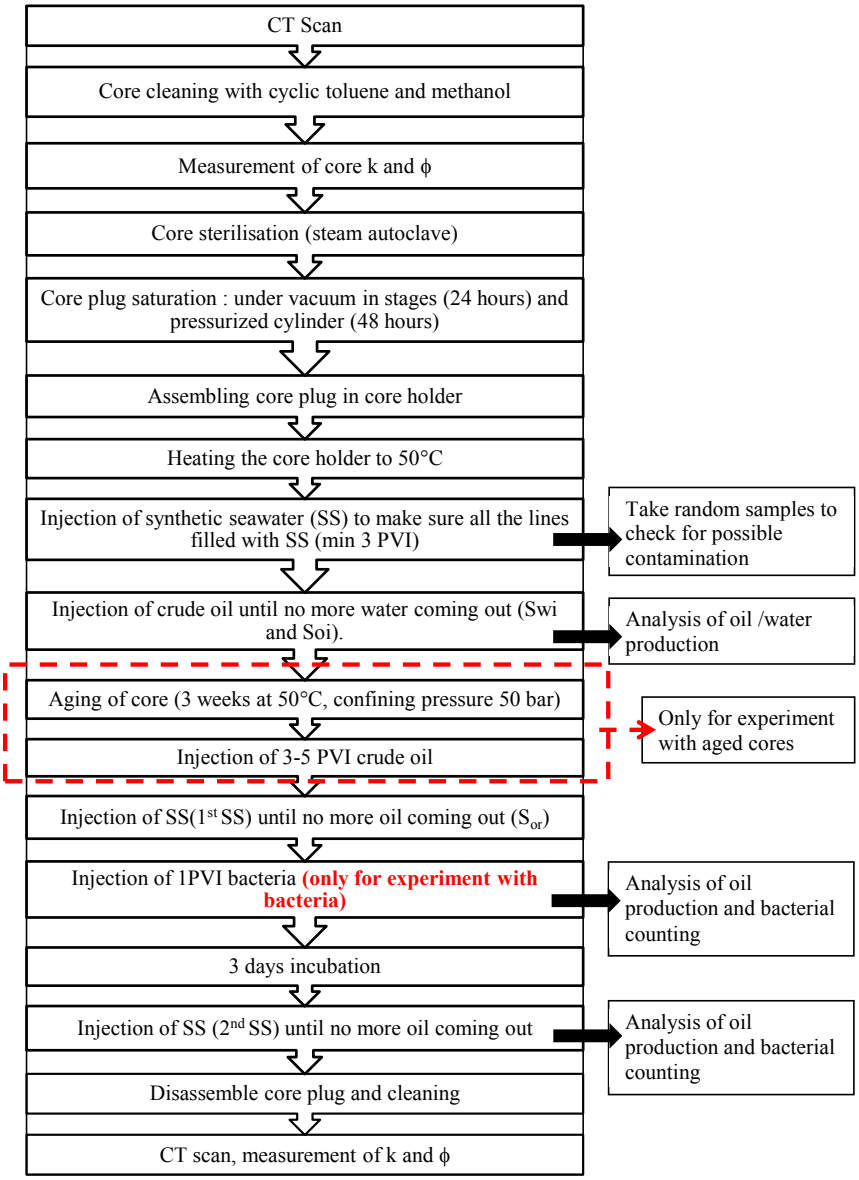

Fig.4.1 Schematic drawing of experimental sequences. In the baseline experiment, no bacteria were injected. In the experiment with aged-cores, the core was aged inside the core holder for 3 weeks at 50°C and confining pressure 50 bars, followed by subsequent injection of crude oil. The cores were re-cleaned after each experiment and re-used for the next experiment.

A 260D ISCO pump was used to keep control over a constant injection rate of 0.1 ml/min throughout the experiment. After the temperature inside the core holder was stabilized, the core was subjected to 3-5 PVI of sterile SS. This was followed by injection of crude oil until no more water was produced, in order to create irreducible water saturation (S_{wi}). The oil saturation under this condition was considered to be original oil in place (OOIP).

In the experiments with aged cores, the inlet and outlet valves were closed to let the core aged in crude oil under static condition for 3 weeks. The temperature was kept constant at 50°C by the heating jacket and confining pressure was kept constant at 50 bar using a 260D ISCO throughout the aging period. Subsequent injection of 3-5 PVI crude oil was performed in the end of aging period.

The core was then flooded with SS (1^{st} SS flooding) until irreducible oil saturation (S_{or}), where no more oil was produced. Then 1 PVI of bacteria in synthetic seawater containing nutrients was injected, and the core was incubated with bacteria for 3 days by closing both inlet and outlet valves. After the 3-days incubation period, the core was again flooded with SS (2^{nd} SS flooding) until no more oil was produced. No bacteria were injected in the baseline experiment and the valves were directly closed after no more oil was produced.

After oil production has stopped in the 2^{nd} SS flooding, the core was re-cleaned with cyclic flooding of toluene and methanol. Subsequently, any possible visible fracture was observed with CT scan and the core properties (porosity and permeability) were measured. This was conducted as confirmation whether the core has not been significantly modified after each experiment and can be re-used for the next experiment.

4.3 Results

A total of eight core flooding experiments, three for baseline-no bacteria (Ho 1_no bacteria, Ho 2_no bacteria and Ho 3_no bacteria), three with injection of 1 pore volume injection (PVI) bacteria in non-aged cores (Ho 1_non-aged, Ho 2_ non-

aged and Ho 3_ non-aged) and two with injection of 1 PVI bacteria in aged cores (Ho 1_aged and Ho 2_aged), were conducted to study the effect of bacterial injection on oil recovery. A summary of core properties before and after experiments is presented in Table 4.1. Furthermore, a summary of the oil production history with different flooding schemes is presented in Table 4.2. All experimental data from core Ho_3 were obtained from Flensborg, J.P., 2015 [7].

Table 4.1 Summary of core plug properties

Core number	Diameter (cm)	Length (cm)	Pore volume (ml)	Ki (mD)	Ka (mD)	ϕi	ϕa
Ho 1_bs	3.8	7.5	27.4	3.2	2.8	30.7	30.7
Ho 1_na	3.8	7.5	27.4	3.2	3.1	31.1	30.7
Ho 1_ag	3.8	7.5	26.7	2.8	3.1	30.7	30.6
Ho 2_bs	3.8	7.5	37.8	6.8	6.4	43.8	43.4
Ho 2_na	3.8	7.5	37.1	6.4	6.4	43.4	43.0
Ho 2_ag	3.8	7.5	37.3	6.4	6.4	43.4	43.0
Ho 3_bs[7]	3.7	5.2	14.1	0.5	0.5	25.0	25.3
Ho 3_ns[7]	3.7	5.2	14.0	0.5	0.5	25.3	25.2

ki = permeability before experiment, ka = permeability after experiment, ϕi = porosity before experiment, ϕa = porosity after experiment
[7] data from Flensborg, J.P., 2015

4.3.1 Core properties

Core permeability and porosity were measured at dry condition by the steady state gas permeameter. There was no significant change in the core properties on both porosity and permeability of the cores before and after experiment (Table 4.1). The results of CT scanning show that cores Ho 1, Ho 2, and Ho 3 are relatively homogenous. No open or partially open fractures were detected in the cores before and after each experiment.

4.3.2 Oil production history

Initial water saturation (S_{wi}) was approximately 0.20 – 0.28 for the non-aged cores (Ho 1_baseline, Ho 1_non-aged, Ho 2_baseline, Ho 2_non-aged, Ho 3_baseline,

and Ho 3_non-aged) and 0.23-0.24 for the aged cores (Ho 1_aged and Ho 2_aged). The cores were flooded with SS (1st SS flooding) until no more oil was produced to establish the residual oil saturation (S_{or}). The 1st SS flooding from the non-aged cores produced more oil as compared to the aged cores. The Ho 1_non-aged produced 3.8% OOIP more than the Ho 1_aged and the Ho 2_non-aged produced 3.6% OOIP more than the Ho 2_aged non-aged.

Application of MEOR as a tertiary oil recovery method: bacterial injection after 1st SS flooding was able to produce additionally 1.0 to 2.3 % OOIP in experiments with non-aged cores and 3.6 to 4.3 % OOIP in experiments with aged cores. It is also important to mention that oil production of the non-aged cores with bacteria injection decreased with decreasing permeability. Table 4.2 summarizes the recoveries achieved at each stage.

In the experiment with core Ho 1_baseline, oil production at the 1st SS flooding stopped after 11.8 PVI. Afterwards, both inlet and outlet valves were closed to simulate the 3-days incubation period and no oil was produced during the 2nd SS flooding (Table 4.2, Fig. 4.2). In the experiment with core Ho 1_non-aged, oil production during the 1st SS flooding stopped at 11.3 PVI. Afterwards 1 PV bacteria were injected, resulting in additional oil recovery 0.9 % OOIP. Additional oil amounting at 1.4 % OOIP was produced during the 2nd SS flooding (Table 4.2, Fig. 4.2). In the experiment with core Ho 1_aged, oil production stopped at 17.6 PVI of the 1st SS flooding. Subsequent injection of 1 PVI bacteria produced 0.4 % OOIP. The 2nd SS flooding after the 3-days incubation period produced additional oil 3.2 % OOIP (Table 4.2, Fig. 4.2).

Table 4.2 Summary of oil production history with different flooding schemes

Core number	S_{wi}	S_{oi}	$S_{or,1st\ SS}$ (%OOIP)	1st SS (%OOIP)	Bacteria (%OOIP)	2nd SS (%OOIP)	Total additional recovery (%OOIP)	Bacterial inoculum (cfu/ml)
Ho 1_bs	0.23	0.77	45.6	54.4	n/a	0.1	0.1	n/a
Ho 1_na	0.25	0.75	44.2	55.8	0.9	1.4	2.3	2.9×10^7
Ho 1_ag	0.23	0.77	43.9	52.0	0.4	3.2	3.6	2.2×10^7
Ho 2_bs	0.28	0.72	39.2	60.8	n/a	1.0	1.0	n/a
Ho 2_na	0.26	0.74	38.0	62.0	0.1	1.8	1.9	2.6×10^8
Ho 2_ag	0.24	0.76	41.6	58.4	0.6	3.7	4.3	8.2×10^8
Ho 3_bs[7]	0.20	0.80	47.9	52.1	n/a	0.1	0.1	n/a
Ho 3_ns[7]	0.24	0.76	43.9	56.1	0	1.0	1.0	7.2×10^8

* The experiments were conducted at overburden pressure 50 bar and temperature at 50°C. Oil density used is 854.2 kg/m^3 @20°C
S_{wi} = initial water saturation, S_{oi} = initial oil saturation, $S_{or,1st\ SS}$ = residual oil saturation after 1st SS flooding, 1st SS = 1st SS flooding, Bacteria = bacteria flooding, 2nd SS = 2nd SS flooding, OOIP = original oil in place, cfu = colony forming unit, bs = baseline experiment (no bacteria), na = experiment with non-aged core, ag = experiment with aged core.
[7] data from Flensborg, J.P., 2015

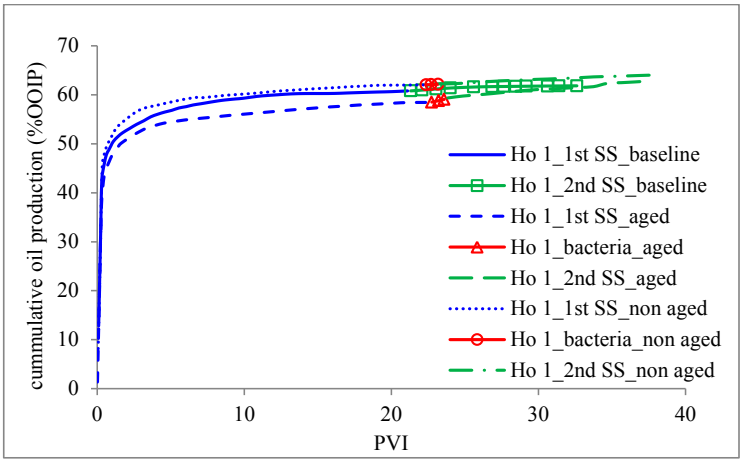

Fig.4.2 Cumulative oil production vs. pore volumes injection for baseline experiment core Ho 1 (no bacteria), experiment with non-aged core Ho 1 and experiment with aged core Ho 1.

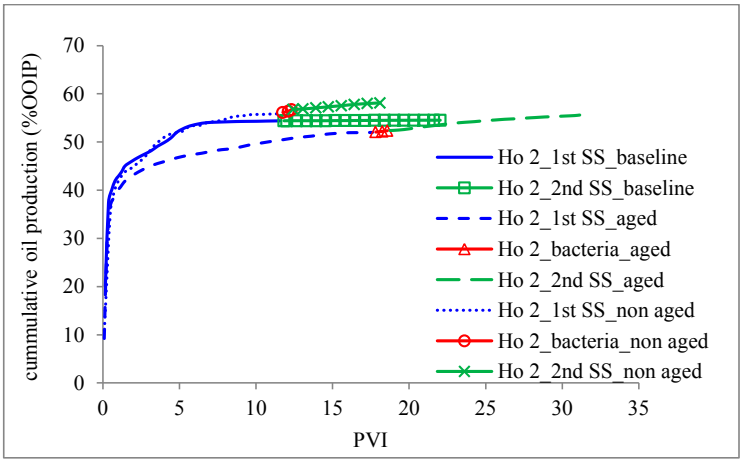

Fig.4.3 Cumulative oil production vs. pore volumes injection for baseline experiment core Ho 2 (no bacteria), experiment with non-aged core Ho 2 and experiment with aged core Ho 2.

In the experiment with core Ho 2_baseline, oil production at the 1st SS flooding stopped after 21.1 PVI. Afterwards, both inlet and outlet valves were closed to simulate the 3-days incubation period and 1.0 % OOIP was additionally produced during the 2nd SS flooding (Table 4.2, Fig. 4.3). In the experiment with core

Ho 2_non-aged, oil production during the 1st SS flooding stopped at 22.2 PVI. Afterwards 1 PV bacteria were injected, resulting in additional oil recovery 0.1 % OOIP. Additional oil 1.8 % OOIP was produced during the 2nd SS flooding (Table 4.2, Fig. 4.3). In the experiment with core Ho 2_aged, oil production stopped at 22.6 PVI of the 1st SS flooding. Subsequent injection of 1 PVI bacteria produced 0.6 % OOIP. Additional oil amounting at 3.7 % OOIP was produced during the 2nd SS flooding (Table 4.2, Fig. 4.3).

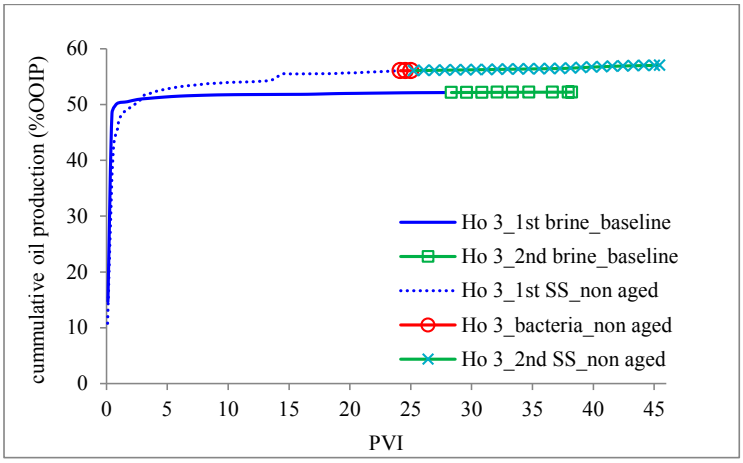

Fig.4.4 Cumulative oil production vs. pore volumes injection for baseline experiment of core Ho 3 (no bacteria) and experiment with non-aged core Ho 3 [7].

In the experiment with core Ho 3_baseline [7], oil production at the 1st SS flooding stopped after 27.7 PVI. Afterwards, both inlet and outlet valves were closed to simulate the 3-days incubation period and 0.1 % OOIP was additionally produced during the 2nd SS flooding (Table 4.2, Fig. 4.4). In the experiment with core Ho 2_non-aged, oil production during the 1st SS flooding stopped at 23.9 PVI. Afterwards 1 PV bacteria were injected and no additional oil was produced. However, additional oil 1.0 % OOIP was produced during the 2nd SS flooding (Table 4.2, Fig. 4.4) [7]. Due to time constrain, experiment with core Ho 3_aged was not conducted.

The webcam at the outlet of the core holder confirmed that when the cumulative oil production curve flattened, no more oil was produced. The camera also captured the moments when small oil fractions were produced at late stages of recovery after bacteria were injected.

4.3.3 Pressure changes and bacteria migration

Ideally, the pressure-tapped core holder is able to monitor bacterial migration by pressure increase/decrease at the different sections of the core. If bacteria plug at a specific section of the core, then pressure will increase at this section (normally near the injection end). Correspondingly, if bacteria migrate to a deeper part of the core, the pressure near the inlet will decrease and pressure at the deeper sections will increase. It is important to mention that, the pressure data for the experiments with bacteria (both aged and non-aged cores), are plotted only after the 1st SS flooding (at the start of bacteria injection). While in the baseline experiments (no bacteria), the pressure is plotted as the last pore volume injection (PVI) of the 1st SS flooding followed by the pressure at the 2nd SS flooding.

The pressure change in the baseline study (no bacteria) is shown in Fig. 4.5a, Fig. 4.6a and Fig. 4.7a for core Ho 1_ baseline, Ho 2_ baseline and Ho 3_ baseline, respectively. The injection pressure (P_injec) at the last PVI of the 1st SS was relatively stable, indicating that water was the dominant fluid flowing in the rock and oil was at residual state. After the 3-days incubation period, there was an increase in the P_injec of core Ho 1_ baseline and Ho 3_ baseline. In the experiment with core Ho 2_ baseline, the pressure increased simultaneously in all the different sections of the core. During the 2nd SS flooding, pressure decreased in the experiments with the three different cores.

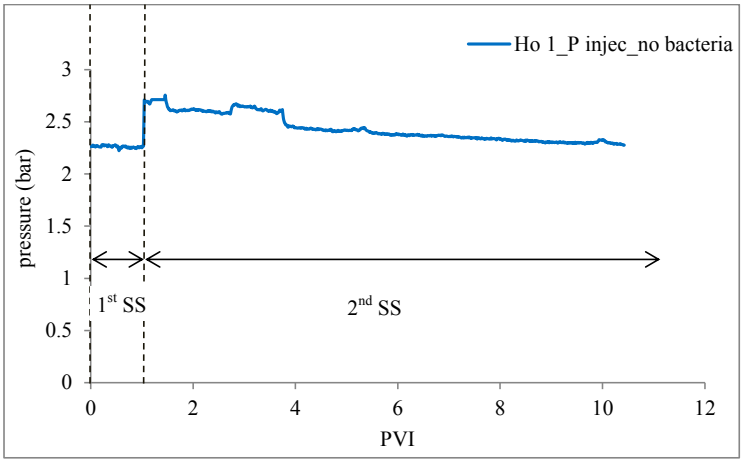

Fig.4.5a Injection pressure of core Ho 1 during different injection schemes in the baseline experiment (no bacteria). Data was plotted as the last pore volume injection (PVI) of the 1st SS flooding, followed by the 2nd SS flooding. There was a 3 days incubation period after 1st SS injection before 2nd SS flooding.

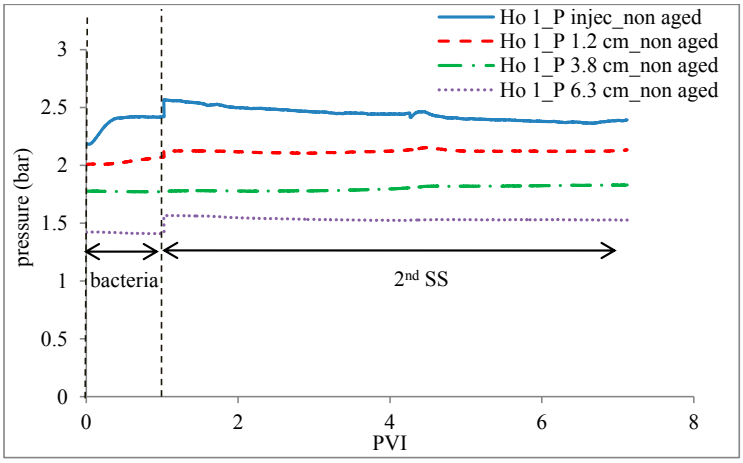

Fig.4.5b Pressure at different positions during different injection schemes in the experiment with non-aged core Ho 1. Data was plotted as pore volume injection (PVI) after 1st SS flooding, start from bacteria injection onwards. There was a 3 days incubation period after bacteria injection before 2nd SS flooding.

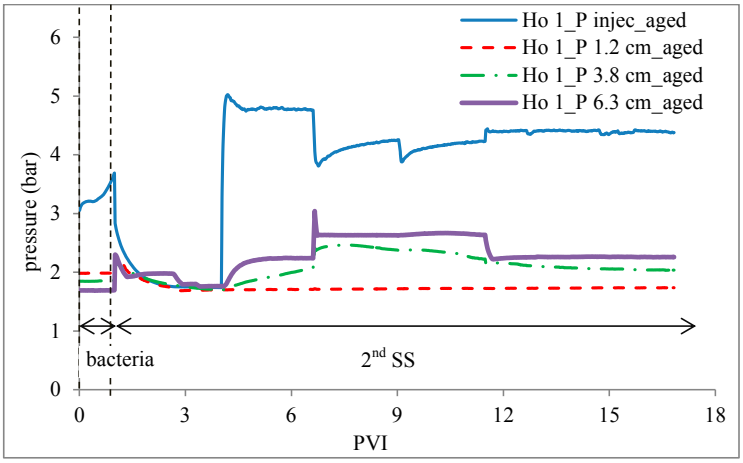

Fig.4.5c Pressure at different positions during different injection schemes in the experiment with aged core Ho 1. Data was plotted as pore volume injection (PVI) after 1st SS flooding, start from bacteria injection onwards. There was a 3 days incubation period after bacteria injection before 2nd SS flooding.

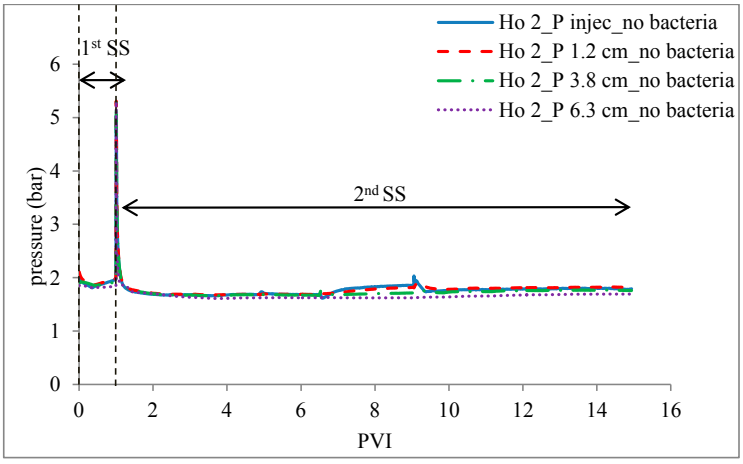

Fig. 4.6a Pressure at different positions during different injection schemes in the baseline experiment (no bacteria) of core Ho 2. Data was plotted as the last pore volume injection (PVI) of the 1st SS flooding, followed by the 2nd SS flooding. There was a 3 days incubation period after 1st SS injection before 2nd SS flooding.

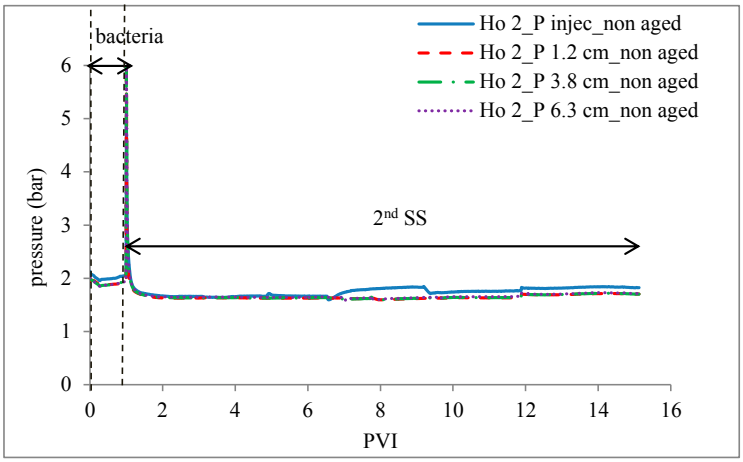

Fig. 4.6b Pressure at different positions during different injection schemes in the experiment with non-aged core Ho 2. Data was plotted as pore volume injection (PVI) after 1st SS flooding, start from bacteria injection onwards. There was a 3 days incubation period after bacteria injection before 2nd SS flooding.

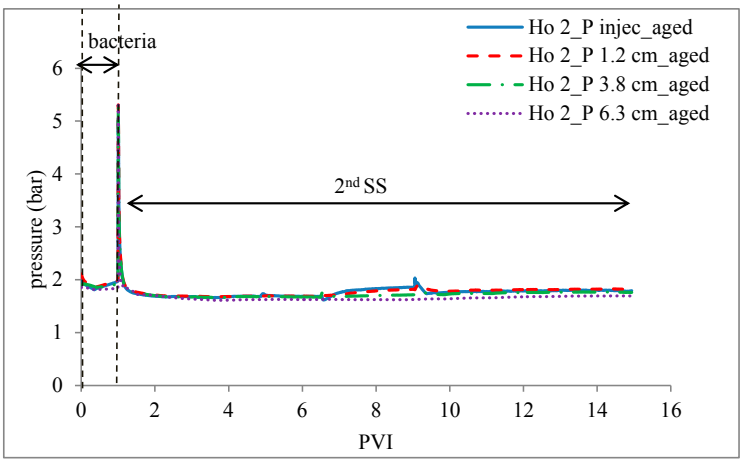

Fig. 4.6c Pressure at different positions during different injection schemes in the experiment with aged core Ho 2. Data was plotted as pore volume injection (PVI) after 1st SS flooding, start from bacteria injection onwards. There was a 3 days incubation period after bacteria injection before 2nd SS flooding.

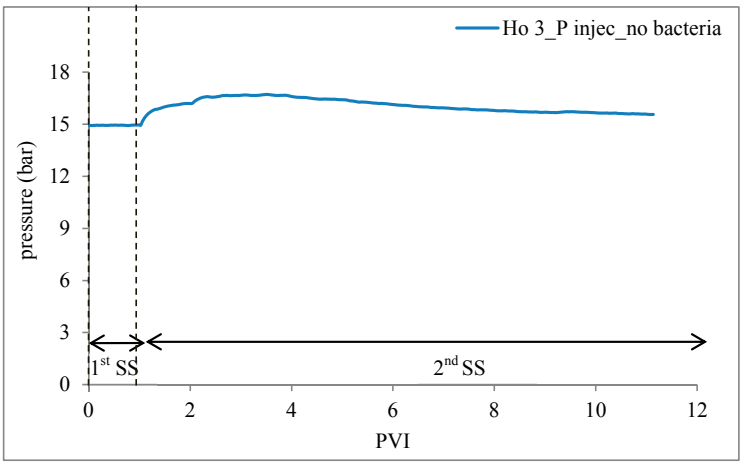

Fig 4.7a Injection pressure of core Ho 3 during different injection schemes in the baseline experiment (no bacteria). Data was plotted as the last pore volume injection (PVI) of the 1st SS flooding, followed by the 2nd SS flooding. There was a 3 days incubation period after 1st SS injection before 2nd SS flooding [7].

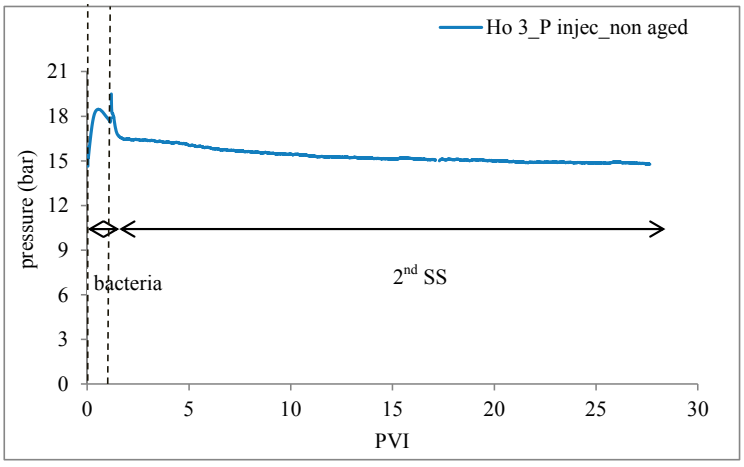

Fig. 4.7b Injection pressure during different injection schemes in the experiment with non-aged core Ho 3. Data was plotted as the last pore volume injection (PVI) of the 1st SS flooding, followed by the 2nd SS flooding. There was a 3 days incubation period after 1st SS injection before 2nd SS flooding [7].

In the experiment with core Ho 1_non-aged (Fig. 4.5b) there was a slight P_injec increase when bacteria were injected into the cores and the P_injec increase was higher in the experiment with core Ho 3_non-aged (Fig. 4.7b). Meanwhile, in the

experiment with core Ho 2_non-aged, P_injec remained relatively stable during bacteria injection (Fig 4.6b). After the 3-days incubation period, there was pressure increase in the experiments with the three different cores. In core Ho 1_non-aged pressure increased at the inlet and outlet of the core (Fig. 4.5b), while in core Ho 2_non-aged pressure increased uniformly at the different sections of the core (Fig. 4.6b). The experiment with core Ho 3_non-aged was run in the ordinary Hassler-type core holder, therefore only P_injec can be monitored. The P_injec of core Ho 3_non-aged also increased (Fig. 4.7b). During 2nd SS flooding, P_injec of core Ho 1_non-aged decreased while the other pressure ports showed relatively stable values (Fig. 4.5b). In the experiment of core Ho 2_non-aged, pressure decreased in all sections of the core during the 2nd SS flooding (Fig. 4.6b). Similarly, P_injec of core Ho 3_non-aged also decreased (Fig. 4.7b).

In the experiment with aged cores, a slight increase of P_injec was observed during bacteria injection in core Ho 1_aged (Fig. 4.5c). Meanwhile, in the experiment with core Ho 2_aged P_injec remained relatively stable during bacteria injection (Fig. 4.6c). After the 3-days incubation period, there was slight pressure increase in the different sections of core Ho 1_aged (Fig. 4.5c) and a significant pressure increase at the different sections of the core Ho 2_aged (Fig. 4.6c). During 2nd SS flooding, P_injec of core Ho 1_aged decreased rapidly and other sections also decreased slightly. However, after approximately 3 PVI, there was a significant increase of P_injec and slight increase of P_3.8 cm and P_6.3 cm of core Ho 1_aged (Fig. 4.5c). In the experiment with core Ho 2_aged, pressure decreased rapidly in the beginning of 2nd SS flooding and remained relatively stable afterwards (Fig. 4.6c).

4.3.4 Cell-to-spore ratio and bacterial enumeration

DAPI staining and image analysis confirmed that the injected bacterial suspension contained above 98% growing cells and hence that the propagation methodology was satisfactorily (data not shown). Quantification of bacterial cells in the effluents gave different results for the five different core flooding experiments with bacteria. In the experiments with core Ho 1_non-aged, core Ho 1_aged and

Ho 3_non aged, no viable bacterial cells were observed in any of the representative effluent fractions (data not shown). In experiments with core Ho 2_non-aged and core Ho 2_aged, a few viable cells were detected in the effluents (Fig. 4.8a and 4.8b). However, in general less than 1% of the injected bacterial cells were found in the effuents of the five different core flooding experiments with bacteria. Hence, most likely the majority of the cells were retained inside the cores. It was not possible to detect bacterial spores in the effluents with the current method. This was due to that the traces of oil dissolved in the collected effluents gave autofluorescence thereby masking the potential presence of spores when the sample was observed under the microscope.

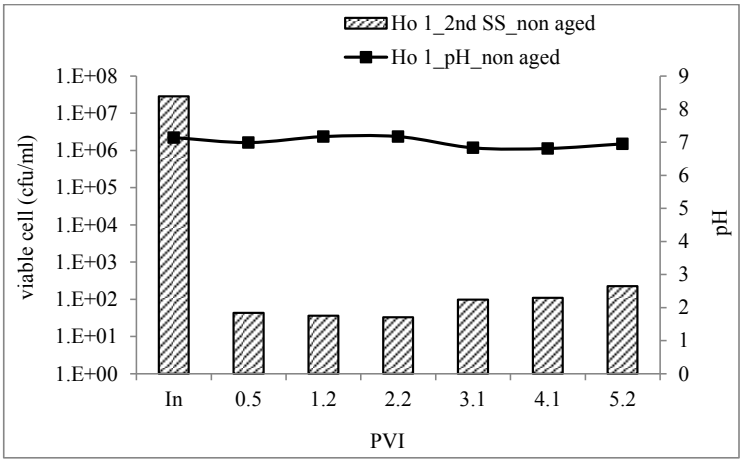

Fig. 4.8a Viable *B. licheniformis* 421 cells detected in the effluent of the 2nd SS flooding, after 3 days incubation in the non-aged core Ho 1 at 50°C (In = concentration of injected bacteria/inoculum).

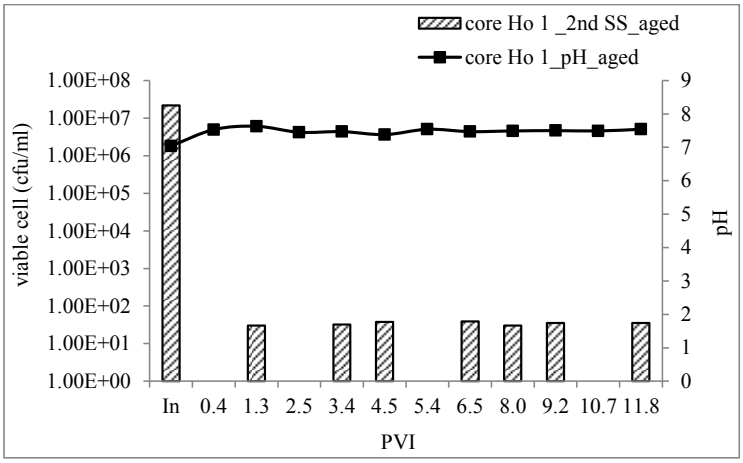

Fig. 4.8b Viable *B. licheniformis* 421 cells detected in the effluent of the 2nd SS flooding, after 3 days incubation in the aged core Ho 1 at 50°C (In = concentration of injected bacteria/inoculum).

4.4 Discussion

It was clearly shown in this study that application of MEOR in homogeneous chalk cores increases oil production as compared to the baseline (no bacteria). In the baseline study, injection of synthetic seawater without bacteria produced maximum 1.0 % OOIP. Meanwhile, injection of bacteria (*B. licheniformis* 421) in the non-aged homogeneous chalk cores produced 1.0-2.3 % OOIP and in the aged homogenous cores oil production was only slightly higher (3.6-4.3 % OOIP). Consequently, wettability alteration by bacterial biomass and/or surfactant does not seem to give significant contributions in MEOR application in chalk. To the best of our knowledge, no core flooding study has earlier been reported in low permeable chalk with aged and non-aged cores aiming to see the effects of microbial biomass and/metabolites to wettability alteration in chalk. Therefore, it was not possible to compare our results with that from other studies.

A subsequent study was conducted to see the oil production when the bacteria were injected as secondary recovery approach (after the first water breakthrough was observed). This study was conducted using core Ho_1 (see Appendix 1). It was shown that the final oil recovery was higher when the bacteria were injected

as secondary method compared with the tertiary method (Appendix 1, table A1). This might indicate that, at early injection of bacteria, higher oil production can be expected. However, as this only one single experiment we cannot take any further conclusions and the experiment should be repeated

In all cases, incubation (shut-in) period after injection of bacteria and/or nutrients was needed to produce more oil. This incubation period allows the bacteria to grow inside the core, and produce some useful substances that may help releasing the trapped oil (Harish *et al.*, 2009; Kaster *et al.*, 2012).

The aim of aging the core in crude oil for 3 weeks is to alter the wettability of the core towards more oil wet system. As previously mentioned (Chapter 1, section 1.3.2.2), bacterial biomass and/or metabolites are believed to be able to change the wettability of minerals [8-12]. However, our results showed no significant oil production difference between aged and non-aged core. This result may be interpreted by comparison with our subsequent study of bacterial growth in Chapter 5; where the bacteria were grown in shake flasks under anaerobic condition and in the presence of different carbon sources (molasses and n-alkanes mixture-to simulate hydrocarbon). The growth study showed that in the presence of hydrocarbons the bacteria grew slower and in significantly lowers numbers. Correspondingly, metabolite production (organic acid and surfactant) was significantly lower in the culture grown with the presence of hydrocarbon (Chapter 5, section 5.B.3.1). This phenomena might be the reason why there was no significant difference of the oil production of the aged and the non-aged cores; there was not enough bacterial biomass/and or metabolites being produced to contribute to the wettability alteration.

Similarly to what has been explained in Chapter 3, each core was cleaned with organic solvents to remove all the organic materials and salts after each experiment before measuring the permeability in the gas permeameter. Therefore, the degree of plugging cannot be observed under such measurements.

The bacterial migration was monitored by pressure changes in the pressure-tapped core holder during different injection schemes. The P_injec increased during bacteria injection in experiment with core Ho 1_non aged and Ho 3_non aged, which indicates that bacteria accumulate and plug the porous medium near the injection point. This result is expected and is similar to observations by other investigators for the different porous media [13-15]. Some possible explanations for pressure increase (P_injec and P_6.3 cm) in core Ho 1_non aged with bacteria, after the 3-days incubation, have been given previously in Chapter 3, section 3.5. In general, during the 2nd SS flooding the P_injec was decreasing for the five different experiments with bacteria (Ho 1_non-aged, Ho 1_ aged, Ho 2_non-aged, Ho 2_ aged and Ho 3_non-aged), which might indicate bacteria migration deeper into the core. We are not able to explain the reason for the pressure behaves irregularly in the experiment with aged core Ho 1 after 3PVI of the 2nd SS flooding. The different behavior of the pressure data in Ho 2 (baseline, non-aged and aged) as compared with core Ho 1 and Ho 3 might be due to the different permeability, pore geometry and tortuosity.

Similar to the results in Chapter 3, we are not able to give a detailed explanation for the results of the bacterial cells enumeration in the effluents. It most likely that tortuosity, instead of permeability, plays an important role in bacteria penetration (Jenneman *et al.*, 1985).

4.5 Conclusions

In this chapter, it was demonstrated that *B. licheniformis* 421, increases the oil production in the low permeable, homogeneous North Sea chalk cores. The oil recovery in the non-aged cores was 1.0-2.3 % OOIP and in the aged cores was 3.6-4.3 % OOIP. The oil recovery difference between non-aged and aged cores was not significant. This may indicate that, in this specific study, metabolites produced by *B. licheniformis* 421 are not contributing to improve oil production in chalk. However, this should be investigated on case by case basis as it was clearly shown in Chapter 5 that the bacteria grew slow in the presence of hydrocarbon;

and thus there might be not enough biomass and/or metabolites present to alter the core wettability.

The conclusions similar to Chapter 3 were also seen in this study: 1) injection of bacteria needed to be followed by an incubation period in order to allow bacterial growth inside the cores, 2) the pressure-tapped core holder was used to monitor the bacterial plugging and/or migration by pressure difference changes at the different sections of the cores, and 3) less than 1% of the injected cells were found in the effluents. This indicates that most of the bacteria were retained inside the cores.

4.6 Future recommendations

Further work should be performed to support our conclusion that wettability alteration does not give a significant contribution to improve oil production in chalk cores. This can be achieved by the Amott test and/or contact angle measurement. Different concentrations of the bacteria biomass and/or metabolites should be tested to verify whether the low number of bacterial biomass and/or metabolites is the reason for no significant wettability alteration and thus no significant improvement in the experiments with aged cores.

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CHAPTER 5
Towards the Understanding of Microbial Metabolism in Relation to Microbial Enhanced Oil Recovery

This chapter deals with *B. licheniformis* 421 growth in batch cultures. The aims of the study are to understand *B. licheniformis* 421 growth in the different media and on different carbon sources; production of metabolites; and the effects of bacterial cells and/or metabolites on the fluid-fluid interactions. This chapter is divided into two sub-chapters, part A and part B. Part A is a preliminary study of the bacterial growth under aerobic conditions to determine the medium composition used in the anaerobic growth experiments (part B) and also to determine the bacterial inoculum age used for the core flooding experiments. Part B is a study of bacterial growth under more realistic scenario: anaerobic conditions, high salinity and in the presence of mixed carbon sources (molasses and n-alkanes). Part B is aimed for publication and is a manuscript under preparation.

Part A
B. licheniformis 421 aerobic growth

5.A.1 Introduction

In this section, modified minimal salt medium containing glucose based on the study by Nazina et al.[1] was used as our baseline medium. First the effect of replacing pure glucose with molasses on the *B. licheniformis* growth was investigated Molasses is a waste product from sugar industry and was tested because it is a more widely used carbon source for MEOR field application since it is cheaper than pure glucose. Later on, the bacterial growth on high salinity was tested by changing the medium to a synthetic seawater based medium to mimic the reservoir conditions that often having high salinities. The experiments were conducted on shake flasks under aerobic conditions. Based on these experiments, the inoculum age used for the core flooding experiments can be determined.

5.A.2 Materials and methods

5.A.2.1 Chemicals

Unless otherwise stated, all chemicals were analytical-grade from Sigma-Aldrich (St. Louis, MO). Water was purified on a Milli-Q system (Millipore, Bedford, MA). The molasses used was as described on section 5.B.2.1. The media used for this study are a minimum salt medium modified from by Nazina et al. [1] (Table 5.1) and synthetic seawater (SS) based media supplemented with either 16.9 g/l molasses or 10 g/l glucose (Table 5.1).

Table 5.2. Media compositions

Chemical	Amount (g/l)			
	SSM	SSG	Minimal salt medium**	Minimal salt medium** (reduced KH_2PO_4)
NaCl	18.00	18.00		
$NaHCO_3$	0.17	0.17		
KCl	0.74	0.74		
$MgCl_2.6H_2O$	9.15	9.15		
$CaCl_2.2H_2O$	1.91	1.91	0.01	0.01
Na_2SO_4	3.41	3.41		
$NaNO_3$	2.00	2.00	2.00	2.00
KH_2PO_4	0.10	0.10	1.00	0.1
Molasses (59% sucrose)	16.90	-		
Vitamins [2]	1% (v/v)	1% (v/v)	1% (v/v)	1% (v/v)
Trace elements [3]	1% (v/v)	1% (v/v)	1% (v/v)	1% (v/v)
MOPS (buffer)	20.00	20.00	20.00	20.00
$MgSO_4.7H_2O$			0.25	0.25
Glucose		10.00	10.00	10.00

SSM = synthetic seawater based medium supplemented with molasses, SSG = synthetic seawater based medium supplemented glucose

**Modified from Nazina et al.[1], the yeast extract was replaced by vitamins

5.A.2.2 Media preparations

The chemicals (Table 5.1) were weighed with an analytical balance. The minimal salt medium was made to final volume of 100 ml medium in 250 ml shake flasks and pH was adjusted to 7 by adding 2 M KOH. Then the flasks were closed with cotton wool plugs and sterilized by steam at 121°C for 20 minutes. Vitamins and trace elements were added after autoclavation. The SSM and SSG media were made in 1L bottles and pH was adjusted to 7 by adding 2 M KOH. The media were then sterilized by filtration with 0.2 µm mixed ester cellulose membrane filter (Advantec®) and aseptically distributed to sterile 250 ml shake flasks. The final volume of each shake flask was adjusted to 100 ml and closed with cotton wool plugs.

5.A.2.3 Bacterial cultivation conditions

Four different conditions were tested: (1) SS supplemented with glucose (SSG), (2) SS supplemented with molasses (SSM), (3) minimal salt medium, and (4) minimal salt medium with reduced KH_2PO_4. Triplicate experiments were performed for each condition. The four experiments with different media were run one after another in the following order: minimal salt medium, minimal salt medium -reduced KH_2PO_4, SSG and SSM.

The media were inoculated with bacterial inoculum prepared in the enrichment media (Table 5.3). Prior to inoculation, the cells were washed 3 times in 0.85% NaCl and then were adjusted to the initial concentration of OD_{600} = 0.4-0.5. The flasks, containing media and bacteria, were incubated in a shaker incubator for 2 days (50°C, 150 rpm). Samples were collected periodically for bacterial growth and pH analysis. The bacterial growth was measured by observing the changes of optical density using UV vis spectrophotometer at wavelength 600 nm and pH was measured with Mettler Toledo™ EL20 Benchtop pH Meter. At the logarithmic and stationary growth phases, samples were taken for dry weight measurements of the cells.

5.A.2.4 Dry weight measurements

The dry weight measurements were performed by filtration through 0.22 µm cellulose acetate membrane (Sartorius). Prior to use, the membrane was dried in microwave oven for 10 minutes (180 W) and let to cool down inside a desiccator for 10-15 minutes. Then, the filter was weighed in an analytical balance and assembled in the filtration unit. The media containing bacteria cells were filtered through the membrane (5ml), followed by 3 times washing with MQ water (5 ml each time). The filter was dried in the microwave oven for 20 minutes (180 W) and was cooled down again inside a desiccator for 20 minutes. The filter was weighed again and thus the difference of the filter before and after bacteria filtration corresponds to the dry weight of the cells. Duplicate measurements were carried out for each sample.

5.A.3 Results

It has earlier been shown by Nazina et al.[1] that *B.licheniformis* 421 grows well and produces useful metabolites for MEOR in minimal salt medium under aerobic conditions. Our experiments confirm this observation and the final dry cell weight concentration was determined to about 2 g/L (Table 5.2). It was also observed that when growing in minimal salt medium, the bacteria turn the medium colour to reddish (Fig. 5.1), indicating formation of secondary metabolites. The effects of reducing KH_2PO_4 to a ten times lower concentration in the minimal salt medium on microbial growth was also tested. The OD was lower when the KH_2PO_4 was reduced (Fig. 5.2); however, the dry weight measurements showed that reduction of KH_2PO_4 did not change growth analysed as dry weight of bacteria (Table 5.2). It was also observed that reduction of KH_2PO_4, led to that the medium colour turned orange instead of reddish. This might be the reason for that the OD was lower in the minimal salt medium with reduced KH_2PO_4, because OD measurements are influenced by the background colour. It was concluded that reducing KH_2PO_4 is not a limiting factor for bacterial growth. In addition, the bacterial growth peaked at the same time, 10 hours after inoculation, for both the minimal salt media and the minimal salt media with reduced KH_2PO_4 concentration.

Fig.5.1 a) minimum salt medium, b) minimum salt medium-reduced KH_2PO_4 and c) SSG media inoculated with *B. licheniformis* 421 after 34 hours of incubation.

Further experiments were performed to investigate the effect of high salinity environment to bacterial growth. The concentrations of nutrients and their internal relative amounts, (C:N:P), supplemented in SSG and SSM were adjusted based on the respective concentration of these compounds in the minimal salt medium. However, it was found that adding phosphate to the synthetic seawater media, caused precipitation after a few hours of incubation at 50°C and hence only the reduced KH_2PO_4 conditions were investigated for the high salinity media. When *B. licheniformis* was grown is SSG media a long lag phase (9 hours) was seen. However, there was no significant difference in final optical density or bacterial biomass determined by dry weight compared to the base-line medium (Fig. 5.2 and Table 5.2). No colour change was observed when the bacteria grew in SSG.

Lastly, an experiment was conducted to check the effect of using molasses as carbon source instead of glucose. The amount of molasses added to the medium was adjusted based on the sucrose content to an equivalent glucose concentration in SSG. It was shown clearly that addition of molasses promoted the bacterial growth as a shorter lag phase (2 hours) and significantly higher OD/biomass dry weight were observed (Fig 5.2, Table 5.2). The bacterial growth peak at 21 hours in SSG and at 18 hours in SSM. The molasses is a complex medium and contains not only sugars, but also a range of other compounds such as amino acids/organic acids (Appendix 3) which could have resulted in the reduced lag time. This bacterial growth results were then used further in the core flooding experiments.

The bacterial inoculum for the core flooding experiments was prepared in SSM media and the inoculum age was adjusted to 18 hours.

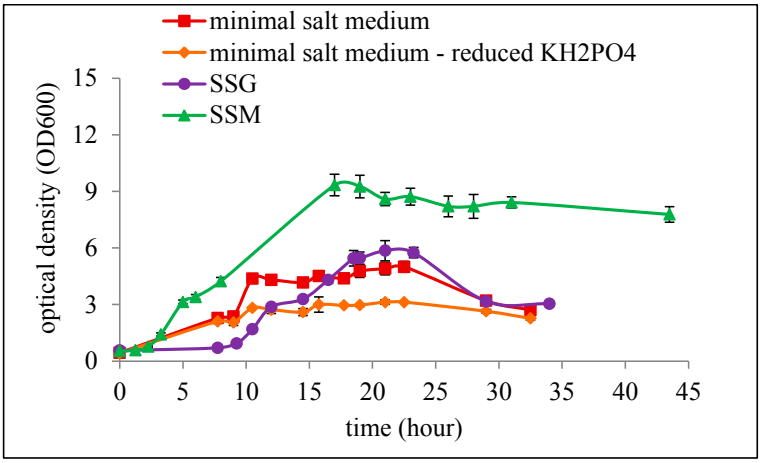

Fig.5.2 Microbial growth under aerobic conditions in (■) minimal salt medium, (◆) minimal salt medium with reduced KH_2PO_4, synthetic seawater based medium supplemented with (●) glucose (SSG), synthetic seawater based medium supplemented with (▲) molasses (SSM). Values presented are mean values of three independent experiments and error bars represent one standard deviation of the mean of triplicates

Table 5.2 Bacterial dry weight under aerobic conditions in different media (g/l)

Time	minimal salt medium	minimal salt medium - reduced KH_2PO_4	SSG	SSM
12	1.9 ± 0.1	2.1 ± 0.2	1.6 ± 0.1	3.5 ± 0.5
15.75	2.1 ± 0.4	2.1 ± 0.0	n/a	n/a
19	n/a	n/a	2.4 ± 0.2	3.8 ± 0.6
29	1.9 ± 0.4	2.0 ± 0.0	1.7 ± 0.1	4.2 ± 0.4

SSG = synthetic seawater based medium supplemented with glucose, SSM = synthetic seawater based medium supplemented with molasses

The pH of the media was rather stable around neutral conditions, with a tendency of increasing pH towards end of incubation period, except for the minimal salt medium cultivations. In these cultivations the pH decreased at the beginning of incubation, hereafter increased rapidly and the final pH was 8.9 (Fig. 5.3).

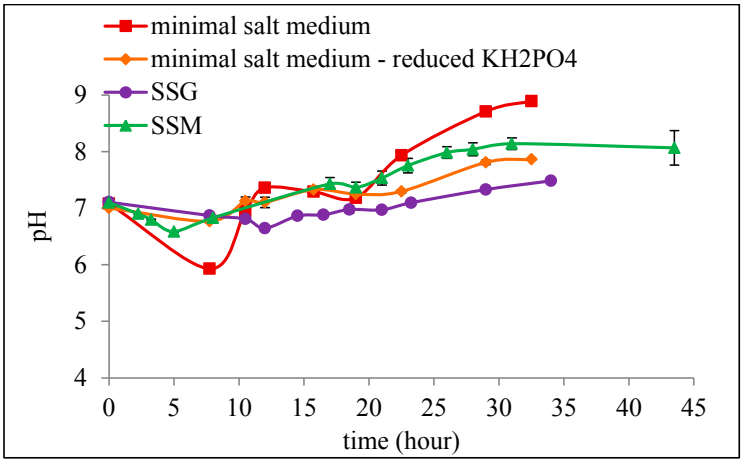

Fig.5.3 Fig. 5.3 pH of (■) minimal salt medium (MSM), (◆) MSM with reduced PO4, synthetic seawater based media supplemented with (●) glucose (SSG), synthetic seawater based media supplemented with (▲) molasses (SSM) during aerobic incubation with *B. licheniformis* 421. Values presented are mean values of three independent experiments and error bars represent one standard deviation of the mean of triplicates

5.A.4 Conclusions

From the results, it was found that *B. licheniformis* 421 is able to grow in high salinity conditions which corresponds to reservoir condition. However, salinity might affect different metabolites production as indicated by the difference in the media colour when the salinity was changed. If there is a concern on salts precipitation due to KH_2PO_4, this compound can be reduced to the current level at 0.10 g/L, because it does not limit the bacterial growth. The bacterium grows very well on glucose, but even better at molasses, and the lag phase was reduced markedly. Therefore, molasses was further used both in the core flooding and anaerobic growth studies. The results from the bacterial growth in SSM were then further used to prepare and determine the inoculum used for the core flooding experiments. It was decided that the age of inoculum for injection in the core flooding experiments was adjusted to 18 hours, which correspond to the late logarithmic phase.

Part B

B. licheniformis 421 anaerobic growth

Abstract

In this study, *Bacillus licheniformis* 421 was used as a model study organism to understand the effects of microbial cell, growth and their metabolic products under anaerobic conditions in relation to microbial enhanced oil recovery. The bacterium was able to grow anaerobically on different carbon compounds, where n-alkanes were preferred over molasses carbohydrates. The bacteria grew slowly when n-alkanes were used as carbon source, however, significant formation of emulsion and reduction of interfacial tension (IFT) were still observed. Furthermore, production of lipopeptides, lichenysin G, was observed. The bacterial cells were mainly present at the interfase of the synthetic seawater medium and n-alkanes. It was proposed that the bacterial cells themselves or the attached metabolites on the cell surface may play an important role in the formation of emulsions and IFT reduction.

5.B.1 Introduction

Production of bacterial biomass and metabolites, such as organic acids, lipopeptide surfactants, polymers, solvents, and gases may be useful to help recovering the residual oil in the depleted reservoirs. The technology is known as microbial enhanced oil recovery (MEOR), which involves injection of nutrients and/or microbes into the reservoir [4-6]. The mechanisms by which the growing microorganisms assist in oil recovery is not well understood and it is most likely that several microbial processes act cooperatively to enhance the flow of oil [5]. Organic acids can increase the permeability and form emulsions [6]; surfactants can promote formation of emulsions, reduce the interfacial tension (IFT) and simultaneously alter the wettability of the rock [6-8]; the bacterial biomass can change rock wettability, selectively block the high permeability zone and thus redirect the fluid flow to low-permeable oil bearing zones [6, 9]. It is important to gain a better understanding of the mechanisms by which microbial cells and the metabolites produced can help to improve oil production in the reservoir.

Particularly, the growth behavior of the bacteria on different nutrient mixtures composed of hydrocarbons and sugars; and which types and quantities of metabolites that are produced.

Biologically produced lipopeptide surfactants are believed to be able to lower the surface tension [10-13] and are useful for microbial enhanced oil recovery applications [10, 12]. Therefore, the study of biologically produced lipopeptide surfactants is important for successful application of MEOR. *B. licheniformis* is one of the Bacillus species that has been widely investigated for MEOR [1, 7, 9, 12, 14-16]. Armstrong and Wildenschild [9] performed pore-scale studies with *B. licheniformis* JF-2 for MEOR in a 3D glass bead pack micromodel. The bacterium is able to improve oil production as a result of both lipopeptide surfactant production and bioclogging of the pore space by the bacterial biomass. Al-Hattali et al. [14] showed that *B. licheniformis* W16 produces 27-30% of the residual oil in core flooding experiments using fractured Indiana limestone. Biria *et al*. [7], studied *B. licheniformis* MS3 that produces a lipopeptide called licheniformin. Oil production was improved due to IFT reduction and wettability alteration mechanism. It is also noted in their investigation that the bacterial cells themselves positively contribute to the addition of the cumulative oil productions. *B. licheniformis* TT33 [17] also produces polymer and surfactant. In addition, the biomass and the polymer can selectively plug the higher permeable zone recovering 27-32 % of the residual oil [17]. Yakimov et *al.*[15] found that *B. licheniformis* BAS50 produces extracellular polymers both under aerobic and anaerobic conditions. The bacillus strain is also able to live over a wide range of temperatures, pressures and salinities. In core flooding experiments they found that 9.3 to 22.1% of the residual oil in sandstone is produced [15].

B. licheniformis JF-2 is the most intensively studied of the oil-degrading *B. licheniformis* strains for MEOR. It has been shown to produce lipopeptide surfactant both aerobically [1, 11, 18] and anaerobically [10, 18]. Further investigations showed that the lipopeptide surfactant production by *B. licheniformis* JF-2 is higher at oxygen-limited conditions (30% dissolved

oxygen saturation) as compared with oxygen sufficient conditions (85% dissolved oxygen saturation) [19]. McInerney et al.[11] found that lipopetide surfactant produced by *B. licheniformis* JF-2 is more effective at high salinity and high temperatures. Increasing the NaCl concentration up to 50g/l or greater and raising the temperature to 50°C, lowers the IFT against octane. These two are the harsh conditions found in many oil reservoirs. This bacterium has also been tested in a cross-flow block system made from Berea sandstone to create layers with different permeability for studying the selective plugging mechanism. Flooding with this bacterium enhanced the oil recovery through selective plugging of the high permeability zone that alter the water path to the low permeable zone [16].

Emulsion formation is also considered to be an important factor that can affect fluid flow through porous media [20]. It was mentioned by Kaster et al. [20] that emulsions can be useful in MEOR as the emulsions may prevent or reduce re-trapping of mobilized oil. Microbes have been shown to form emulsions, either by production of surfactant [21, 22] or by attaching and surrounding hydrocarbon [23]. Kaster *et al.* [20] mentioned that in their investigation emulsification is related to the presence of biomass itself and that the amount of emulsification is propositional to the amount of biomass. In addition, studies by Rosenberg and Rosenberg [24] revealed that some microorganisms can emulsify hydrocarbons even without cell growth or uptake of hydrocarbons.

This work was conducted to understand the microbial processes that play a role in improving oil production and to support our core flooding results [25]. *B. licheniformis* 421 was used because it has been shown to reduce the IFT and form emulsions in the previous study by Nazina et al. [1]. However, the experiments conducted by Nazina et al. [1] was done in a medium with low salinity and under aerobic conditions. In our experiment, we modified the medium to mimic the North Sea reservoir environment with high salinity and the experiments were done anaerobically. These are considered to be important factors that have to be taken into consideration because the reservoir is anaerobic

by nature and often has a high salinity. In addition, it is most likely that MEOR is applied in reservoirs that have already been flooded with seawater.

5.B.2 Materials and methods

5.B.2.1 Chemicals and Solvents

Unless otherwise stated, all chemicals were analytical-grade and solvents were high-performance liquid chromatography (HPLC)-grade from Sigma-Aldrich (St. Louis, MO). Water was purified on a Milli-Q system (Millipore, Bedford, MA). Crude oil was obtained from Hess South Arne (HSA) field, North Sea. The molasses, which was kindly supplied by Nordic Sugar, contained 59% per total solids (TS) of sucrose and 8.46% per TS of various organic acids, where acetic acid and lactic acid were the most abundant acids (1.7% per TS and 2.5% per TS, respectively), and 32.54% per TS of various salts.

5.B.2.2 Bacteria sample

The strain *Bacillus licheniformis* 421 was obtained from Professor T. Nazina (Winogradsky Institute of Microbiology Russian Academy of Sciences). This bacterium is a facultative anaerobic, Gram positive, motile, spore forming, rod-shaped bacterium. It is isolated from a high temperature oil field in China and has previously been reported having a potential for MEOR application under aerobic conditions [1].

5.B.2.3 Media preparation

The chemicals (Table 5.3) were weighed with an analytical balance. The KH_2PO_4 and $NaHCO_3$ solutions were made as separate concentrated stock solutions in serum bottle vials. Medium pH was adjusted to 7 by adding 2 M KOH. The n-alkanes mixture was prepared volumetrically by mixing equal volume of C6-C10 compounds. The media were then boiled and flushed with nitrogen to generate anaerobic conditions. The bottles were sealed with butyl rubber stopper (Glasgerätebau Ochs GmbH, Bovenden, Germany) and closely tightened with Schott screw cap. Afterwards, the media were autoclaved at 121°C for 20 minutes

and stored at room temperature until use. Prior to inoculation, 0.5 ml $NaS.9H_2O$ (0.2M) was added as oxygen scavenger.

Table 5.3. Synthetic seawater (SS) based media composition

Chemical	Amount (g/l)			
	SSM	SSMA and Control	SSA	Enrichment media
NaCl	18.00	18.00	18.00	2.00
$NaHCO_3$	0.17	0.17	0.17	
KCl	0.74	0.74	0.74	
$MgCl_2.6H_2O$	9.15	9.15	9.15	
$CaCl_2.2H_2O$	1.91	1.91	1.91	
Na_2SO_4	3.41	3.41	3.41	
$NaNO_3$	2.00	2.00	2.00	
KH_2PO_4	0.10	0.10	0.10	
Molasses (59% sucrose)	16.90	16.90	-	
Vitamin [2]	1% (v/v)	1% (v/v)	1% (v/v)	
Trace elements [3]	1% (v/v)	1% (v/v)	1% (v/v)	
MOPS (buffer)	20.00	20.00	20.00	
n-alkanes	-	6% (v/v)	6% (v/v)	
Glucose				1.00
Yeast extract				2.50
Bacto® tryptone				5.00

SSM = synthetic seawater based media supplemented with molasses, SSMA = synthetic seawater based media supplemented with molasses and n-alkanes, SSA = synthetic seawater based media supplemented with n-alkanes

5.B.2.4 Bacterial cultivation conditions

The media used in this experiment were synthetic seawater (SS) based media supplemented with nutrients, vitamins, trace elements and different carbon sources (Table 5.3). Four different conditions were tested: (1) SS without bacteria (Control), (2) SS supplemented with molasses (SSM), (3) SS supplemented with molasses and n-alkane mixtures of C6-C10 (SSMA), and (4) SS supplemented with alkane mixtures (SSA). Hereafter, they will be referred to as Control, SSM,

SSMA and SSA, respectively. Triplicate experiments were performed for each condition. The bacterial inoculum was prepared in the enrichment media (Table 5.3) and then inoculated into SSM, SSMA and SSA media; and were adjusted to the initial concentration of OD_{600}=0.2. The 1L bottles, containing 950 ml medium and bacteria, were incubated in a shaker incubator for 7 days (50°C, 150 rpm). Samples were collected twice per day from the water phase for bacterial growth analysis and once per day for metabolite analysis. The bacterial growth was measured based on the changes of optical density using UV mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan) at the wave length 600 nm. Samples for organic acids (1ml) and sugar analysis (1ml) were filtered through 0.22 µm Qmax® syringe filter (Frisenette Aps., Knebel, Denmark) and directly frozen in -20°C until analysed. Samples for lipopeptide analysis (1ml) were directly frozen in -20°C until further analyses were performed. The bottles were purged with N_2 for 10 minutes every time samples were taken for analysis to keep the system anaerobic. Nitrate in the water phase was monitored by Merkoquant® nitrate strips (Merck KGaA, Darmstadt, Germany).

5.B.2.5 Analysis of sugars, hydrocarbons, metabolic products and bacteria-fluids interactions

Sugar concentrations were analysed using IC5000 ion chromatography system (Dionex, Sunnyvale, CA, USA) with PAD detection with AminoPac PA10 column and a standard gold electrode. A linear gradient concentration of 0-80% NaOH (250 mM) was applied as mobile phase and the flow rate was set to 0.25 mL min^{-1}.

Samples for organic acids analysis were purified by solid phase extraction (SPE) on bond elute® C8 column (Varian, Harbor City, CA, USA). Prior to use, the C8 SPE column were conditioned with 1 ml methanol followed by 1 ml MQ water. Sample, 1 ml, was loaded on to the C8 SPE column and eluted with further 3 ml water. A subsample of the 4 ml was then transferred into auto sampler vials and were analysed by Agilent 1100 High Performance Liquid Chromatography (HPLC) (Agilent Technologies, CA, USA), with refractive index detector (RID

Agilent 1200, Agilent Technologies, CA, USA) and UV detector (Agilent Technologies, CA, USA) set to 210 nm. Pyruvate, succinate, glycerol, acetate, lactate and ethanol were analysed using an Aminex HPX-87H cationic-exchange column (BioRad, Hercules, CA, USA) eluted at 60°C, with 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1}.

Unless otherwise stated samples for lipopeptide analysis were extracted using 2-butanol (1:2 v/v) in 15 ml polypropylene centrifuge tubes (VWR, Philadelphia, PA, USA). The tubes were put in a shaking table for 15 min to allow the samples to be in contact with 2-butanol. The samples were subsequently centrifuged (4000xg, 2 min, 20°C). Later on, 2 ml of the upper phase of the supernatant from each vial was transferred into a new tube and dried under nitrogen. The samples were re-dissolved in 1ml isopropanol, mixed with a vortex and centrifuged (4000xg, 2 min, 20°C). Approximately 0.5 ml of the samples in each vial was transferred into an auto sampler vial.

Lipopeptides were analysed by ultra high performance liquid chromatography (UHPLC) on a Dionex Ultimate 3000 UHPLC system (Thermo Scientific) equipped with a 100 x 2.1 mm, 2.6 µm, Kinetex C_{18} or C_8 column (Phenomenex, Torrance, CA, USA) held at a temperature of 60°C and eluted using a linear water-acetonitrile gradient system at a flow of 0.4 ml/min. The gradient started at 30 % acetonitrile and was increased to 100% in 10 min, holding this for 3 min before reverting to start conditions in 1 min and equilibrating for 4 min. UHPLC was interfaced to a maXis HD quadrupole time of flight mass spectrometer (QTOFMS) (Bruker Daltonics, Bremen, Germany). The instrument was equipped with an electrospray ionization source operated in positive mode, and was either tuned in low mass range *m/z* 100–1,000 or high mass mode *m/z* 500–3000 [26].

Lipopeptides were identified from their $[M+H]^+$ and $[M+Na]^+$ ions and quantified from peak areas from the extracted ion chromatograms of the $[M+H]^+$ ± 5 mDa. The concentrations of lipopeptides were calculated based on a standard curve

prepared for surfactin (Sigma-Aldrich, St Louis, MO, USA). Five concentration levels were made from a stock solution of 1mg/ml surfactin.

Total weight fraction of n-alkanes was analysed using Agilent 7890A gas chromatograph with flame ionization detector (GC-FID), equipped with 10 m x 0.53 mm id, 2.65 µm film, 125-10HB (DB-1) column (Agilent, Santa Clara, CA, USA). The analysis was set according to ASTM method D2887 [27]. Calibration of the retention time was conducted using Agilent 5080-8768 containing solution of C5-C18 (Agilent, Santa Clara, CA, USA)

Synthetic seawater media vs. n-alkane mixtures were analysed under Olympus BX40 phase contrast microscopy to confirm the presence of microbes at the interfase. The microscope was equipped with a CoolSNAP camera connected to a computer. Approximately 1 ml of sample was taken from the interfase of the synthetic seawater media and n-alkanes. One drop of crystal violet was added into the sample and gently mixed with a micropipette for one minute. Subsequently, 10 µl of the sample was put on a glass slide, covered with a cover slide and observed under the microscope. At least 20-30 different random spots on the glass slide were observed for each sample.

5.B.2.6 Density, viscosity, interfacial tension (IFT) and emulsifying activity analysis

The n-alkanes density was measured by DMA 4100 density meter (Aston Paar GmbH, Graz, Austria) at 50 °C and viscosity was measured by AMV200 microviscometer (Aston Paar GmbH, Graz, Austria) equipped with a water bath at 50°C. The interfacial tension (IFT) between crude oil and brine was measured by the drop volume method using a 500 µl syringe (Hamilton, Bonaduz, Switzerland) connected to an inverted needle (Hamilton, Bonaduz, Switzerland) and a goniometer (Mitutoyo, Hiroshima, Japan) at 20°C. IFT measurements of brine against n-alkane mixtures from the flask were conducted every day, except for day 3 due to some logistic issue. In addition, on day 0, 4, 5 and 7 the IFT of the spent media (media from incubations containing bacteria and metabolites) was

measured against Hess South Arne (HSA) crude oil. The emulsifying activity was analysed at the end of experiment using the spent media and HSA crude oil as described by other investigators [1, 28]. Emulsifying activity was expressed as the ratio of the emulsion volume to the total volume of the mixture in per cent [1, 28].

5.B.3 Results

5.B.3.1 Microbial growth, substrate degradation and metabolite production

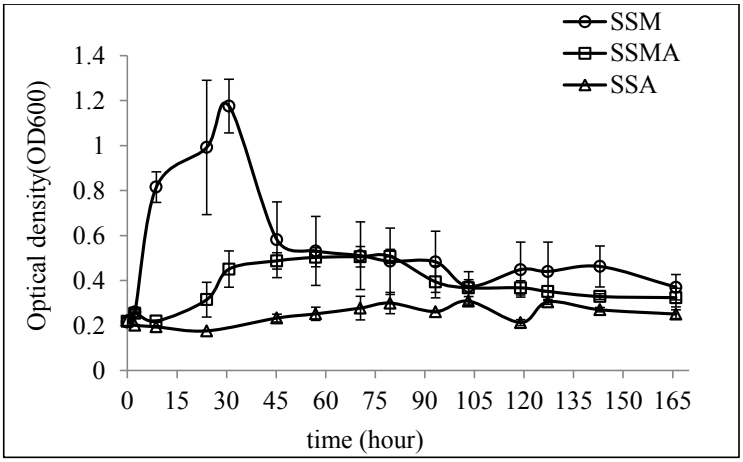

Fig.5.4 Microbial growth based on OD measurements under anaerobic conditions in synthetic seawater based medium supplemented with 2 g/l $NaNO_3$, 0.1 g/l KH_2PO_4, and different carbon sources: (○) molasses (SSM), (□) molasses + n-alkanes mixture (SSMA), (△) n-alkanes mixture (SSA).

Bacillus licheniformis 421 grew differently in the in the presence of different carbon sources (Fig. 5.4). In the two media containing n-alkanes, both in synthetic seawater medium supplemented with molasses and n-alkane mixtures (SSMA) and synthetic seawater medium supplemented with n-alkane mixtures (SSA), lower bacterial growth and lower final biomass were observed based on the OD measurements. In the synthetic seawater supplemented with molasses (SSM), the bacterial growth peaked at 30 hours and rapidly decreased afterwards. It was noted that in SSM cultivations after 24 hours of incubation, clumping of bacterial cells was observed directly by eye and the clumps disappeared again after 45

hours. In the SSMA and the SSA cultivations, clumping of bacterial cells was only seen in the interfase of the water and n-alkanes under microscope.

There was a lag phase for 15 hours in the bacterial growth in the SSMA although the growth also reached its peak at 30 hours. The bacterial growth in SSMA then remained relatively stable until 80 hours and hereafter declined. In the SSA, the optical density remained relatively stable throughout the incubation period, indicating that there was no significant change in cell numbers throughout the incubation. However, a small OD decrease was noted at 119 hours (Fig.5.4).

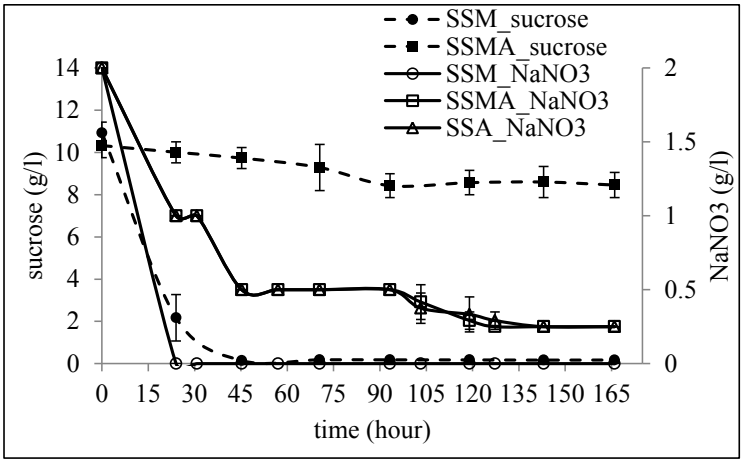

Fig.5.5a Sucrose and $NaNO_3$ consumption under anaerobic conditions by B. licheniformis 421 in synthetic seawater based media supplemented with (○, ●) molasses (SSM), (□, ■) molasses + n-alkanes mixture (SSMA), and (△) n-alkanes mixture (SSA), where full and empty symbols refer to sucrose and $NaNO_3$, respectively.

The sugar analysis performed in SSM and SSMA showed that sucrose from the molasses was hydrolysed into glucose and fructose (Fig 5.5a). In SSM, sucrose was rapidly hydrolysed within the first 24 hours of incubation, with an average rate of 0.37 g l^{-1} h^{-1} and no sucrose was detected after 45 hours of incubation. In SSMA, sucrose was hydrolysed slowly until 93 hours, with an average rate of 0.02 g l^{-1} h^{-1}. Hereafter, the sucrose concentration remained relatively stable at 8.5 g l^{-1}. The bacteria were able to use both fructose and glucose as indicated by no

accumulation of these monosaccharides (Fig. 5.5b). Nitrate was used as electron acceptor in all the three incubations (Fig. 5.5a) as indicated by the decrease of nitrate concentration in the media. Nitrate was rapidly consumed and disappeared after 24 hours in SSM. In SSMA and SSA, nitrate was consumed within the first 45 hours, but remained relatively stable from 45 hours to 90 hours. Hereafter, nitrate slightly decreased again.

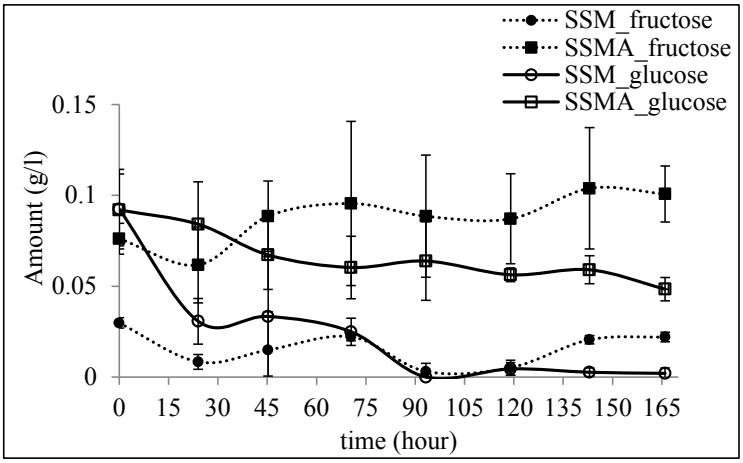

Fig. 5.5b Fructose and glucose consumption under anaerobic conditions by *B. licheniformis* 421 in synthetic seawater based media supplemented with (○, ●) molasses (SSM), and (□, ■) molasses + n-alkanes mixture (SSMA), where full and empty symbols glucose and fructose concentration, respectively.

In the SSA incubation, concentrations of hexane (C6) and heptane (C7) decreased rapidly in the first 93 hours. An increase of hexane and heptane was noted at 119 hours, but hereafter they decreased again. In SSMA, both C6 and C7 also decreased overtime (Fig. 5.5c). The consumption rate of C6 was slightly faster in SSMA compared with SSA, but overall the growth on hydrocarbon was slower than on molasses. No decrease of C8-C10 in both SSMA and SSA (data not shown). In control experiment, there was a minor decrease in C6 while C7-C10 remained relatively stable (Fig. 5.5c).

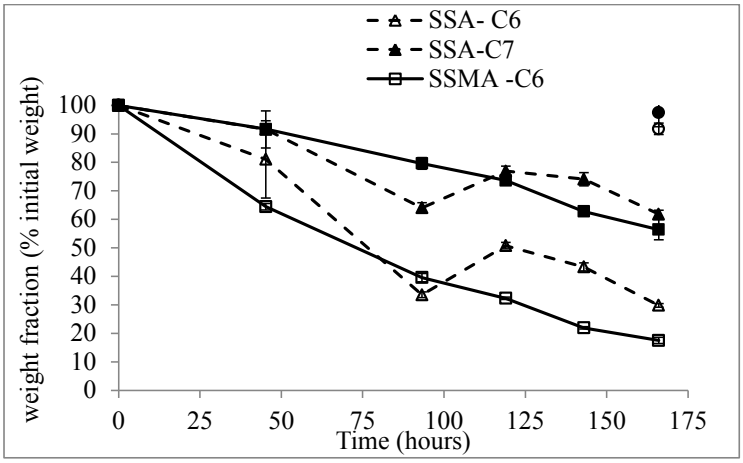

Fig. 5.5c Weight fraction of n-hexane (C6) and n-heptane (C7) during anaerobic incubation by *B. licheniformis* 421 in synthetic seawater based media supplemented with (□, ■) molasses + n-alkanes mixture (SSMA), (△, ▲) n-alkanes mixture (SSA), and (○, ●) control, where empty and filled symbols refer to C6 and C7, respectively.

The HPLC analyses confirmed that organic acids were produced (Fig. 5.6a and 5.6b) in the medium containing molasses. In SSM, lactate and acetate were the main fermentation products (Fig. 5.6a and 5.6b). Acetate was produced only within the first 24 hours, with a rate of 0.06 g l^{-1} h^{-1} and lactate (Fig. 5.6a) and was produced only in the first 45 hours with a rate of 0.09 g l^{-1} h^{-1} (Fig. 5.6b). Glycerol was produced in very low amount, with a rate of 0.001 g l^{-1} h^{-1} in 119 hours and no production thereafter (Fig 5.6c). In SSMA, only acetate was produced, however, in a very low amount, with an average rate of 0.002 g l^{-1} h^{-1} in the first 45 hours and afterwards no more acetate was produced (Fig. 5.6a). There was no pyruvate, succinate, and ethanol production both in SSM and SSMA. There were no organic acids, glycerol and ethanol detected in SSA and control (data not shown). The synthetic seawater media supplemented with molasses (SSM and SSMA) contained noticeable traces of lactate from the molasses (Fig 5.6b). There was a slight pH decrease in SSM, from 7 to 6.5. The pH was relatively stable near neutral conditions in SSMA and SSA (Fig 5.6d).

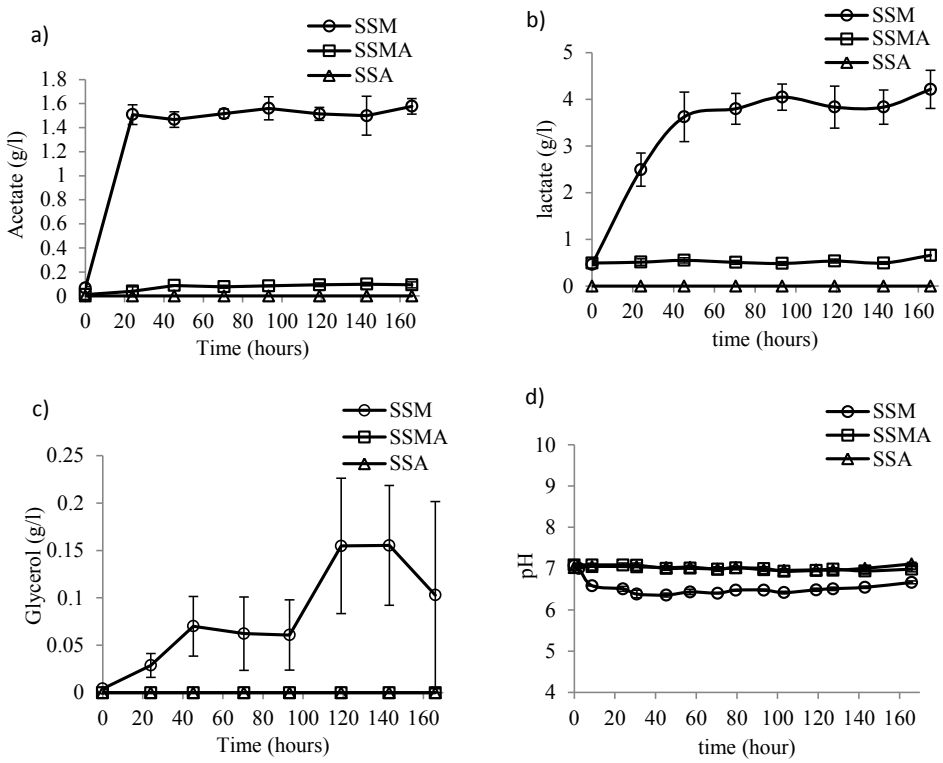

Fig.5.6 Production of a) acetate, b) lactate and c) glycerol and d) pH changes by *B. licheniformis* 421 under anaerobic conditions in synthetic seawater based media supplemented with ○ molasses (SSM), □ molasses + n-alkanes mixture (SSMA), and △ n-alkanes mixture (SSA).

The UHPLC-QTOFMS analysis revealed that the lipopeptide produced in the three different conditions was lichenysin G, however, it was produced with different rates (Fig 5.7). Extraction of the lipopeptides from the water phase showed that in SSM, there seemed to be a delay in lipopeptide production before the concentration peaked at 9.6 mg l^{-1} after 45 hours. In SSMA and SSA, lipopeptide surfactant was produced already from the beginning, where it was detected in the water phase within the first 3 hours. However, the concentration was in almost four times lower compared to SSM. In SSMA, the lipopeptide concentration peaked at 2.5 mg l^{-1} after 24 hours. In SSA, the highest

concentration at 2.1 mg l^{-1} was found after 70 hours. There was a tendency of lipopeptide disappearance towards the end of the incubation period.

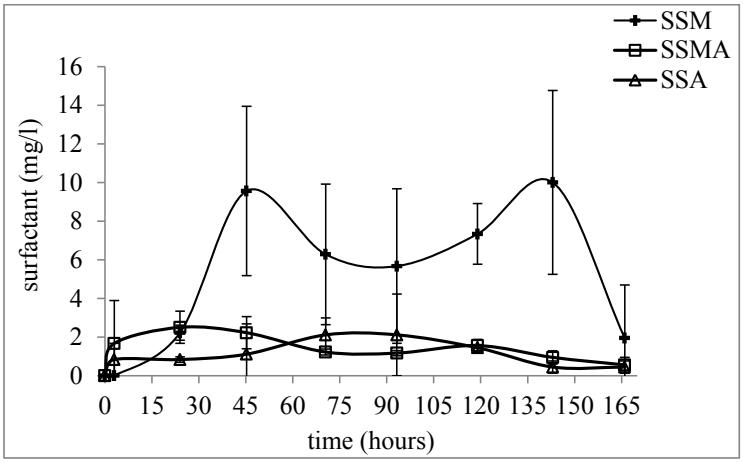

Fig.5.7 Surfactant produced under anaerobic conditions by *B. licheniformis* 421 at 50°C. Surfactant extracted from water phase of the synthetic seawater based media supplemented with (○) molasses (SSM), (□) molasses + n-alkanes mixture (SSMA), and (△) n-alkanes mixture (SSA).

Analyses of the lipopeptide samples from the emulsions/oil phase of the media supplemented with n-alkane mixtures (SSMA and SSA) at the last time point showed that lipopeptide was detected in the emulsion phase and it was found in larger amounts when compared relatively to the amount detected in the oil phase (Table S1).

Gas production was observed in all flasks in the three different incubations. This was indicated by overpressure every time samples were taken for analysis. Furthermore, visible emulsions were seen in SSMA and SSA (Fig 5.8). In SSA, visible emulsions started to form after 3 hours of incubation and the media colour turned from colourless into yellow. In SSMA, visible emulsions were only observed after 45 hours of incubation. It was also noted that in SSMA the media colour turned from light brown to dark brown. In the control experiment, no emulsions were formed and the colour did not change (Fig 5.8).

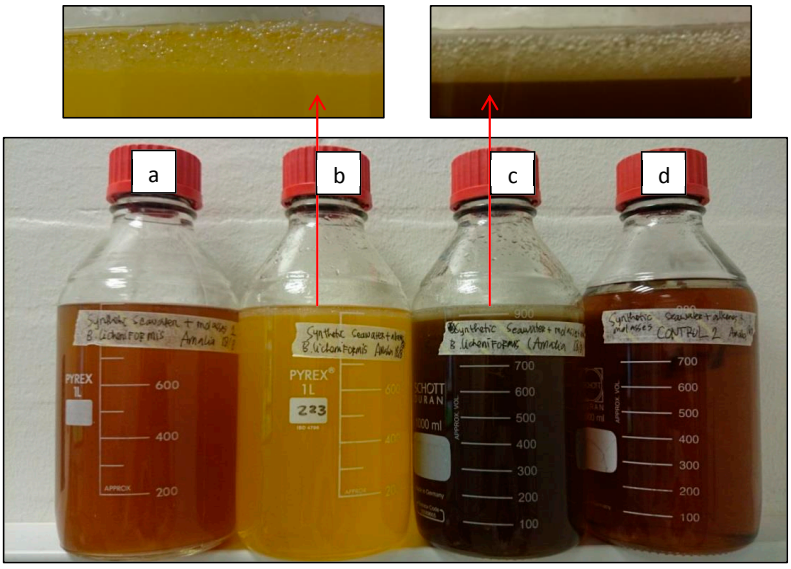

Fig.5.8 Synthetic seawater based media supplemented with (a) molasses (SSM), (b) molasses + n-alkanes mixture (SSMA), (c) n-alkanes mixture after 166 hours of incubation with *B. licheniformis* 421 and (d) control-no bacteria. Insert pictures on the top are magnification of the emulsions in SSA (left) and SSMA (right).

5.B.3.2 Effect of bacterial growth and metabolites on synthetic seawater/n-alkanes mixture

There was no significant change in crude oil density (Table 5.4) and viscosity (Table 5.5) before and after incubation in the three different incubations. This indicates that bacteria did not produce significant biomass and/or metabolites to modify the synthetic seawater and n-alkanes. In SSA, there was a major change in the interfacial tension (IFT) of the synthetic seawater vs. n-alkanes mixture from the incubation. In SSA, the IFT was 26.3 mN/m at the beginning of the incubation, and rapidly decreasing to 0.6 mN/m after 93 hours of incubation. However, IFT increased again towards the end of the incubation (Fig. 5.9). In SSMA, IFT of the synthetic seawater vs. n-alkanes mixture was more fluctuating with an initial value of 21.2 mN/m and an end value 16.0 mN/m (Fig. 5.9).

Table 5.4. Density of synthetic seawater and n-alkanes mixtures at 50°C

	Synthetic seawater density (kg/m3)		n-alkanes density (kg/m3)	
	T0	T166	T0	T166
Control	1032.7 ± 0.0	1030.9 ± 0.0	710.3 ± 0.0	707.5 ± 0.0
SSM	1032.4 ± 0.0	1031.4 ± 0.0	n/a	n/a
SSMA	1033.0 ± 0.0	1030.9 ± 0.0	707.2 ± 0.0	708.8 ± 0.0
SSA	1032.7 ± 0.0	1029.9 ± 0.0	707.5 ± 0.0	707.5 ± 0.0

SSM = synthetic seawater based media supplemented with molasses, SSMA = synthetic seawater based media supplemented with molasses and n-alkanes, SSA = synthetic seawater based media supplemented with n-alkanes

Table 5.5. Viscosity of synthetic seawater and n-alkanes at 50°C

	synthetic seawater viscosity (cP)		n-alkanes viscosity (cP)	
	T0	T166	T0	T166
Control	0.70 ± 0.0	0.69 ± 0.0	0.48 ± 0.0	0.48 ± 0.0
SSM	0.67 ± 0.0	0.70 ± 0.0	n/a	n/a
SSMA	0.68 ± 0.0	0.68 ± 0.0	0.50 ± 0.0	0.54 ± 0.0
SSA	0.69 ± 0.0	0.68 ± 0.0	0.49 ± 0.0	0.54 ± 0.0

SSM = synthetic seawater based media supplemented with molasses, SSMA = synthetic seawater based media supplemented with molasses and n-alkanes, SSA = synthetic seawater based media supplemented with n-alkanes

Subsequently, the IFT was measured again between the synthetic seawater vs. Hess South Arne (HSA) crude oil (Fig. 5.9). This was done because we observed that at the start of incubation, bacteria present as single cells in the synthetic seawater media (Fig. 5.10a and Fig. 5.10b) and there was a tendency of bacteria being present at the interfase of the two liquids in the incubations containing n-alkanes (Fig. 5.10c and 5.10d). In addition, the bacteria also tend to form clumps in the presence of n-alkanes (Fig. 5.10c and 5.10d). Another advantage of repeating the measurement using HSA crude oil is data from the SSM incubation can be included for comparison. There was only a minor reduction when the IFT was measured against HSA crude oil in the three different incubations.

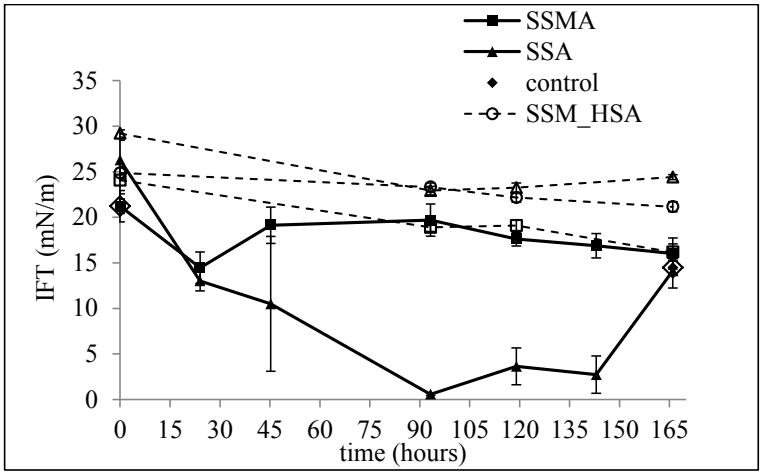

Fig.5.9 Interfacial tension (IFT) changes by *B. licheniformis* 421 under anaerobic conditions in synthetic seawater based media supplemented with (□, ■) molasses + n-alkanes mixture (SSMA), (△, ▲) n-alkanes mixture (SSA), (○) molasses (SSM) and (◇, ◆) control. Full lines and dashed lines refer to IFT of synthetic seawater media vs. n-alkanes mixtures from incubation and synthetic seawater media vs. HSA crude oil, respectively.

The emulsifying activity tests showed that the spent media used and HSA crude oil formed emulsions. Emulsifying activity in control experiment was relatively stable from the start of incubation until end of incubation after 166 hours. In SSM and SSMA, increased emulsifying activity was observed (Table 5.6). The emulsifying activity in SSM was increasing from 6% to 20% and in SSMA from 5% to 29%. On the contrary, in SSA there was only a decrease in emulsifying activity from 7% to 3%.

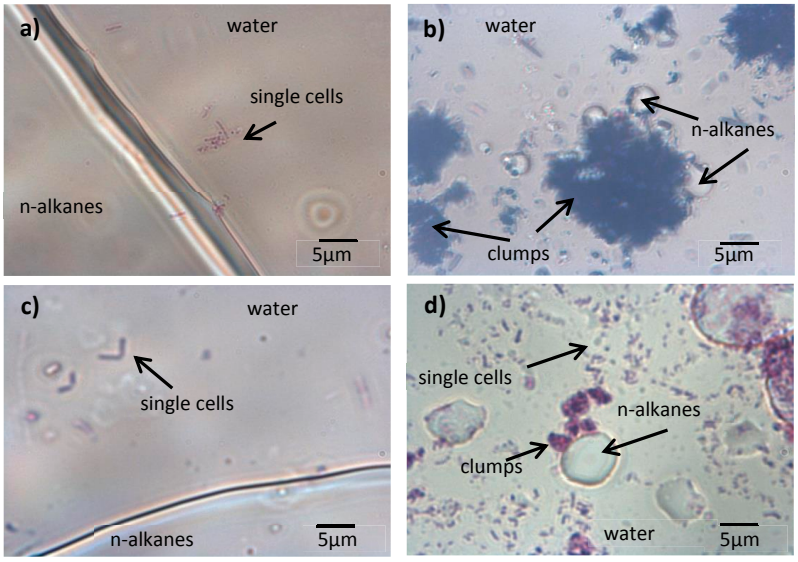

Fig.5.10 Bacteria cells stained with crystal violet under phase contrast microscope in synthetic seawater based media supplemented with n-alkanes (SSA) at (a) T0 and (b) at T166 hours; in synthetic seawater based media supplemented with molasses and n-alkanes (SSMA) at (c) T0 and (d) at T166 hours. There was a tendency of bacterial accumulation at the water/n-alkanes interfase and formation of clumps when bacteria attached to the n-alkanes.

Table 5.6. Emulsifying activity of the synthetic seawater vs. HSA crude oil at 20°C

	Emulsifying activity (%)	
	T0	T166
Control	4.69 ± 0.53	4.21 ± 0.65
SSM	5.79 ± 0.44	19.86 ± 1.92
SSMA	4.93 ± 0.28	28.93 ± 3.10
SSA	7.16 ± 1.12	3.09 ± 1.67

SSM = synthetic seawater based media supplemented with molasses, SSMA = synthetic seawater based media supplemented with molasses and n-alkanes, SSA = synthetic seawater based media supplemented with n-alkanes

5.B.4 Discussion

Our results showed that *B. licheniformis* 421 can grow in high salinity environment under anaerobic conditions using both molasses and n-alkanes as carbon source. In the presence of the n-alkanes, the bacteria grew at significantly lower rate. However, the phase contrast microscope showed *B. licheniformis* 421 preferred to stay at the interface meaning that there was a possibility that a fully representative sample of cells was not obtained from the media containing n-alkanes for determination of cell optical density. The bacteria formed visible clumps in synthetic seawater media supplemented with molasses (SSM). Formation of bacterial clumps in the water phase was not observed when the bacteria were grown aerobically in the same media (not shown). Likewise, the phenomena that the bacteria form clumps in the presence of hydrocarbon is also observed by Afrapoli et al. [29]. The authors found that *Rhodococcus* sp. 094 culture grown on dodecane tend to form clumps and the culture grown on acetate are present as single cells. However, unlike our study, different carbon sources change the ability of *Rhodococcus* sp. 094. to produce the surface-active compounds claimed to be surfactants. In the absence of hydrocarbon (dodecane), the bacterium lost its ability to produce surfactant [29]. The bacteria used in our study produced a lipopeptide, lichenysin G, in both presence and absence of hydrocarbons (n-alkane mixtures). Lichenysin G has been reported to be a potential surfactant [30] having a CMC 22 mg/l, which is similar to surfactin with CMC at 20-40 mg/l [31]. The pH remained relatively stable in SSM despite the fact that organic acids were produced in considerable amounts; this could be attributed to the efficient MOPS buffer system used.

Microbial fermentation products such as organic acids and solvents are believed to contribute to some of the possible MEOR mechanisms that can improve oil recovery [4-6]. The question is whether these compounds are produced in sufficient amounts in the oil reservoir to induce an effect. It was shown in the experiment with synthetic seawater supplemented with molasses and n-alkanes (SSMA) that only small amounts of organic acids were produced. Correspondingly, it is clear that only small amounts of sucrose from the molasses

was used. This was attributed to the fact that *B. licheniformis* 421 prefers to use hydrocarbon (n-alkanes) instead of sugar compounds when they are present in the same batch under anaerobic conditions (Fig. 5.5a - 5.5c). The results also confirmed that the bacterium, *B. licheniformis* 421, used in this experiment is a hydrocarbonoclastic type bacterium that is able to degrade hydrocarbons [22], as shown by the decreasing amount of C6 and C7 (Fig. 5.5c). In addition, the bacteria used nitrate as electron acceptor in the absence of oxygen. The ability of facultative, nitrate-reducing strains to degrade hydrocarbon was not surprising since it has been mentioned in several reports [22, 32-34].

It was also observed in our study that the bacteria stayed at the interface between synthetic seawater and n-alkanes. According to Rojo [22], bacteria need to adhere to hydrocarbon droplets to gain access to the medium- and long-chain-length n-alkanes. The n-alkanes metabolism by microorganism is challenging due to the low water solubility nature of the n-alkanes, and thus, they tend to accumulate in the cell membranes [22]. If microbial cells lyse, these compounds might be released again. We speculated this could be the reason for the increase of C6 and C7 in the synthetic seawater supplemented with n-alkane mixtures (SSA) incubation at 119 hours. In addition, the bacterial growth data also support this hypothesis, since the OD reduction was noted observed at 119 hours, indicating a decrease in the bacterial cell number.

In literature, many bacterial effects are proposed to contribute to IFT reduction, such as production of lipopeptide surfactants [4, 6, 10] and solvents [10]. It has been investigated widely that surfactant produced by microbes contribute to the reduction of IFT [10, 11, 13]. Javaheri [10] mentioned that alcohols (solvent), a commonly bacterial end product, can act as cosurfactant to enhance the effectiveness of surfactants due to alcohol-surfactant interactions. No solvent (alcohol) was detected in our study; therefore, the reason for IFT reduction was to some extent due to lichenysin G. The question is whether microbes produce enough surfactant or other surface-active compounds such as lipopetides that can facilitate a markedly reduction of the IFT. In oil recovery, the IFT should be

decreased at least up to two orders of magnitude in order to achieve a significant change in the capillary number to mobilize the trapped-oil in formation [35]. Our results showed that only low amounts of lipopetide surfactant were produced in combination with a limited reduction of IFT when sugar was present. The low lipopetide surfactant production might be due to the fact that this compound is not related to the primary metabolism of the cell [12].

Still, IFT of the synthetic seawater vs. n-alkanes decreased up to two orders of magnitude in SSA incubation, but the IFT increased again towards the end of incubation period (after 93 hours onwards). A similar phenomenon is also observed in the studies by Lin et al. [13] and Yakimov et al. [18]. It was mentioned by Lin et al. [13] that reduction of the lichenysin B concentration in the culture broth (water phase) the reason for this phenomenon. In our study, it was not clear what could be the main reason. This finding emphasized the importance of suitable incubation time before the disappearance of surfactant from the 'water phase'. This will give important input on the approximate time for the 'shut in' period during core flooding experiment.

The lipopeptide found in the emulsion phases were also confirmed to be lichenysin G. It is most likely that the hydrophobic lipopeptides are found on proteins and the surface of the cell. The fact that the bacteria prefer to stay in the interface and form emulsion, makes it no surprise that this compound was found in the emulsion phase.

The emulsifying activity is higher in the SSM and SSMA media. This could be because in SSA, n-alkanes mixture is the sole carbon source and the bacteria prefer to stay at the interface; and thus not a sufficient number of cells was present in the water phase of the spent medium. On the other hand, cells in the SSMA were distributed both at the interface and in the water phase entailing that a fraction of the cells from the water phase were present during the emulsifying activity test.

Formation of emulsions was also observed in our experiments. The emulsion formation and IFT reduction are closely related. Kowalewski et al.[8] stated that the emulsification indicates a local reduction of IFT in the limited zones where microbes grow. Rojo, F. [22] mentioned that most n-alkanes degraders secrete different surfactants that facilitate emulsification of the hydrocarbons. However, in our study, whether the lipopetide, lichenysin G, is fully responsible for the emulsification or act as surfactant seems to be questionable with the low concentration detected.

Kaster et al. [20] stated that microbial cells themselves play an important role in changing the oil/brine interactions, not necessarily their metabolites. In SSMA and SSA, there is noticeable emulsion formation even though only small amounts of bacterial metabolites were produced. Microbes can behave as fine solid particles at the interfaces and facilitate the formation of stable emulsions [23]. In our study, the emulsification occurred even when low lipopeptide concentrations were detected in the water phase in combination with limited IFT reduction. This could be attributed to the bacteria themselves behaving as particles or that local IFT reductions take place due to surfactants such as lipopeptides could be attached to the bacterial surface. However, the emulsifying activity in our experiment was not as high as what has been reported by Nazina et al. [1]. Our experiments were carried out under anaerobic conditions evoking differences in the metabolism and results in a lower number of bacterial cells [1].

5.B.5 Conclusions

Our results demonstrated that the model study bacterium, *B. licheniformis* 421, was able to use various carbon sources under anaerobic conditions. The bacteria preferred to use hydrocarbon (n-alkanes) instead of sugar compounds, and nitrate was used as electron acceptor. Correspondingly, only small amounts of organic acids were produced in the media containing n-alkanes. In the presence of n-alkanes, the bacteria formed clumps and accumulated at the hydrocarbon-water interface. Under anaerobic conditions, the bacteria produced lichenysin G, a lipopeptide that may act as surfactant and contribute to formation of emulsions

even though its concentration in the water phase was low. The IFT was markedly reduced and emulsions were formed when the bacteria were grown on n-alkanes (SSA). In addition, bacteria themselves may play an important role in formation of emulsions, even in the cases where limited IFT reduction takes place. The results showed that for the bacteria being able to use hydrocarbons, emulsion formation and IFT reduction are mechanisms that may be important for MEOR.

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Table S1. Peak area of the surfactant from the emulsion/oil phase of the media supplemented with n-alkanes

Samples	Peak area
SSMA (emulsion phase)	$1.5 \times 10^5 \pm 7.6 \times 10^4$
SSA (emulsion phase)	$7.2 \times 10^4 \pm 2.4 \times 10^4$
SSMA (oil phase)	n/a
SSA (oil phase)	1.0 ± 0
Control	ND

SSMA = synthetic seawater based media supplemented with molasses and n-alkanes, SSA = synthetic seawater based media supplemented with n-alkanes. n/a = samples was not available because stable emulsions were formed and not possible only take samples of oil phase, ND = not detected.

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Paper 3

Halim, A., D.S. Pedersen, S. M. Nielsen and A. E. Lantz, 2015. Profiling of Indigenous Microbial Community Dynamics and Metabolic Activity during Enrichment in Molasses-Supplemented Crude Oil-Brine Mixtures for Improved Understanding of Microbial Enhanced Oil Recovery. *Applied Biochemistry and Biotechnology*. April 2015, DOI:10.1007/s12010-015-1626-y

CHAPTER 6

Profiling of Indigenous Microbial Community Dynamics and Metabolic Activity during Enrichment in Molasses-Supplemented Crude Oil-Brine Mixtures for Improved Understanding of Microbial Enhanced Oil Recovery

Abstract

Anaerobic incubations using crude oil and brine from a North Sea reservoir were conducted to gain increased understanding of indigenous microbial community development, metabolite production and the effects on the oil-brine system after addition of a complex carbon source, molasses, with or without nitrate to boost microbial growth. Growth of the indigenous microbes was stimulated by addition of molasses. Pyrosequencing showed that specifically *Anaerobaculum*, *Petrotoga* and *Methanothermococcus* were enriched. Addition of nitrate favored the growth of *Petrotoga* over *Anaerobaculum*. The microbial growth caused changes in the crude oil-brine system: formation of oil emulsions, and reduction of interfacial tension (IFT). Reduction in IFT was associated with microbes being present at the oil-brine interphase. These findings suggest that stimulation of indigenous microbial growth by addition of molasses has potential as microbial enhanced oil recovery (MEOR) strategy in North Sea oil reservoirs.

Key words: MEOR, anaerobic, oil emulsion, pyrosequencing, microbial enrichment

6.1 Introduction

The study of microbes for enhanced oil recovery was credited to Beckman in 1926, who found that microorganism could be used to release oil from porous media [1-3]. In 1947, Zobell and his group started a series of systematic studies on the application of microorganism for enhanced oil recovery (EOR) [2, 4]. The microbial enhanced oil recovery (MEOR) research was boosted by the oil crisis in the 1970s and later became a substantial EOR method supported by research projects worldwide. Despite its long history, MEOR is still not widely accepted in the industry. This is because of unpredictable and contradictory results of MEOR

due to lack of published scientific data on field reports and analysis [1, 4], as well as limited cooperation between microbiologist, reservoir engineers, geologists, economists and owner operators [2, 4]. Despite several reviews on the field application, there is still lack of knowledge on microbial metabolic processes in order to generate useful products or activities in sufficient amounts and rates needed for significant oil recovery [4]. Moreover, many of the published results used mixtures of hydrocarbon degraders or unspecified bacteria or "biocatalysts" making it difficult to provide a scientific assessment of the technology [4].

The MEOR technology relies on growth of microbes either indigenous [2, 3, 5, 6] or exogenous [2, 3, 6-9] to the reservoir, that can produce useful substances [2-4, 6-10]. These substances, such as gasses, organic acids, solvents, and surfactants, are able to affect the properties of the formation water, oil, and gas mixture(s), as well as change the properties of the matrix of the reservoir and thus play a significant role in the recovery of residual oil [6]. Growth of microbes is stimulated by addition of nutrients (carbon, nitrogen, phosphorus). The most commonly used carbon source is molasses [2, 3, 6, 10] and it is recommended that the sugar content in the molasses should be no less than 40% [6]. Molasses is a waste product from sugar cane processing and hence is a low cost substrate [2]. Molasses fermentation typically yields a large quantity of metabolites (90%) and a small amount of biomass (10%) [3].

The present work is an investigation to evaluate the potential of the indigenous microbes of the Dan oil field in the Danish North Sea sector for MEOR application. The Dan field is located in the southwestern corner of the Danish North Sea, 205 km west of the port of Esbjerg and approximately 150 km south of the Ekofisk fields in the Norwegian sector of the North Sea. The reservoir rock type is mainly chalk with high porosities and very low permeabilities. Oil recovery from the field is based on the simultaneous production of oil and injection of water to maintain reservoir pressure. Water injection was initiated in 1989, and has gradually been extended to the whole field [11]. The published data on profiling of indigenous microbial communities in North Sea oil reservoirs has

mainly focused on investigation of microbial souring mitigation [12-17]. It has been reported that microbes from the genera *Thermotoga*, *Caminicella*, *Thermoanaerobacter*, *Archaeoglobus*, *Thermococcus*, and *Methanobulbus* are native inhabitants of a high temperature and fractured chalk Ekofisk oil reservoir in the North Sea [12]. The bacterial community in Dan oil field produced water has been shown to include members of *Firmicutes*, *Deltaproteobacteria*, *Desulfotomaculum* and some unidentified bacteria, with *Firmicutes* being the most abundant group (70%) [16]. The native archaeal community in the same produced water consisted of members of the orders *Thermococcales*, *Methanococcales* and *Archaeoglobus*, with *Thermococcales* being the most abundant group (60%).

The objective of this work was to determine and quantify what metabolites, useful for MEOR, that are produced by the indigenous microbes and to observe the development of the microbial community after addition of molasses with and without nitrate. The effect of nitrate was investigated because nitrate can in addition to being a nitrogen source also be used as an alternate electron acceptor and thus alter the microbial community composition [10]. Furthermore, to verify how microbial activity affects properties important for improved oil recovery, interfacial tension (IFT) and emulsification were analyzed throughout the incubations.

6.2 Material and Methods

6.2.1 Sample description

The brine and crude oil were sampled from the Dan oil field. The brine was sampled from a high pressure separator; therefore it contained microbes from various wells with seawater breakthrough and without seawater breakthrough.

6.2.2 Molasses

The molasses, which was kindly supplied by Nordic Sugar, contained 59% per total solids (TS) of sucrose and 8.46% per TS of various organic acids, where acetic acid and lactic acid were the most abundant acids (1.7% per TS and 2.5%

per TS, respectively), and 32.54% per TS of various salts. The molasses was diluted with Milli-Q water (1:1) and serial filter sterilized using Millipore^{TM} sizes 5 µm, 3 µm, 1.2 µm, 0.8 µm, 0.45 µm and final filtration with 0.2 µm mixed ester cellulose membrane filter (Advantec®).

6.2.3 Enrichment test incubation conditions

The enrichment tests were conducted anaerobically in 500 mL bottles, capped with butyl rubber stopper (Glasgerätebau Ochs, Germany) and closely tightened with Schott screw cap. Approximately 425 ml brine was transferred from the plastic container used for sampling using silicon tubing, then 50 ml crude oil was added and the bottles were closed and purged with pure N_2 for 10 minutes. In order to ensure that the system was anaerobic, 0.025% (w/v) Na_2S was added using a syringe. Bottles were incubated for 28 days (55°C, 1atm) and were shaken at 150 rpm. Three different conditions were carried out: (A) Control without any nutrient addition, (B) addition of 2% (v/v) molasses, and (C) addition of 2% (v/v) molasses and 2 mM sodium nitrate (Fluka). Hereafter, they will be referred to as incubation A, incubation B and incubation C, respectively. Samples were collected for analysis at day 0, 3, 7, 14, 19 and 28. Triplicate experiments were performed for each incubation and the replicate number was put in the brackets after the sampling time to point out variation that might have taken place at the different incubations throughout the experiment. Hence sample 3 (2), correspond to the sample taken at day 3 from replicate no 2. The bottles were purged with N_2 for 5 minutes every time samples were taken for analysis to keep the system anaerobic. Nitrate in incubation C, was monitored by Merkoquant® nitrate strips (Merck KGaA Germany, cat no 1.10020.001). When low concentration of nitrate was detected, a concentrated nitrate solution was added to keep nitrate concentration in incubation C at ca. 2.5 mM.

6.2.4 Analysis of sugars, hydrocarbon and metabolic products

Sugar content was analyzed using a Dionex IC5000 ion chromatography system with PAD detection with AminoPac PA10 column and standard gold electrode. A

linear gradient concentration of 0-10% NaOH (250 mM) was used as mobile phase and the flow rate was set to 0.3 mL min^{-1}.

Organic acids were analyzed using Agilent 1100 High Performance Liquid Chromatography (HPLC) systems with DAD and RI detection. Pyruvate, succinate, glycerol, acetate and ethanol were analyzed using an Aminex HPX-87H cationic-exchange column (BioRad, Hercules, CA, USA) eluted at 60°C, with 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1}.

Total weight fraction of hydrocarbons was monitored using Agilent 7890A gas chromatograph with FID detector, according to ASTM method D6352. This method covers hydrocarbons with boiling points up 700 °C, which is the boiling point of C94. The method is not applicable to fractions with carbon numbers lower than C10. External standard was used for calibration of the retention time for C10-C94. Calibration was done in two steps in order to identify the retention times of the fractions with the carbon numbers ranging from C10 to C94. Firstly, a known solution of C5-C18 (Agilent 5080-8768) was injected into the gas chromatograph, from which the corresponding retention time could be obtained. Secondly, a solution of 10 mg of Polywax 655 (Agilent 5188-5317) was injected with 1.5 ml of toluene to identify the retention times of carbon numbers C20 to C94. All carbon numbers below C10 was calculated as C10 with this particular method.

6.2.5 Crude oil analysis

The crude oil density was measured by DMA 4100 density meter (Aston Paar) at 55 °C. The crude oil viscosity was measured by AMV200 microviscometer (Aston Paar) equipped with a water bath at 55 °C. Viscosity of samples from incubation B – with addition molasses, at day 19 (2); and incubation C – with addition of molasses and sodium nitrate, at day 19 (1), 19 (2), 28 (1), and 28 (3) could not be measured because the microbial biomass aggregates/crude oil emulsion plugged the capillary glass tubing used for the measurement. At the last sampling point, day 28, crude oil viscosity was also measured after removal of

microbial biomass. In addition, crude oil-brine mixtures were also analyzed under Olympus phase contrast microscopy to confirm the presence of microbes in the interphase. At least 20 different random spots were observed, however, pictures were only taken from the spots where meaningful results were noticed. The interfacial tension (IFT) between crude oil and brine was measured by the drop volume method using a 500µl syringe (Hamilton-Bonaduz, Schweiz) connected to an inverted needle (Hamilton) and a goniometer (Mitutoyo, Japan) equipped with a water bath at 55 °C. IFT measurements were only conducted at day 0 and day 28. On day 28, the IFT was measured both with and without microbes' presence at the crude oil-brine interphase. For the cell-free viscosity and IFT measurements, microbes were removed by centrifugation at 10.000xg for 5 minutes at 4°C. In addition, general observations such as formation of emulsion and gas were done during sampling. Emulsion was clearly seen as a layer of thin oil droplets in between brine and oil phases and gas production was observed by over-pressure during sampling.

6.2.6 DNA extraction and qPCR amplification

Subsamples of 3-10 ml of the water phase from the incubations were filtered through a 0.2 µm filter in order to collect microbial cells on the filter surface. DNA was subsequently extracted from the cells using the FastDNA™ SPIN Kit for Soil (Cat# 6560-200; MP Biomedicals) as described by manufacturer, which includes mechanical disruption of filter and cells by bead beating. The DNA concentration was evaluated on Nanodrop ND-1000 spectrophotometer (Saveen Werner) and diluted to a concentration of 10-20 ng/µl. Bacteria 16S rRNA genes were quantified by quantitative polymerase chain reaction (qPCR) performed in an Mx-3005P cycler (Agilent Technologies) using forward primer 341F [18] and reverse primer 1390R [19]. Archaea 16S rRNA genes were quantified with forward primer 806F [16] and reverse primer 958R [16]. Duplicate 20 µl qPCR reactions were performed containing: 10 µl Brilliant II SYBR® QPCR Master Mix with Low Rox (Cat# 600830, Agilent Technologies) containing polymerase and dNTPs, 2 µl of 10 µg/µl BSA (Sigma), 0.88 µl 10 µM of each forward and reverse primer, 2 µl template DNA and 4.24 µl autoclaved distilled water. The

PCR was performed under the following conditions: Initial denaturation at 95 °C for 10 min, followed by 40 cycles of 30 sec at 95°C, 1 min at 55°C and 1 min at 72 °C. Bacterial and archaeal 16S rRNA copy numbers were calculated from a reference standard curve on plasmid, containing one copy of the 16S rRNA gene of either *Eschericia coli* or *Methermicoccus shengliensis*. Due to logistic constraint only end-point samples (day 28) were collected in triplicates for microbiological analyses (qPCR and sequencing). At the remaining sampling time points (day 3-19) only one sample from each incubation was collected. However, on incubation C – with addition of 2% (v/v) molasses and 2 mM sodium nitrate, duplicate or triplicate samples were taken when variations in chemical data among triplicates were observed.

6.2.7 Microbial diversity (sequencing)

The DNA extracted for qPCR was also sequenced. Archaea 16S rRNA and bacteria 16S rRNA genes were amplified in two successive PCRs. In the first PCR, primer 341F [17] and 1390R [18] were used for amplification of the bacteria 16S rRNA gene, and primer Arch8F [19] and 958R [15] for amplification of archaea 16S rRNA genes. The PCR was performed in a G-Storm GS1 thermocycler (Sopachem) in 25 µl reactions containing: 0.5 µl Hotstar High Fidelity (Qiagen), 5 µl supplied reaction buffer, 1.25 µl of 10 µM solution of each primer, 2 µl DNA extract and 15 µl autoclaved distilled water. The PCR was carried out under the following conditions: Initial denaturing at 95°C for 5 minutes followed by 20-35 cycles (sample variation) of denaturation at 95 °C for 15 sec, annealing at 55 °C for 30 sec, elongation at 72 °C for 30 sec, and a final elongation at 72 °C for 10 min. After confirming the presence of PCR products by agarose gel electrophoresis, a second PCR was performed on the amplicons to introduce adaptors and specific MID sequences. Primer pyroA-926R-XX, with modification of adaptor and MID sequence from Liu *et al.*[20] and pyroB-343F, with modification of adaptor sequence from Nossa *et al* [21] were used for bacterial amplicons. Primer pyroA-519F, with modification of adaptor and MID sequence from Wang *et al.* [22] and pyroB-915R, with modification of adaptor sequence from Stahl *et al*. [23] were used for archaeal amplicons. Detailed primer

specifications are available as supplementary materials (Table S1). The second PCR was performed as described above for the first PCR except for using 1 µl of 10 µM solution of each primer and increasing the amount of autoclaved distilled water. 2 µl of 1:100 or 1:1000 diluted DNA from the first PCR was used as template. Amplicons containing adaptors were subsequently processed at Macrogen (Korea) where DNA was purified, pooled in equimolar amounts and subjected to emulsion PCR and subsequent to 454-pyrosequencing using Roche GS FLX sequencing platform. Samples from incubation C – with addition of molasses and nitrate, at day 28 (2) and day 28 (3) could not be amplified with neither bacteria nor archaea pyrosequencing primers; samples from incubation B – with addition of molasses, at day 7 (3) and day 14 (3) could not be amplified with archaea pyrosequencing primer. Samples from incubation A-control, at day 7 (3), day 28 (3) and incubation C – with addition of molasses and nitrate, at day 3 (3), day 7 (3) for the bacterial population analysis and samples from incubation A-control at day 28 (3) and incubation B – with addition of molasses, at day 19 (3) and day 28 (1) for the archaeal population analysis resulted in poor number of sequence reads and were therefore discarded for further analysis.

6.2.8 Analysis of sequence data

The sequence reads obtained were uploaded into the software package Quantitative Insights Into Microbial Ecology v1.6.0 (QIIME) [20] and processed using default parameters unless otherwise stated. Sequence reads less than 400 bp and/or a quality score below 25 were discarded. In order to compare samples at equal sequencing depth, samples were normalized using the rarefaction script to 1118 bacteria reads and 1772 archaea reads in each sample, samples with lower number of reads were discarded. Sequence reads were binned into operational taxonomic units (OTUs) using UCLUST [21] with shared identity threshold of 97%, as this level of identity is considered to represent species level. One representative sequence from each OTU was aligned to the Greengene classifier [22] with PyNAST with a minimum identity of 75%. UniFrac distances were calculated and generated data summaries of the proportions of taxa present and

PCoA plots based on UniFrac distances. Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier 2.2 [23].

6.3 Results

6.3.1 Microbial growth, substrate degradation and metabolite production

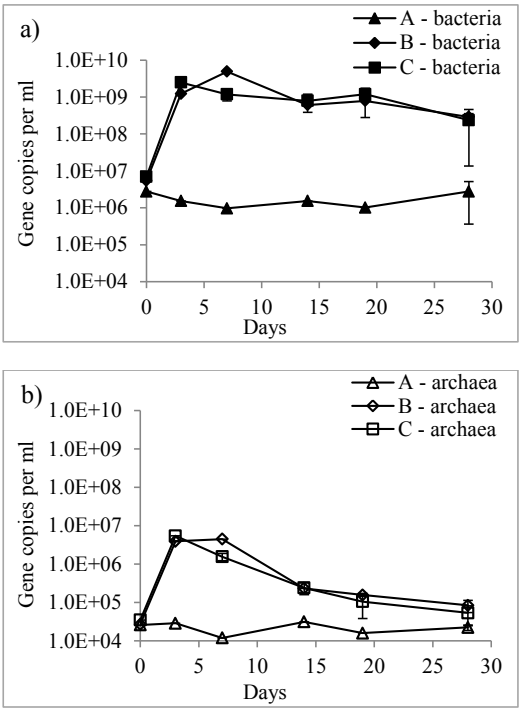

Fig.6.1 Abundance of a) bacteria 16S rRNA genes and b) archaea 16S rRNA genes in samples retrieved from incubations A (filled triangle, empty triangle), control; incubations B (filled diamond, empty diamond), with addition of molasses; and incubations C (filled square, empty square), with addition of molasses and nitrate. The filled and empty symbols refer to bacteria and archaea, respectively. At each sampling time point, qPCR was performed in duplicates on DNA extracted from one of the replicates of incubations A, B, and C, except for day 28 where qPCR was performed in duplicates on DNA extracts from all three replicates

The initial microbial population and the microbial growth during incubation were analyzed with qPCR targeting the 16S rRNA genes of bacteria and archaea,

respectively. It was observed that the initial number of bacteria 16S rRNA genes was two orders of magnitude higher than the number of archaea 16S rRNA genes. Addition of molasses to the crude oil-brine mixture stimulated growth of the indigenous microbes as a significant increase in numbers of bacteria and archaea 16S rRNA genes occurred in incubations B- with addition of molasses and incubation C with addition of molasses and nitrate, within the first 3 days of incubation (Fig. 6.1a and 6.1b). The abundance of bacteria and archaea 16S rRNA genes peaked at day 7 in incubation B-with addition of molasses and at day 3 in incubation C-with addition of molasses and nitrate; and hereafter declined throughout the incubation period. In incubation A-control, the numbers of bacteria and archaea 16S genes were relatively stable throughout the incubation period, indicating that there was no overall increase in cell numbers.

Correspondingly, the substrate analysis revealed that sucrose from the molasses was hydrolyzed into glucose and fructose in incubations B-with addition of molasses and incubation C-with addition of molasses and nitrate. Sucrose hydrolysis was fastest in the first 3 days of incubation (Fig. 6.2a). Incubation B-with addition of molasses, had an average sucrose hydrolysis rate of 0.26 g l^{-1} day^{-1} until day 3; hereafter the hydrolysis rate decreased over time and reached 0.10 g l^{-1} day^{-1} on day 28. Incubation C-with addition of molasses and nitrate, had an average sucrose hydrolysis rate of 0.34 g l^{-1} day^{-1} up to day 3; where after the hydrolysis rate decreased over time and reached 0.02 g l^{-1} day^{-1} on day 28. After day 7, glucose and fructose accumulated in both incubations (Fig. 6.2b). This is most likely resulting from that the enzyme produced to hydrolyze sucrose was available at a faster rate compared to the subsequent uptake of fructose and glucose by microbes.

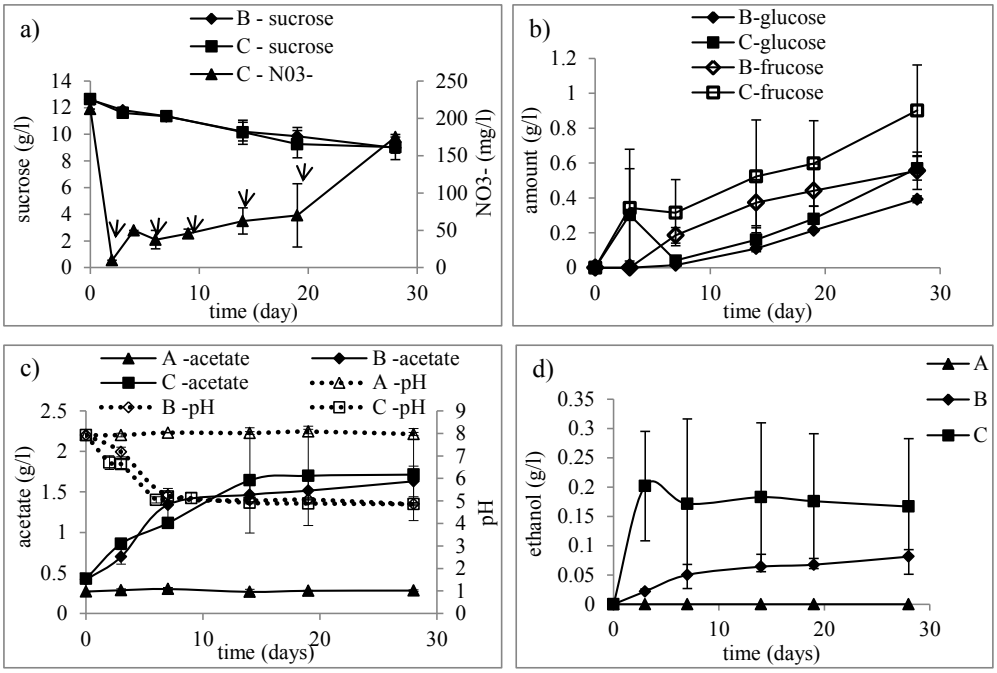

Fig.6. 2 a) Sucrose concentration inincubations B (filled diamond), with addition of molasses, and incubations C (filled square), with addition of molasses and nitrate. Nitrate concentration in incubation C (filled triangle) over time. Black arrows show the time when depleted nitrate concentration was observed and concentrated nitrate was added to keep nitrate level at ca. 2.5 mM. **b)** Glucose and fructose concentration in incubations B (filled diamond, empty diamond), with addition of molasses, and incubations C (filled square, empty square), with addition of molasses and nitrate, where empty and filled symbols refer to fructose and glucose, respectively. **c)** Acetate concentration and pH in incubations A (filled triangle, empty triangle), control; incubations B (filled diamond, empty diamond), with addition of molasses; and incubations C (filled square, empty square), with addition of molasses and nitrate, where empty and filled symbols refer to pH and acetate, respectively. No acetate was produced in incubation A. **d)** Ethanol concentration in incubations A (filled triangle), control; incubations B (filled diamond), with addition of molasses; and incubations C (filled square), with addition of molasses and nitrate. No ethanol was produced in incubation A. Value presented are mean values of three independent experiments and error bars represent one standard deviation of the mean of triplicates

In incubation C-with addition of molasses and nitrate, it was observed that nitrate was depleted over time (Fig. 6.2a). Concentrated nitrate was added on day 2, 6, 9, 14, 19 (black arrow) to maintain nitrate in the incubation bottles at a concentration of ca. 2.5 mM. Gas production was observed in all flasks for incubations with molasses added (incubation B and incubation C) as overpressure every time samples were taken for analysis. Furthermore, a visible stable crude oil emulsion was seen in all samples from these incubations. The whole oil layer turned into stable emulsion on day 3 of incubation and the crude oil color changed from dark brown into light brown. The emulsion layer was approximately 0.5-0.7 cm for the triplicates of incubation B-with addition of molasses and 0.8-1.0 cm for the triplicates of incubation C-with addition of molasses and nitrate. Further visual observation showed that after day 9, the oil turned into small droplets hence the crude oil-brine became one phase when shaken. There was no visible stable emulsion observed in incubation A-control flasks without nutrient addition. However, microscopy investigation showed that some formation of crude oil-brine emulsion also took place in incubation A (Fig. S1).

The brine used for the incubations contained traces of acetate (0.2 g/l) as evident from the acetate detected in incubation A- control (Fig. 6.2c). There was no production of acetate and ethanol in incubation A-control (Fig. 6.2c-d). Acetate and ethanol production were detected in flasks from incubations B-with addition of molasses and incubation C-with addition of molasses and nitrate. This indicates that fermentative microbes were growing in these flasks. The highest acetate production rate in incubation B-with addition of molasses, was between day 4-7 with a rate at 0.16 g l^{-1} day^{-1}; and in incubation C-with addition of molasses and nitrate, between day 0-3 with a rate of 0.14 g l^{-1} day^{-1} (Fig. 6.2c). The highest ethanol production rate in incubation B-with addition of molasses, took place between day 0-7 with a rate of 0.007 g l^{-1} day^{-1}; and in incubation C-with addition of molasses and nitrate, between day 0-3 with a rate of 0.067 g l^{-1} day^{-1} (Fig. 6.2d). Concentration of acetate and ethanol in incubation C-with addition of molasses and nitrate, replicate no 2 is slightly higher than replicate no 1 and 3. Therefore, the average concentration in incubation C is slightly higher than in

incubation B-with addition of molasses, even though there was no significant difference in microbial growth in incubation B-with addition of molasses and C-with addition of molasses and nitrate. There was no pyruvate, succinate, lactate or glycerol detected in any of the samples from incubations B-with addition of molasses and incubation C-with addition of molasses and nitrate. The pH was stable at 8.0 ± 0.1 in incubation A-control, while the pH decreased to 4.8 in incubations B-with addition of molasses and incubation C-with addition of molasses and nitrate (Fig. 6.2c). The analysis of formation of metabolites confirmed the qPCR data that microbial growth did not take place in incubation A-control.

The total carbon weight fraction of the crude oil was measured on day 0, 3, 7, 14, 19, and 28. In general no significant change of the oil composition was observed (Fig. S2).

6.3.2 Density, viscosity and interfacial tension of the crude oil

Crude oil density (Fig. 6.3a) and viscosity (Fig. 6.3b) increased over time in all flasks from incubations A-control, B-with addition of molasses, and C-with addition of molasses and nitrate. After day 19 viscosity measurement became problematic, and for some samples even impossible to be measured, as the microbial biomass /crude oil emulsion caused plugging of the measurement equipment. Removal of biomass made it possible to measure the crude oil viscosity and it was observed that the viscosity value was the same with and without the presence of microbial biomass (Fig. 6.3b, day 28).

The interfacial tension (IFT) of the crude oil vs. brine was up to one order of magnitude lower when microbes were present in the crude oil-brine interphase. The IFT at the start of experiment (day 0) were 19.29 ± 0.12 mN/m, 18.79 ± 0.18 mN/m, 18.60 ± 0.9 mN/m for incubation A-control, B-with addition of molasses, and C-with addition of molasses and nitrate, respectively. The IFT at the end of experiment (day 28) with biomass were 17.70 ± 1.48 mN/m, 2.34 ± 0.15 mN/m,

2.96 ± 0.43 mN/m for incubation A-control, B-with addition of molasses, and C-with addition of molasses and nitrate, respectively.

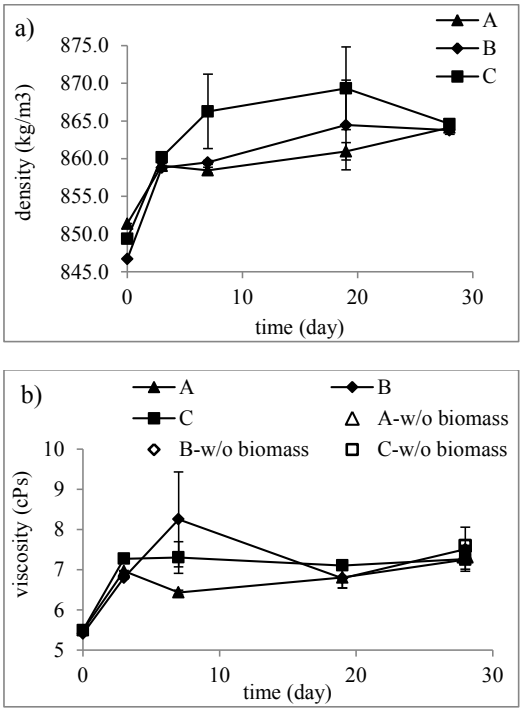

Fig.6.3 a) Crude oil density in incubations A (filled triangle), control; incubations B (filled diamond), with addition of molasses; and incubations C (filled square), with addition of molasses and nitrate. **b)** Crude oil viscosity in incubations A (filled triangle, empty triangle), control; incubations B (filled diamond, empty diamond), with addition of molasses; and incubations C (filled square, empty square), with addition of molasses and nitrate, where empty and filled symbols refer to viscosity without and with microbial biomass, respectively. Values are mean values of three independent experiments and error bars represent one standard deviation of the mean of the triplicates

The IFT at the end of experiment (day 28) without biomass were 15.20 ± 0.32 mN/m, 21.65 ± 0.15 mN/m, 22.60 ± 0.88 mN/m for incubation A-control, B-with addition of molasses, and C-with addition of molasses and nitrate, respectively. It was also observed under phase contrast microscope that in the incubation A-control without any additional carbon source, microbes had a tendency to form

clumps or aggregates. Addition of an easy accessible carbon source, such as molasses, in incubations B-with addition of molasses and C-with addition of molasses and nitrate resulted in microbes being present as single cells (Fig. S6.1).

6.3.3 Microbial diversity and dynamics deducted from 16S rDNA gene sequencing

The microbial community structure and dynamics during the 28 days of incubation was analyzed by pyrosequencing of the bacteria and archaea 16S rRNA genes in samples collected from the incubations. An overview of the development in bacterial and archaeal community structure during the incubation period is shown in Fig. 6.4 and Fig. 6.5. The bacterial population is presented at phylum level. Since all archaea detected belonged to the phylum *Euryarchaeota*, the archaeal community is presented at a lower taxonomic rank; the order level. The most abundant bacterial and archaeal OTUs in the samples are summarized in Table S6.2 – S6.5 (available as supplementary materials).

At incubation start, bacteria belonging to the phyla *Firmicutes* and *Proteobacteria* dominated the community composition in the crude oil-brine mixture of all incubations (Fig. 6.4a-6.4c). Since the oil and brine of all incubations originated from the same sampling bottle that was mixed before transferring to incubation bottles, the difference in relative proportions of *Firmicutes* and *Proteobacteria* in incubation A-control, incubation B-with addition of molasses, and incubation C-with addition of molasses and nitrate mainly represents variation due to the applied methods. The members of *Firmicutes* and *Proteobacteria* were marine and petroleum reservoir habitats comprising genera such as *Desulforomonas* and *Marinobacter* (both *Proteobacteria*) as well as *Halanaerobium* and members of the *Clostridicea* family (both *Firmicutes*) that could not be resolved at genus level (Table S6.2-S6.5). Members of the phyla *Thermotoga, Synergistetes, Spirochaetes, Chloroflexi, Bacteroidetes, Actinobacteria* and *Acidobacteria* were detected as minor populations (up to 5%) in samples from day 0.

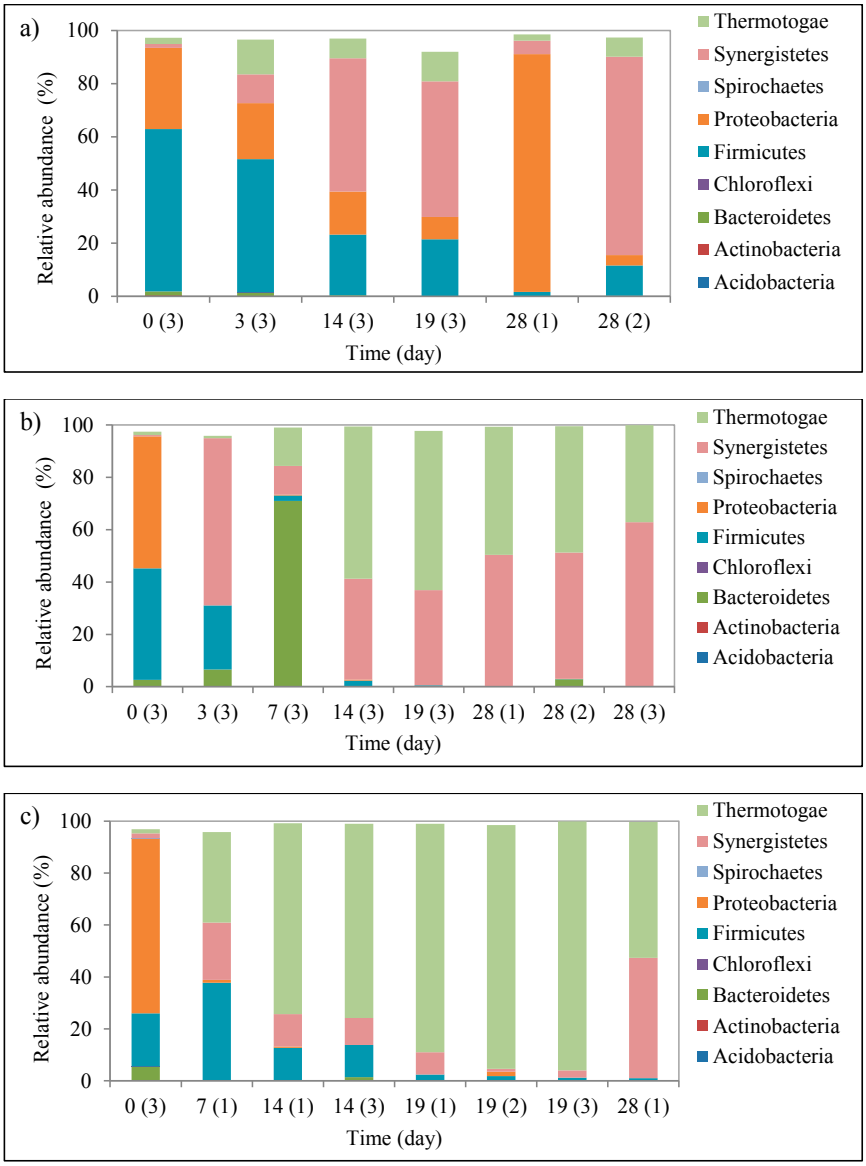

Fig.6.4 Development in bacterial community at phylum level after normalization of sequence reads in **a)** incubations A, control; **b)** incubations B, with addition of molasses; and **c)** incubations C, with addition of molasses and nitrate. White space represents unclassified sequence reads and low-abundance phyla. The number in the brackets following the sampling time refers to the specific flask where the replicates are taken form

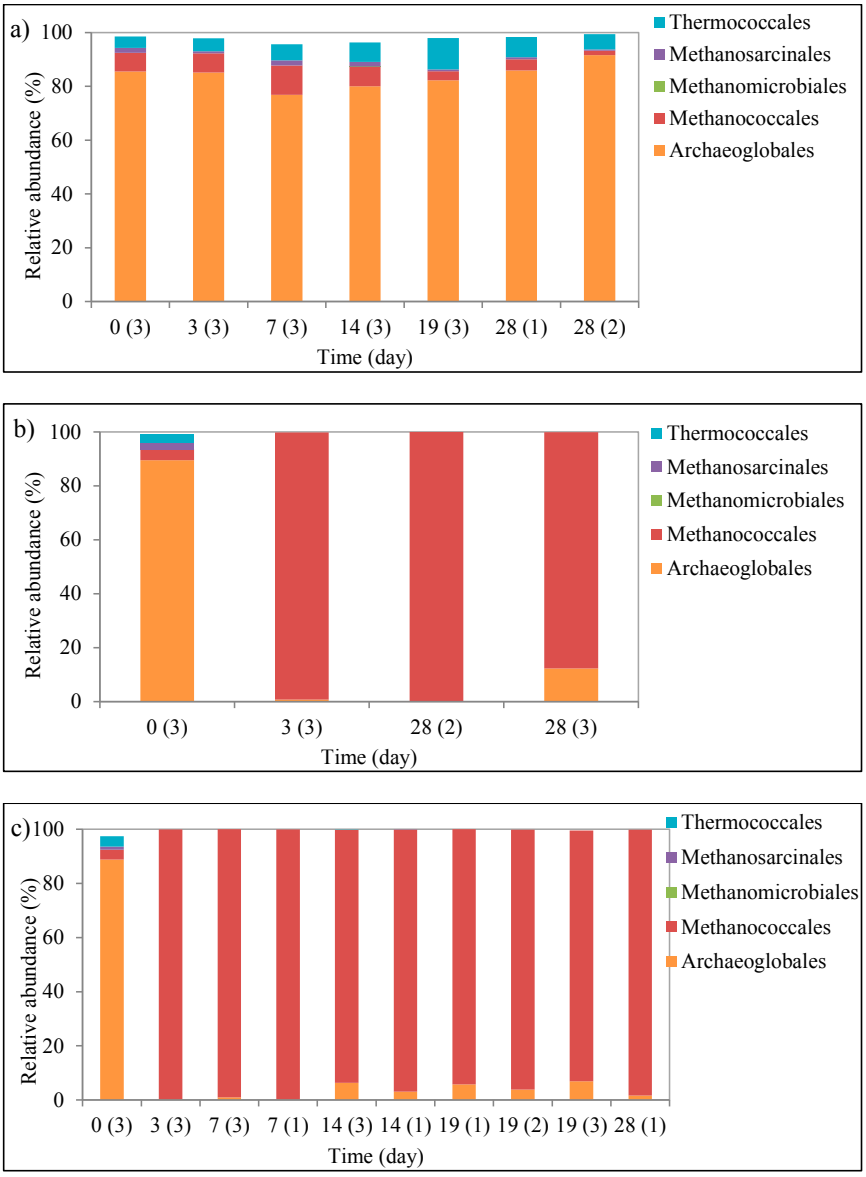

Fig.6.5 Development in archaeal community at order level after normalization of sequence reads in **a)** incubations A, control; **b)** incubations B, with addition of molasses; and **c)** incubations C, with addition of molasses and nitrate. White space represents unclassified sequence reads and low-abundance phyla. The number in the brackets following the sampling time refers to the specific flask where the replicates are taken form

In incubation A-control, although there was no overall increase in the number of 16S rRNA genes, the relative community composition did change during the 28 days of incubation (Fig. 6.4a and Table S6.2). In replicates no 2 and no 3 the bacteria dominating at the beginning of incubation were out-competed by members of the family *Thermovirgaceae* (phylum *Synergistetes*). The characterized strain with highest sequence identity (93-94 %) was *Thermovirga*. In replicate no 2, *Anaerobaculum* (phylum *Synergistetes*) was also abundant at the end of incubation. *Thermovirga* and *Anaerobaculum* are fermenting bacteria, and whereas *Anaerobaculum* is able to ferment a wider variety of substrates such as starch, sugars, proteins and amino acids [24-26], *Thermovirga* is restricted to proteins, some single amino acids and a limited number of organic acids [27] . In replicate no 1, the population developed differently, as the sulfate-reducing *Proteobacteria, Desulfacinum* dominated the bacterial population after 28 days of incubation.

In incubation B-with addition of molasses (Fig. 6.4b and Table S6.3), a more dynamic bacterial population change was observed. Within 3 days of incubation, *Synergistetes* out-competed *Proteobacteria* primarily due to an increase in the genus *Thermovirga.* In the sample retrieved at day 7, *Bacteroidetes* temporarily dominated the community due to growth of bacteria of the family *Marinilabiaceae.* At day 14 and throughout the incubation period, members of *Synergistetes* and *Thermotogae* dominated the bacterial population. Their abundance was primarily due to an increase in two genera; *Anaerobaculum* (*Synergistetes*) and *Petrotoga* (*Thermotogae*). *Petrotoga* has been found exclusively in oil reservoirs [28-31], ferments a range of sugars, and is able to degrade long-chain hydrocarbons of crude oil into shorter-chain hydrocarbons.

Overall, the development in the bacterial community composition was comparable in incubations B-with addition of molasses and C-with addition of molasses and nitrate since both incubations were enriched for *Petrotoga* and *Anaerobaculum* belonging to the *Thermotogae* and *Synergistetes* phyla, respectively. In incubation C-with addition of molasses and nitrate, however, growth of *Petrotoga* was

favored over *Anaerobaculum* as in this incubation significant growth of *Anaerobaculum* was only seen at the end of incubation (Fig. 6.4c and Table S6.4).

At the beginning of the incubation, the archaeal community was dominated by the hyperthermophilic, sulfate-reducing archaea *Archaeoglobus fulgidus,* which belongs to the order *Archaeoglobales* (Fig. 6.5a-6.5c) (Table S6.5). *Methanococcales* and *Thermococcales* were the second most abundant archaeal orders, and *Methanosarcinales* was present as minor population (< 3%) (Fig. 6.5a-6.5c). In incubation A-control, the archaeal community composition stayed relatively unchanged throughout the incubation period (Fig. 6.5a). In incubations B-with addition of molasses (Fig. 6.5b) and incubations C-with addition of molasses and nitrate (Fig. 6.5c), the archaeal population changed within 3 days. From day 3 and onwards, the community consisted almost exclusively of *Methanothermococcus,* which belongs to the order *Methanococcales. Methanothermococcus* are methanogenic archaea known to obtain energy for growth by reducing carbon dioxide to methane using hydrogen or formate as electron donors.

6.4 Discussion

In this study we evaluated the effect of adding molasses with and without nitrate to crude oil-brine incubations to promote growth and metabolite production of indigenous microbes for enhanced oil recovery. Not only did addition of molasses stimulate microbial growth (Fig. 6.1a and 6.1b), but it also altered the microbial community population. The growth rate was highest during the first 3 days of incubation. The drop in pH from 8 to 5 within the first 7 days of incubation may have been a limiting factor for further microbial growth as well as a factor affecting the population dynamics throughout the incubation period (Fig. 6.4a-c and Fig 6.5a-c). The drop in pH might be due to organic acids and dissolved CO_2, which were produced by fermenting microorganisms. Pyrosequencing revealed that *Anaerobaculum*, *Petrotoga* and *Methanothermococcus* were enriched compared to the incubation A-control, without molasses. An earlier investigation by Gittel *et. al*. [16] saw similar bacterial development as growth of

Anaerobaculum, *Petrotoga,* and *Thermovirga* was stimulated during anaerobic incubations with brine from the Dan oil field amended with substrate consisting of lactate, acetate, glucose and yeast extract, but without crude oil. Likewise the initial bacterial population diversity we observed is in accordance with findings by Gittel *et al.* [16], who have reported that the Dan oil field was predominantly inhabited by bacteria from the *Firmicutes* (70%) and *Deltaproteobacteria* (10%) phyla, hereby confirming that these bacteria are indigenous inhabitants of the Dan oil field. Furthermore, we observed the same archaeal inhabitants as Gittel *et al* [10], although they identified *Thermococcales* as the most abundant archaeal order in the brine from Dan oil field (60%), followed by *Methanococcales* (20%) and *Archaeaoglobales* (20%), whereas, in our study, members of *Archaeoglobales* was the most abundant archaeal order (> 85% at T0).

There was a significant difference in the IFT of the samples with molasses (incubation B and C) compared to samples without molasses (incubation A-control). The IFT decreased for samples containing molasses. These samples also have an increased level of microbial biomass and metabolites as compared to the incubation A-control. It was further revealed that microbes themselves play an important role in reducing IFT by formation of emulsions. When microbial biomass was removed from the interphase by centrifugation an increase in IFT was observed. The underlying cause for the reduction in IFT in the presence of microbial biomass may be the presence of cell-associated emulsifiers as has been observed by Acevedo and McInerney for thermophilic and extremely thermophilic archaea [32]. Methanogenic archaea have been found to be effective emulsifying microorganisms and the emulsifying activity was assigned to cell-associated proteins [32]. In our experiment, the mechanisms still remain unclear, as it could be the microbial biomass itself, surface compounds, or secondary metabolites within the biomass that causes the reduction of IFT. In incubation A-control, there was no significant change in IFT, before and after removal of microbes. This could be due to two reasons; microbes were not present in sufficient numbers to reduce IFT as the qPCR results showed there was no overall increase in microbial

number or microbes that were present in the incubation A-control did not contribute to the reduction of IFT.

Microbial morphology was strongly influenced by the type of carbon source. When crude oil was present as the only carbon source, microbes had a tendency to form aggregates. When molasses was added the microbes mainly existed as single cells. This is important information as chalk has pore throat sizes that are comparable to the sizes of microbial cells. Microbial aggregates may be unwanted in chalk due to the risk of plugging of pore throats, especially in the near-wellbore area. Afrapoli, *et. al* [33] have reported that cell hydrophobicity was strongly influenced by a carbon source in the media, similar to our observations. In their study two different carbon sources, dodecane and acetate, were used to cultivate *Rhodococcus* sp. strain 094. When dodecane was used, the cells were hydrophobic and tended to form aggregates. The cells grown on acetate were hydrophilic and travelled as individual cells.

We observed a continuous consumption of nitrate in the incubation C-with addition of molasses and nitrate and hence additional nitrate was added several times during the incubation period. *Methanothermococcus* has been reported to be able to use nitrate [34, 35], whereas characterized strains of *Petrotoga* [28-31] and *Anaerobaculum* [24-26] have not been reported to utilize nitrate. Therefore, *Methanothermococcus* was most likely the cause of nitrate depletion. However, the reason for enrichment of *Petrotoga* over *Anaerobaculum* when nitrate was added remains unclear.

Petrotoga has previously gained attention in relation to MEOR. This is due to its ability to degrade longer-chain alkanes of crude oil into shorter-chain alkanes when grown in oil-brine mixtures amended with yeast extract, thus resulting in reduction of oil viscosity by 40% after 14 days of incubation. Further, under specific CO_2 conditions, longer chain alkanes were degraded more selectively [36]. However, in our study there was no reduction in oil viscosity and no significant difference in total carbon weight fractions of the crude oil at start and

end of the incubations with and without molasses. Instead, the crude oil density and viscosity of all incubations increased from day 0 to day 28. This might be due to formation of oil emulsions [37]. In incubation A-control, emulsions were only seen under the microscope while without magnification the brine and oil was still seen as two distinct phases. The unchanged carbon weight fraction might be due to the fact that i) we measure the total weight fraction of the hydrocarbon and not specifically the alkanes and/or ii) molasses was used as substrate by *Petrotoga* instead of the oil components (in incubations B-with addition of molasses and C-with addition of molasses and nitrate).

Instead of degradation of complex hydrocarbons by specific microbes, other aspects of using molasses to stimulate microbial growth for MEOR purposes were observed; production of acid may be useful for dissolving the carbonate precipitate from pore throats and to increase permeability [1, 2, 10], and production of ethanol (solvent) improves the effective permeability [1, 10] and alters wettability [10]. Furthermore, gas production was also observed in incubations containing molasses, and has also been implicated in MEOR [1, 2, 10]. However, the type of gas and the amount produced was not analyzed. The gas is likely CO_2 produced by *Petrotoga* and methane produced by *Methanothermococcus.* The growth of *Methanothermococcus* was potentially fueled by fermentation products from the bacteria. In addition, CO_2 may be used by *Methanothermococcus,* which grows on hydrogen and carbon dioxide [35]. *Methanothermococcus* should be further investigated for its potential to form emulsion and reduce the IFT.

Our results demonstrate that injection of molasses is a promising MEOR strategy; however, further investigations are needed to confirm that microbial growth and activity is simulated at reservoir conditions (e.g. high pressure, porous media). It is also important to investigate the role of biomass in reduction of IFT, whether it is the biomass itself, metabolites attached to the surface, and/or intracellular metabolites that reduce IFT.

Acknowledgements

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Supplementary Materials

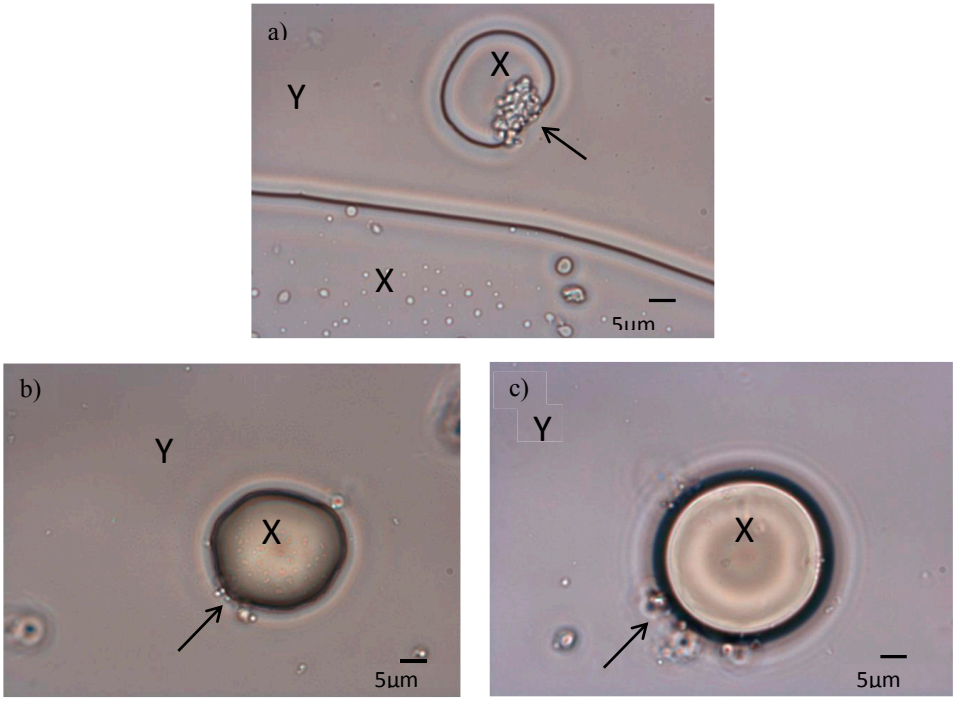

Fig.S6.1 Microbes (black arrow) in oil droplets under phase contrast microscope, **(a)** microbes were present as aggregates in incubation A-control and as single cells in **(b)** incubation B-with addition of molasses and **(c)** incubation C-with addition of molasses and nitrate. The X sign refers to the crude oil and Y sign refers to the brine phase.

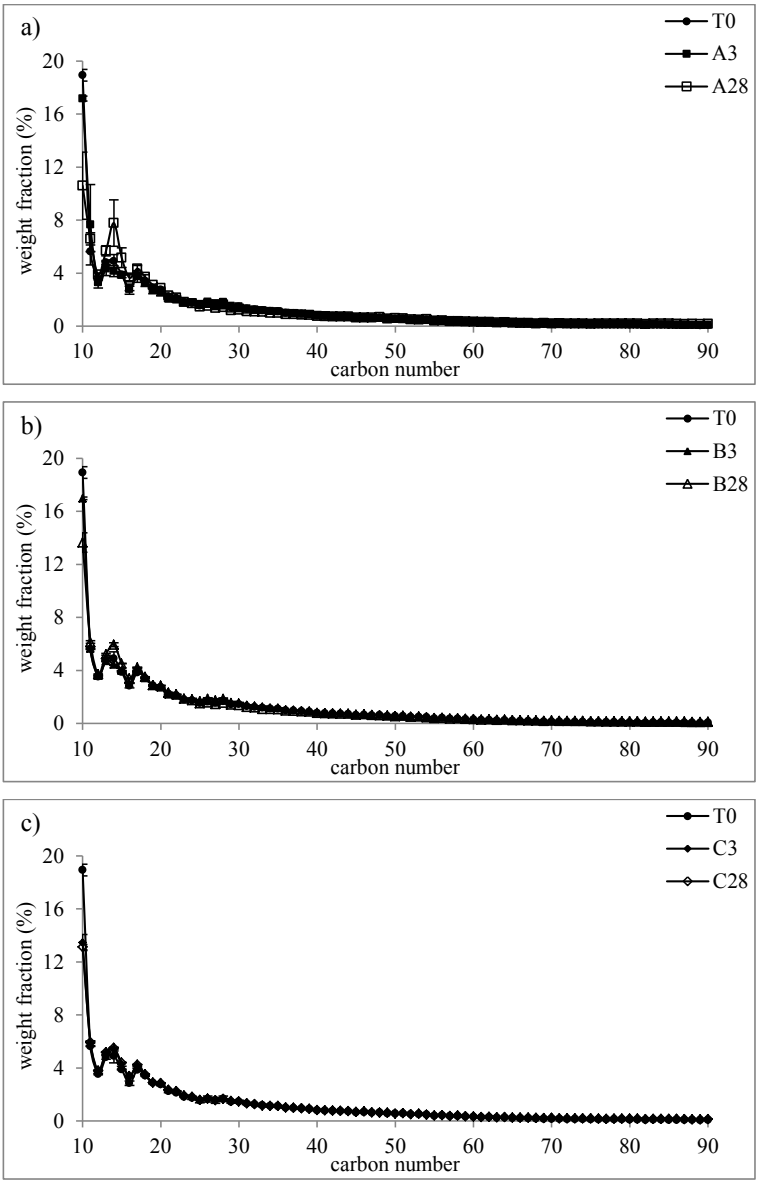

Fig.S6.2 Total carbon weight fraction of crude oil in **(a)** incubation A-control, **(b)** incubation B-with addition of molasses, and **(c)** incubation C-with addition of molasses and nitrate. No significant change in composition was observed in the total carbon weight fraction. Values are mean values of three independent experiments and error bars represent one standard deviation of the mean of the triplicates

Table S6. 1 Primer specification used for qPCR and pyrosequencing.

Primer name	Primer sequence (5´- 3´)	Target	Reference
341F	CCT ACG GGA GGC AGC AG	16S, bacteria	[17]
1390R	GAC GGG CGG TGT GTA CAA	16S, universal	[18]
Arch8F	TCC GGT TGA TCC TGC C	16S, archaea	[19]
806F	ATT AGA TAC CCS BGT AGT CC	16S, archaea	[15]
958R	YCC GGC GTT GAM TCC AAT T	16S, archaea	[15]
PyroA-926R-XX	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG AAC GCA CGC TAG CAC CGT CAA TTC MTT TRA GT (unique MID sequence is underscored)	16S, bacteria	Modified with adaptor and MID sequence from Liu *et al*. [20]
PyroB-343F	CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG TCT ACG GRA GGC AGC AG	16S, bacteria	Modified with adaptor sequence from Nossa *et al.* [21]
PyroA-519F-XX	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG ACG TAC TCA GTG CAC AGC MGC CGC GGT AAH ACC (unique MID sequence is underscored)	16S, archaea	Modified with adaptor and MID sequence from Wang *et al.* [22]
PyroB-915R	CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG TCG TGC TCC CCC GCC AAT TCC T	16S, archaea	Modified with adaptor sequence from Stahl *et al.*[23]

Table S6. 2 The abundance of bacterial OTUs after normalization in incubation A-control.

Phyla		*Firmicutes*					*Proteobacteria*				*Synergistetes*				*Thermotogae*	
Sequence reads assigned to OTUs (f=family, g=genus)		*Clostridiacea* (f) (OTU1024)	*Clostridiaceae* (f) (OTU6500)	*Halanaerobiaceae* (f) (OTU5598)	*Halanaerobium* (g) (OTU3255)	*Sporotomaculum* (g) (6063)	*Desulfacinum* (g) (OTU4931)	*Desulforomonadales* (o) (OTU4500)	*Desulfuromonas* (g) (OTU6576)	*Marinobacter* (g) (OTU2530)	*Thermovirgaceae* (f) (OTU1135)	*Thermovirgaceae* (f) (OTU988)	*Thermovirga* (g) (OTU5825)	*Anaerobaculum* (g) (OTU4633)	*Petrotoga* (g) (OTU6539)	*Thermosipho* (g) (OTU1079)
Time (days)	0(3)[1]	**6.35**	**29.3**	0.09	3.67	2.77	0.00	3.76	**8.68**	4.65	0	0.09	0.81	0	0.09	1.52
	3(3)	1.70	**22.7**	0.09	**5.28**	4.38	0.18	**4.83**	3.94	3.13	3.85	2.77	1.25	0	**5.64**	3.31
	14(3)	1.34	**8.59**	2.24	2.68	0.72	1.88	2.95	2.77	1.79	**26.10**	**10.90**	2.86	1.61	3.67	1.25
	19(3)	0.09	**7.51**	2.06	1.43	0	0.81	1.70	0.89	0.54	**15.00**	**9.75**	1.16	0.63	**7.78**	0.09
	28(1)	0	0.98	0.09	0.09	0.09	**75.80**	0.09	0.27	0.36	**2.15**	0.81	0	0.36	1.16	0
	28(2)	0.54	6.35	0.63	1.52	0.27	0.45	0.36	0.72	0.54	**27.8**	**13.60**	2.33	**18.40**	4.38	0.27

Numbers are given in percentages out of the total number of OTUs in the samples[1]. Only OTUs representing more than 5% of the normalized number of OTUs summarized across samples are shown.

[1] Naming of samples is described in M&M section. As an example 0 (3) corresponds to the sample taken at day 0 from flask no 3.

Table S6.3 The abundance of bacterial OTUs after normalization in incubation B- with addition of molasses.

Phyla		*Firmicutes*		*Proteobacteria*		*Synergistetes*				*Thermotogae*			*Bacteroidetes*	
Sequence reads assigned to OTUs (f=family, g=genus, o=order)		*Clostridiales* (o) (OTU6086)	*Clostridiaceae* (f) (OTU6500)	*Desulfuromonas* (g) (OTU6576)	*Marinobacter* (g) (OTU2530)	*Thermovirga* (g) (OTU5825)	*Anaerobaculum* (g) (OTU4633)	*Anaerobaculum* (g) (OTU99)	*Anaerobaculum* (g) (OTU5321)	*Petrotoga* (g) (OTU6539)	*Petrotoga* (g) (OTU1920)	*Petrotoga* (g) (OTU5834)	*Marinilabiaceae* (f) (OTU6585)	*Bacteroidales* (o) (OTU342)
Time (days)	0 (3)[2]	0	**21.20**	**11.40**	**10.20**	0.45	0	0	0	0.27	0	0	0	0
	3 (3)	**15.80**	0	0	0	**49.50**	0	0	0	0.18	0	0	6.17	0.30
	7 (3)	1.07	0	0	0	6.26	0.81	0	0	**9.66**	0	0	**61.70**	5.30
	14 (3)	2.06	0	0	0	**11.40**	**15.80**	2.59	0.40	**42.20**	0.45	0.90	0.09	0
	19 (3)	0.18	0	0	0	0.81	**18.90**	4.92	2.40	**43.30**	4.20	5.70	0	0.10
	28 (1)	0	0	0	0	0.36	**39.70**	4.83	3.20	**36.40**	4.38	3.20	0	0
	28 (2)	0	0	0	0	0.36	**38.00**	4.38	3.00	**32.60**	3.40	1.70	2.68	0.20
	28 (3)	0.18	0	0	0	0.81	**51.90**	1.97	2.50	**32.60**	2.42	1.20	0	0

Numbers are given in percentages out of the total number of OTUs in the samples[1]. Only OTUs representing more than 5% of the normalized number of OTUs summarized across samples are shown.

[2] Naming of samples is described in M&M section. As an example 0 (3) corresponds to the sample taken at day 0 from flask no 3.

Table S6.4 The abundance of bacterial OTUs after normalization in incubation C- with addition of molasses and nitrate

Phyla		*Firmicutes*		*Proteobacteria*				*Synergistetes*		*Thermotogae*						*Bacteroidetes*
Sequence reads assigned to OTUs (f=family, g=genus, o=order)		Clostridiales (o) (OTU6086)	Clostridiaceae (f) (OTU6500)	Desulforomonadales (o) (OTU4500)	Desulfuromonas (g) (OTU6576)	Marinobacter (g) (OTU2530)	Marinobacter (g) (OTU1720)	Anaerobaculum (g) (OTU4633)	*Thermovirga (g) (OTU5825)*	Petrotoga (g) (OTU6539)	Petrotoga (g) (OTU5076)	Petrotoga (g) (OTU1597)	Petrotoga (g) (OTU4060)	Petrotoga (g) (OTU331)	Geotoga (g) (OTU2078)	Marinilabiaceae (f) (OTU609)
Time (days)	0(3)[3]	0.18	**5.55**	**5.81**	21.10	12.50	**5.10**	0	0.81	0.09	0	0	0	0	0.09	**5.64**
	7(1)	**33.1**	0	0	0	0	0	0.63	**11.6**	**14.00**	0.45	0.89	0.98	1.07	6.17	0
	14(1)	**8.23**	0	0	0	0	0	1.88	**7.33**	**58.60**	2.50	1.07	0.81	1.34	1.34	0
	14(3)	**9.21**	0	0	0	0	0	2.33	**4.29**	**49.00**	0.63	1.88	2.50	0.98	10	0
	19(1)	1.97	0	0	0	0	0	2.86	1.43	**73.30**	3.49	0.45	0.27	0.98	0.09	0
	19(2)	0	0	0	0	0	0	0	0.36	**88.20**	0.72	0	0.18	0.89	0.09	0
	19(3)	1.34	0	0	0	0	0	1.16	0.45	**78.40**	1.43	2.33	1.7	0.54	1.52	0
	28(1)	0.45	0	0	0	0	0	**38.50**	0.63	**45.20**	1.16	0	0.09	0.36	0.09	0

Numbers are given in percentages out of the total number of OTUs in the samples[1]. Only OTUs representing more than 5% of the normalized number of OTUs summarized across samples are shown.

[3] Naming of samples is described in M&M section. As an example 0 (3) corresponds to the sample taken at day 0 from flask no 3.

Table S6.5 The abundance of archaeal OTUs after normalization in incubation A-control, B- with addition of molasses and nitrate and C-with addition of molasses and nitrate

Order			*Archaeoglobales*									*Methanococcales*			*Methanosarcinales*	*Thermococcales*	
Sequence reads assigned to OTUs (f=family, g=genus. s=species)			*Archaeoglobus fulgidus* (s) (OTU219)	*Archaeoglobaceae* (f) (OTU407)	*Archaeoglobus* (g) (OTU549)	*Archaeoglobus fulgidus* (s) (OTU723)	*Archaeoglobus* (g) (OTU 590)	*Archaeoglobaceae* (f) (OTU349)	*Archaeoglobus* (g) (OTU66)	*Archaeoglobus fulgidus* (s) (OTU341)	*Archaeoglobus fulgidus* (s) (OTU120)	*Methanothermococcus* (g) (OTU920)	*Methanothermococcus* (g) (OTU567)	*Methanothermococcus* (g) (OTU224)	*Methermicoccaceae* (f) (OTU742)	*Thermococcaceae* (f) (OTU640)	*Thermococcaceae* (f) (OTU501)
Time (days)	Incubation A	0(3)[1]	**66.00**	**7.70**	1.50	2.00	0.80	0.80	0.70	0.60	1.00	4.70	1.60	0	1.10	2.10	2.00
		3(3)	**61.10**	**9.00**	2.40	1.20	1.10	1.20	0.80	0.80	0.70	4.60	2.30	0	0.50	2.70	1.70
		7(3)	**55.40**	**6.30**	2.30	2.40	1.20	1.20	1.20	0.80	0.30	7.50	2.40	0	1.00	3.50	2.00
		14(3)	**60.90**	**10.80**	1.10	0.70	2.20	0.30	0.70	0.40	0.60	5.40	1.50	0	1.50	4.80	1.90
		19(3)	**63.40**	**9.80**	1.00	0.50	1.40	0.80	0.30	0.70	0.50	2.60	0.60	0	0.60	**8.50**	2.00
		28(1)	**71.40**	**7.30**	1.20	0.50	1.00	0.50	1.00	0.30	0.50	3.70	0.30	0	0.60	5.20	1.80
		28(2)	**80.40**	**6.20**	0.30	0.10	0.40	0.20	0.80	0.80	1.20	1.50	0.10	0	0.40	4.20	0.70
	Incubation B	0(3)	**65.60**	**10.40**	1.80	1.00	1.60	1.50	0.70	0.90	0.40	2.30	1.10	0	1.70	1.40	1.40
		3(3)	0.30	0.10	0	0.20	0	0	0	0	0	**97.30**	0.20	0.50	0	0	0
		28(2)	0.10	0.10	0	0	0	0	0	0	0	**98.60**	0	0.70	0	0	0
		28(3)	3.40	**7.60**	0	0.20	0	0	0	0.10	0	**86.70**	0.20	0.20	0	0	0
	Incubation C	0(3)[4]	**57.10**	**15.70**	2.10	0.50	0.70	1.80	0.60	0.80	1.10	2.10	1.40	0	0.60	2.10	1.4
		3(3)	0.20	0	0	0	0	0	0	0	0	**98.40**	0.10	0.60	0	0	0
		7(1)	0	0.10	0	0.10	0	0	0	0.10	0	**98.40**	0	0.30	0	0	0
		7(3)	0.60	0.20	0	0.10	0	0	0.10	0	0	**97.00**	0.10	0.70	0	0	0
		14(1)	1.10	1.40	0	0.20	0	0	0	0.10	0.10	**95.00**	0.20	0.20	0.10	0	0
		14(3)	3.50	1.60	0	0.70	0	0.10	0	0	0	**91.20**	0.80	0.40	0	0.10	0.0
		19(1)	2.70	2.40	0	0.10	0	0	0	0.10	0	**93.10**	0.10	0.50	0	0	0
		19(2)	1.60	2.00	0	0.10	0	0	0	0	0	**94.40**	0.30	0.50	0	0	0
		19(3)	3.70	1.10	0	1.10	0.10	0	0	0	0	**90.60**	0.70	0.30	0	0	0
		28(1)	1.30	0	0	0.40	0	0	0	0	0	**96.30**	0.30	0	0.60	0	0

[4] Naming of samples is described in M&M section. As an example 0 (3) corresponds to the sample taken at day 0 from flask no 3.

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CHAPTER 7
Conclusions and Recommendations for Future Work

7.1 Conclusions

This thesis investigates the possibility of MEOR application in chalk reservoirs. Microbial transport in chalk rock was studied. The selected microorganism, *Bacillus licheniformis* 421, was used further to identify which mechanisms might play a role in improving oil production. Core flooding experiments were carried out to study fluid-rock interactions, while enrichment batch culture experiments were conducted to study fluid-fluid interactions. In addition, the nutrient screening was also conducted using *B. licheniformis* 421. The selected nutrient was then applied to the indigenous microbes from the North Sea oil field in batch culture experiments. The results obtained for *B. licheniformis* 421 and for the indigenous microbial cultures were then compared to identify similar phenomena. Once the selected phenomena are identified, it gives an idea about the possible mechanisms for MEOR application in chalk reservoirs. More detailed conclusions that address the research objectives presented in Chapter 1 are summarized in the sub-sections below.

Bacteria Penetration Study

- The injected bacteria were able to penetrate and to be transported through the chalk rock, even though the permeability was below 4 mD and the pore sizes were comparable to bacterial sizes.
- Significantly higher numbers of the spore-forming bacteria *B. licheniformis* 421 were found in the effluents compared with the non-spore forming *P. putida* K12, indicating that spore-forming bacteria have a higher chance of survival and deep penetration.
- *B. licheniformis* 421 predominantly penetrates in form of spores while the *P. putida* K12 penetrates as vegetative cells, however, smaller in size and of a different shape than during normal growth conditions, due to high salinity of the brine.

- The number of cells being able to penetrate the core is related to the bacteria inoculum concentration. The higher the injected inoculum, the higher number of cells was found in the effluent.
- Retention of bacteria and, probably, of produced metabolites in the cores cause irreversible permeability damage. A starvation period of 12 days did not allow the permeability to return to initial condition

Core flooding experiments: Does injection of bacteria improve oil production? What are the mechanisms that play a role: Selective plugging or wettability alteration?

- Injection of *B. licheniformis* 421 increases oil production compared with injection of synthetic seawater.
- It was demonstrated that the main mechanism of the MEOR action is selective plugging on the small-scale level. *B. licheniformis* 421 can selectively block some permeable channels of the reservoir chalk cores, alter the water paths and thus improve oil production. The bacteria, when injected into low permeable homogeneous chalk cores, produced additionally 1.0-2.3 % original oil in place (OOIP). Meanwhile, injection into the heterogeneous chalk cores with microfractures resulted in a noticeably higher additional recovery of 6.9-8.8% OOIP.
- This proposed mechanism was also supported by the pressure data as the increase of the pressure gradient was much higher in the heterogeneous cores.
- The core flooding experiments showed that the additional oil recovery from the non-aged cores (water-wet system) was 1.0-2.3 % OOIP and from the aged cores (more oil-wet system) was 3.6-4.3 % OOIP. This indicates that wettability alteration may not be a key player in improving oil production.
- Another possible explanation could be that insufficient amount of biomass and metabolites that were not produced in situ to alter the core wettability. This explanation is in correlation with the subsequent anaerobic growth study, which showed that the bacteria grew slowly in the presence of hydrocarbons and produced limited amount of metabolites.

- Both the selective plugging and the wettability experiments showed similar general trends: 1) most of the injected bacteria were retained inside the cores and only less than 1% was detected in the effluent, 2) the pressure-tapped core holder could be used to monitor the bacterial plugging by pressure difference increase around the different sections of the cores, 3) in all cases, injection of bacteria and nutrient needed to be followed by an incubation period in order to allow bacterial growth inside the cores.

***B. licheniformis* 421 Growth Experiments**

- *B. licheniformis* 421 grew better on molasses as compared with pure glucose. This could be due to the fact that molasses do not only contain sugars, but also some organic acids and other minerals.
- Bacterial growth rate was higher in the media with increased salinity, which means that it would grow well under salinities found in reservoirs
- *B. licheniformis* 421 was able to use various carbon sources under anaerobic conditions and produced lichenysin G, a lipopeptide biosurfactant.
- The bacteria preferred to use hydrocarbons (n-alkanes) instead of sugar compounds in molasses when both were present in the same batch.
- Nitrate was used as electron acceptor under anaerobic condition.
- Only small amounts of organic acids and solvents were produced in the media containing n-alkanes. Therefore, it is unlikely that production of organic acids and solvents are the mechanisms that will work to improve oil production.
- In the presence of n-alkanes, the bacteria formed clumps and accumulated at the hydrocarbon-water interphase.
- IFT was significantly reduced and emulsions were formed when the bacteria were grown in media containing only n-alkanes.
- There is a strong indication that bacteria themselves may play an important role in formation of emulsions, even in the case where no significant IFT reduction takes place.

Enrichment Test of Dan Field Indigenous Microbes

- The quantitative real-time polymerase chain reaction (qPCR) results showed that addition of molasses (with and without nitrate) significantly increased the indigenous bacteria and archaea populations.
- The data of pyrosequencing showed that addition of molasses altered the microbial populations. At the start of incubation, bacteria belonging to the phyla *Firmicutes* and *Proteobacteria*; and sulfate-reducing archaea *Archaeoglobus fulgidus* were dominating the microbial populations. Addition of molasses specifically enriched the growth of bacteria from genera *Anaerobaculum* and *Petrotoga*; and archaea from genera *Methanothermococcus*.
- Nitrate could be used as electron acceptor by the indigenous microbes.
- Addition of nitrate favoured the growth of *Petrotoga* over *Anaerobaculum*.
- The microbial growth caused changes in the crude oil-brine system: formation of oil emulsions, and reduction of IFT, indicating that injection of molasses is a promising strategy to boost the indigenous microbial population that might be beneficial for MEOR.

Comparison of model study microbes (*B. licheniformis* 421) vs. indigenous microbes (batch culture experiments) show that:

- Molasses is a promising nutrient that can boost the microbial growth.
- Under anaerobic conditions, nitrate can be used by bacteria as electron acceptor.
- The hydrocarbon degrading microbes tend to stay in the interface between oil and water, affecting fluid-fluid interactions.
- The fluid-fluid interaction studies show that formation of oil emulsions and reduction of IFT are the possible MEOR mechanisms. This is true for both indigenous and model microbes.
- Unlike the model study microbes, the indigenous microbes produce more organic acid (acetate) and solvent (ethanol), even when molasses and hydrocarbons are present in the same batch. This indicates that if the

indigenous microbes are used in the fluid-rock system, organic acid and solvent production might give additional advantages to improve oil production.

7.2 Recommendations for future studies

- The core flooding experiments demonstrate that selective plugging is a major contributor that improves oil production when exogenous bacteria, *B. licheniformis* 421 were injected into chalk rock. This mechanism should be tested with indigenous microbes
- Compatibility and survival test of the exogenous microbes against the indigenous microbes needs to be conducted to investigate the growth behaviour of exogenous microbes in the presence of indigenous microbes.
- Experiments on wettability alteration should be explored further, with application of the Amott test or contact angle measurements with microbial cells and metabolites. This is especially important, since the indigenous microbes produced more metabolites (acetate and ethanol) compared with the model study microbe, *B. licheniformis* 421.
- Further investigation on emulsion formation is requied, with the application of microbial cells and metabolites (especially the lipopeptide surfactant). This is needed to confirm whether microbial cells themselves or the lipopeptide surfactant in the solution play a role in the emulsification. It could also be of interest to produce a mutant bacterium that does not produce lipopeptide, to further investigate the role of microbial cells in formation of emulsions.
- Due to the limitations in the applied ultra-high performance liquid chromatography quadrupole time of flight mass spectrometer (UHPLC-QTOFMS) methods, it was not possible to analyse the lipopeptide when crude oil was present in the enrichment experiment of the indigenous microbes. However, the pyrosequencing revealed the indigenous microbial cultures that were enriched by molasses. Therefore, pure culture studies of these indigenous microbes should be conducted to investigate and identify a possibility for production of lipopeptide or other surface-active compounds.
- Development of mathematical and thermodynamic models that can quantify bacterial growth in porous media and the amounts of bacterial biomass and

metabolites needed in order to transfer the laboratory results to reservoir scale and to investigate the effects of rock heterogeneity and selective plugging.

Appendix 1: Additional Experiment on Application of MEOR as Secondary Oil Recovery Method

This section covers the investigation whether injection of bacteria as secondary oil recovery method, directly after water breakthrough or after 1 pore volume injection (PVI) of the 1st synthetic seawater (SS) will recover more oil compared to injection of bacteria as tertiary oil recovery method.

A1.Materials and methods

Materials used in the experiments (crude oil, reservoir rocks, bacteria samples, chemicals, growth media and core flooding apparatus) are similar to the ones used in Chapter 3. The homogeneous core Ho 1 was used in this study.

The experimental sequence is presented in Fig. A1 and detailed explanations of the procedure are similar to the description in Chapter 3. The part that is different from the descriptions in Chapter 3 is the experimental sequence which will be described below.

A 260D ISCO pump was used to keep control over a constant injection rate of 0.1 ml/min throughout the experiment. After the temperature inside the core holder was stabilized, the core was subjected to 3-5 PVI of sterile SS. This was followed by injection of crude oil until no more water was produced, in order to create irreducible water saturation (S_{wi}). The oil saturation under this condition was considered to be original oil in place (OOIP).

The core was then flooded with 1 PVI of SS (1st SS flooding) or after the water breakthrough was observed. Then 1 PVI of bacteria was injected, and the core was incubated with bacteria for 3 days by closing both inlet and outlet valves. After the 3-days incubation period, the core was again flooded with SS (2nd SS flooding) until no more oil was produced. Subsequently, 1 PVI of nutrient was injected followed by 7 days incubation. Later on, the core was flooded again with SS (3rd SS flooding) until no more oil was produced.

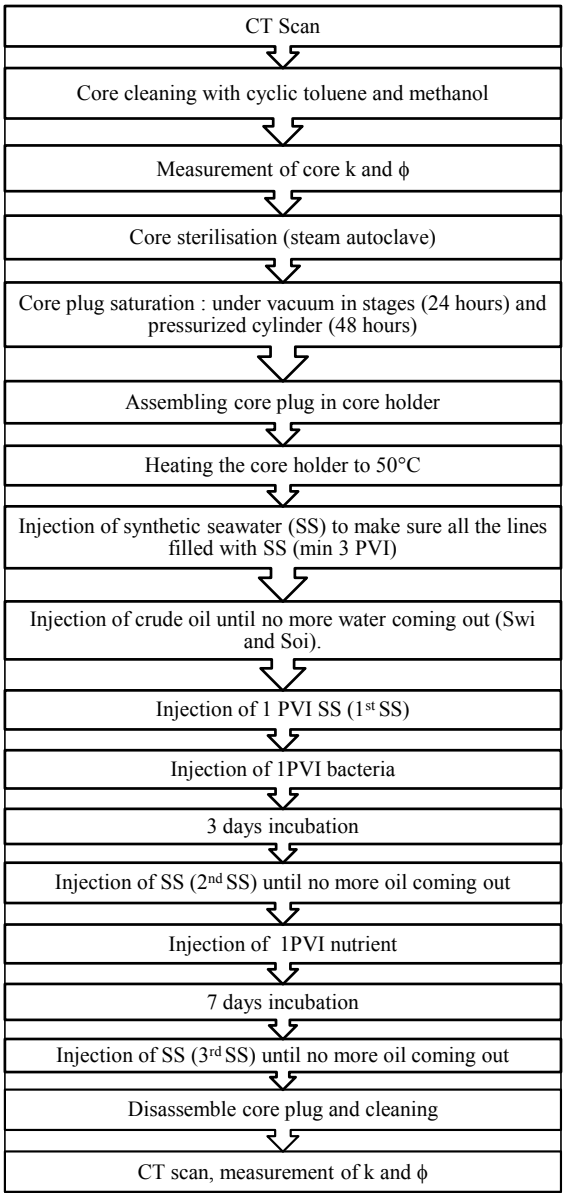

Fig.A.1 Schematic drawing of experimental sequences. The bacteria were injected as secondary oil recovery method (after water breakthrough)

A2.Results

A2.1 Core properties

Core permeability and porosity were measured at dry condition by the steady state gas permeameter. There was no significant change in the core properties on both porosity and permeability of the cores before and after experiment (Table A1). The results of CT scanning show that cores Ho 1 is relatively homogenous. No open or partially open fractures were detected in the cores before and after each experiment.

Table A1 Summary of core plug properties

Core number	Diameter (cm)	Length (cm)	Pore volume (ml)	Ki (mD)	Ka	ϕi	ϕa
Ho 1_2nd	3.8	7.5	27.4	3.1	3.2	30.7	31.7

ki = permeability before experiment, ka = permeability after experiment, ϕi = porosity before experiment, ϕa = porosity after experiment

A2.2 Oil production history

Initial water saturation (S_{wi}) was approximately 0.15. The core was flooded with 1 PVI SS (1st SS flooding) and produced 43.3% OOIP. Injection of 1 PVI bacteria produced 5.2% OOIP. Additional oil amounting at 10.4 % OOIP was produced during the 2nd SS flooding (Table A2, Fig. A2). Subsequent injection of 1PVI nutrient only produced 0.8% OOIP and the 3rd SS flooding produced additionally 1.9% OOIP (Table A2, Fig A2).

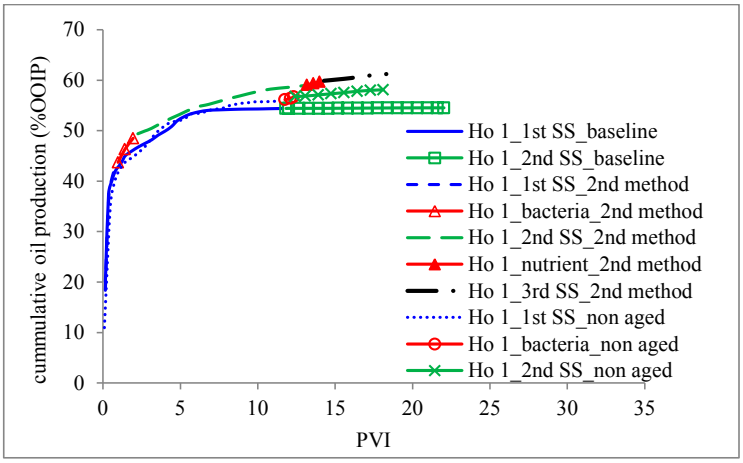

Fig.A.2 Cumulative oil production vs. pore volumes injection for baseline experiment core Ho 1 (no bacteria), experiment with non-aged core Ho 1 as tertiary oil recovery method and experiment with non-aged core Ho 1 as secondary oil recovery method.

A2.3 Cell-to-spore ratio and bacterial enumeration

DAPI staining and image analysis confirmed that the injected bacterial suspension contained above 98% growing cells and hence that the propagation methodology was satisfactorily (data not shown). Quantification of bacterial cells in the showed that viable cells were detected in the representative fractions that were analysed (Fig A3a and A3b). In the 2nd SS flooding only less than 1% of the injected bacterial cells were found in the effuents (Fig. A3a). Meanwhile in the 3rd SS flooding approximately 10% of the injected bacterial cells were detected (Fig A3b). It was not possible to detect bacterial spores in the effluents with the current method. This was due to that the traces of oil dissolved in the collected effluents gave autofluorescence when the filter was observed under the microscope.

Table A2 Summary of oil production history with different flooding schemes*

Core number	S_{wi}	S_{oi}	$S_{or,1^{st}\ SS}$ (%OOIP)	1st SS (%OOIP)	Bacteria (%OOIP)	2nd SS (%OOIP)	3rd SS (%OOIP)	Nutrient (%OOIP)	Total additional recovery (%OOIP)	Total oil produced (%OOIP)	Bacterial inoculum (cfu/ml)
Ho 1_bs	0.23	0.77	45.6	54.4	n/a	0.1	n/a	n/a	0.1	54.5	n/a
Ho 1_na	0.25	0.75	44.2	55.8	0.9	1.4	n/a	n/a	2.3	58.1	2.9×10^7
Ho 1_2nd	0.15	0.85	56.7	43.3**	5.2	10.4	0.8	1.9	n/a	61.5	3.6×10^7

* The experiments were conducted at overburden pressure 50 bar and temperature at 50°C. Oil density used is 854.2 kg/m^3 @20°C

** S_{or} after 1PVI of 1st SS injection (after water breakthrough)

S_{wi} = initial water saturation, S_{oi} = initial oil saturation, $S_{or,1^{st}\ SS}$ = residual oil saturation after 1st SS flooding, 1st SS = 1st SS flooding, Bacteria = bacteria flooding, 2nd SS = 2nd SS flooding, OOIP = original oil in place, cfu = colony forming unit

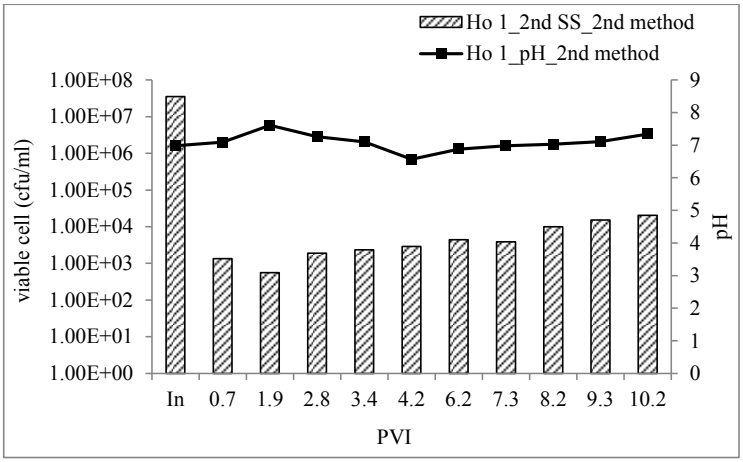

Fig.A.3a Viable *B. licheniformis* 421 cells (bar chart) detected in the effluent of the 2nd SS flooding, after 3 days incubation in the non-aged core Ho 1 as secondary method at 50°C. In = concentration of injected bacteria/inoculum. Effluent pH (■) at different collected fractions.

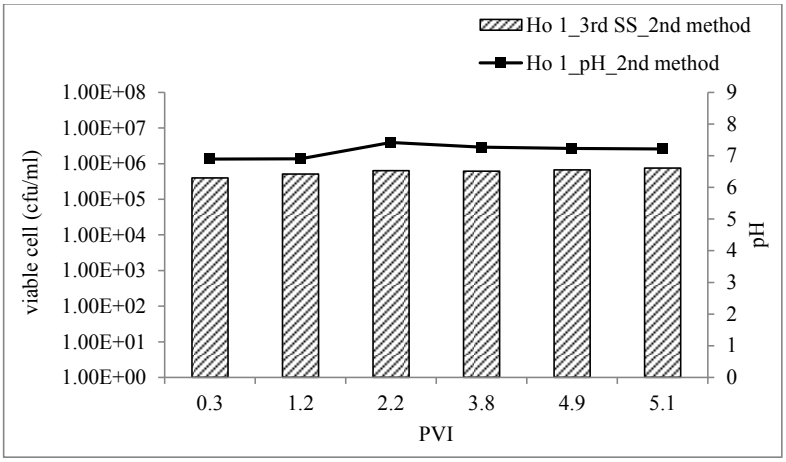

Fig.A.4b Viable *B. licheniformis* 421 cells (bar chart) detected in the effluent of the 2nd SS flooding, after subsequent nutrient injection and 7 days incubation in the non-aged core Ho 1 as secondary method at 50°C. Effluent pH (■) at different collected fractions.

A3. Discussion

This additional test showed a potential of higher oil recovery production if the bacteria was injected before the oil production reached residual stage, or when the first water breakthrough was observed. Similar finding was mentioned by Cresente et al.[1], who conducted serial of core flooding experiment in sandstone. The authors found that when bacteria were injected before the core reached residual state, higher final oil recovery was achieved. The bacterial counting ta the effluents showed that there is a strong indications with longer incubation period, more bacteria cells are produced and detected in the effluents.

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Appendix 2: 77th EAGE Conference article

Quantification of the recovered oil and water fractions during water flooding laboratory experiments

K. Katika* (Technical University of Denmark), A. Y. Halim (Technical University of Denmark), A. Shapiro (Technical University of Denmark) & I. L. Fabricius (Technical University of Denmark)

Summary

During core flooding experiments where water is injected in residual oil saturated core plugs, the fluids are often produced in small amounts. Oil and water come out of the core and are collected in glass vials using a fraction collector. Quantification of these fluids is often difficult since the volume might be less than a few microliters. In this study, we approach the determination of the oil volumes in flooding effluents using predetermined amounts of the North Sea oil with synthetic seawater. The UV/visible spectroscopy method and low-field NMR spectrometry are compared for this determination, and an account of advantages and disadvantages of each method is given. Both methods are reproducible with high accuracy. The NMR method was capable of direct quantification of both oil and water fractions, while the UV/visible spectroscopy quantifies only the oil fraction using a standard curve.

Introduction

Core flooding experiments are commonly used for testing different flooding strategies on the laboratory scale. During these experiments, the oil-saturated core plugs are subjected to water injection. As a result, both oil and water are produced out of the cores. The fluids, that need to be monitored, are collected in glass vials that are connected to the water flooding device and it is a challenge to quantify the oil fraction of these produced fluids (Figure 1).

During secondary recovery schemes, after the injection of several pore volumes of water, the amount of oil recovered from the core can be only a few hundreds of microliters. This oil is distributed among the vials, and the smallest amounts may be only few microliters of oil per vial. This oil may be dispersed (attached to the walls, form drops or films etc.). Meanwhile, in the experiments where the methods of enhanced oil recovery are investigated, these amounts should precisely be accounted for, since in such methods the struggle is only for few per cent of additional recovery.

Several methods for determining small amounts of oil during water flooding experiments have been proposed, including radioactive tracers, image analysis and weight estimations, but they can be expensive, untrustworthy or complex (Shi and Winslow, 1991; Dijs et al., 2006; Dugstad et al., 2011).

Low field nuclear magnetic resonance (NMR) spectrometry is commonly used to determine petrophysical properties of reservoir rocks (Kenyon, 1997). NMR spectrometry can also be applied to provide information about the quantities and the properties of the fluids under investigation. This method can directly measure the density of hydrogen nuclei in reservoir fluids and determine the presence and quantities of the different fluids (water, oil, and gas) (Coates, 1999). Low field NMR has been successfully applied for the determination of water and heavy oil compositions in emulsions at reservoir conditions (Allshop et al., 2001). We suggest application of NMR spectrometry to quantification of the small amounts of oil in the flooding experiments.

The UV/visible spectroscopy method is applicable to study oil-in-water samples in which suspended oil contains asphaltene fractions or functional groups that absorb radiation in the UV region. Evdokimov et al. (2003), investigated molecular aggregation of toluene solutions of crude oil and of solid asphaltene. Hence, UV/visible spectroscopy can also be applied to quantify the oil fractions of the produced fluids during the water flooding laboratory experiments.

In the present study, we set up NMR and UV/visible spectroscopy experiments for determining the small amounts of water and oil that are collected in glass vails. The samples were manually prepared to resemble the amounts of oil and water that come out of the cores under waterflooding. We were able to obtain a good correlation of the amount of oil added in the glass containers and the amount of oil detected from both techniques, in over 99% of all cases.

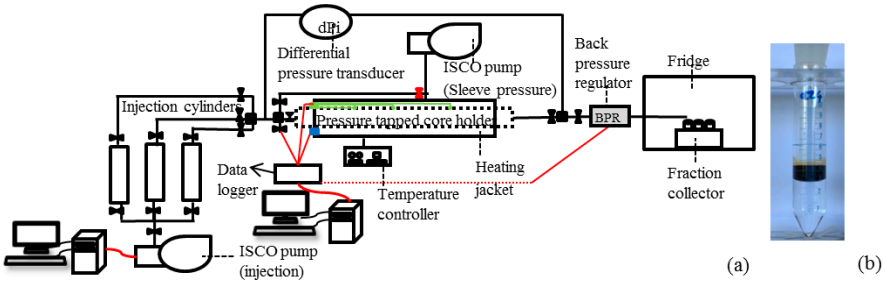

Figure 1 *Flooding device (a) and vial with produced fluids (b).*

Methods

Low-field NMR spectrometry. The low-field NMR spectrometry method involves a series of manipulations of the hydrogen nuclei found in pore fluids. The NMR measurements consist of the alignment and realignment of the hydrogen nuclear spin by action of static and oscillating magnetic fields. After each NMR measurement, an inversion technique converts the perturbation decay curve into distribution of T_2 (where, $1/T_2$, is the transverse relaxation rate). In the case of fluid samples containing both water and oil, the T_2 distribution provides information concerning the origin of the hydrogen atoms emitting the NMR signal. This NMR signal is related to the bulk relaxation, which is the intrinsic relaxation property of a fluid and is controlled by its physical properties, such as viscosity and chemical composition (Coates, 1999). In our experiments, the hydrogen nuclear spin in crude oil relax faster than in the water, hence, the T_2 distribution may be divided into the two individual areas. The peaks with T_2 less than 1 second correspond to the oil signal from a sample, while the peak in later times represents the water signal (Figure 2). The area under the curves may be

translated into the signal produced from those cases, and finally, into the volume of fluid emitting this signal.

The NMR measurements were carried out at a temperature of 30°C, with the GeoSpec2 NMR Core Analyzer. The T_2 relaxation curves were measured by applying a recycle delay (repetition time) of 40 s, the number of echoes 32768, CPMG inter echo spacing (τ) 50 μs and signal-to-noise ratio (SNR) approximately 400. The π/2 and π pulses were 9.75 μs and 19.5 μs, respectively. The T_2 relaxation spectra were generated using the WinDXP software (Oxford Instruments, UK).

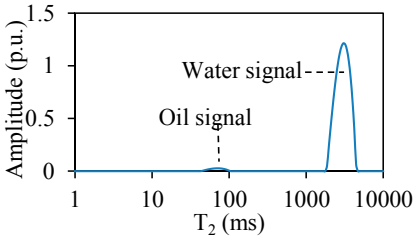

Figure 2 *Typical T_2 distribution of an oil/water sample (0.05 ml oil and 3.06 ml water).*

UV/visible spectroscopy. The samples were studied by application of UV/visible spectroscopy as described by Evdokimov et al. 2003. Approximately 3 ml of toluene (Sigma-Aldrich) was added to each sample. The sample was then shaken by hand in order to mix and to dissolve the oil. After the oil mixed with toluene, the sample was put steadily in a vial holder to let the liquid separate into two phases. The upper phase, containing the dissolved oil-in-toluene, was transferred into a quartz glass cuvette (path length 10 mm), after which the optical density was measured using the UV-VIS spectrophotometer (UV mini 1240, Shimadzu) at λ = 750 nm. When the spectrophotometer showed an absorbance value above 1 the sample was diluted in order to improve the accuracy of the measurement. The glass cuvette was rinsed with toluene (Sigma-Aldrich) and ethanol (CCS healthcare AB) followed by drying with compressed air before reusing to measure next samples. Only pure grade toluene and ethanol were used in the experiment. The absorbance data were plotted against the oil concentration.

Samples preparation. One set of pre-determined North Sea crude oil (density = 0.8453 g/cc ± 0.001) and synthetic seawater (density = 1.0191 g/cc ± 0.0005) samples were prepared in glass vials. The crude oil and synthetic seawater were weighted in a 10 ml glass vial using an analytical balance. The total volume was calculated on the basis of known mass and density. The volume of oil varied from 0 to 1 ml, and the combined volume of an oil/water sample was kept approximately constant at 3.1 ml. In total, 10 samples were prepared.

Results and discussion

Figure 3 presents the oil and water volumes detected in the NMR experiments against the known volume of oil within the glass vial. The NMR spectrometry was able to quantify the amount of oil and water with high accuracy. Figure 4 shows the absorbance data plotted against oil concentration for the UV/visible spectroscopy. Again, the correlation coefficient is over 99% as indicated on the Figures 3 and 4. By comparison with the standard curve for the composition of this oil, one may quantify the amount of oil.

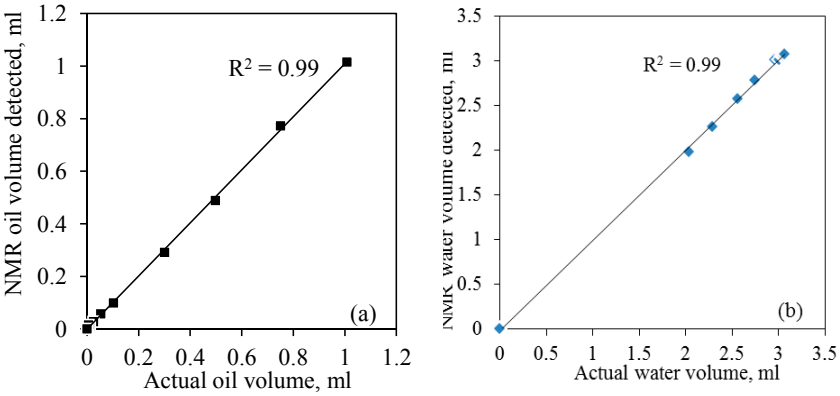

Figure 3 *a) Volume of oil and b) water detected with low-field NMR spectrometry vs. the actual volume of oil and water within the sample.*

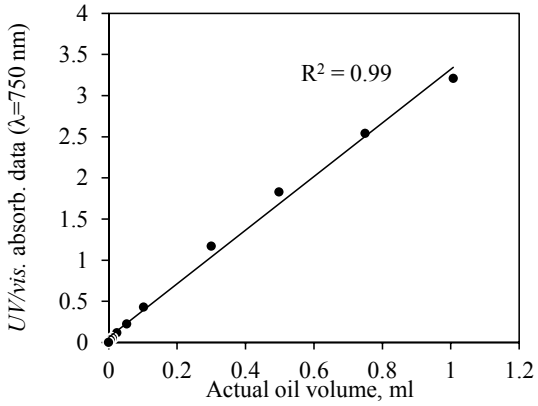

Figure 4 *UV/visible spectroscopy absorbance data at λ = 750 nm vs. volume of oil within the sample.*

In summary, low-field NMR spectrometry and UV/visible spectroscopy both give simple and direct analysis of the oil-in-water samples. The advantages and disadvantages of each method are listed in Table 1.

Conclusions

Low field NMR spectrometry and UV/visible spectroscopy were successful in accurate measurements of the oil contents in the oil-water samples in a full range of oil and water compositions. Both techniques may be applied to quantification of the amounts of oil produced in small amounts during core flooding experiments. The low field NMR spectrometry may additionally quantify the water fraction.

Table 1. *Advantages and drawbacks of low-field NMR spectrometry and UV/visible spectroscopy.*

Parameters	Low-field NMR spectrometry	UV/visible spectroscopy
Cost:	High	Low
Time consuming:	High	Low
Destructive:	No	Yes
Limitations in volume:	No – within probe detection range	No
Limitations in concentration:	No	Yes
User dependent:	No	Yes
Preparation:	No	Solvents
Standard curves:	No	Yes
Conditions of sample:	No – within probe detection range	No
Measures water fraction:	Yes	No
Other:	Limitation on the height of samples: detection of the signal is only possible within 5 cm range.	For oil samples above 200 ml an error can be introduced by diluting and pipetting.

Acknowledgments

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Appendix 3: Molasses Composition

Nordzucker

Analyser for dansk melasse, %/TS

Betaine	5,50%	PCA	3,50%	Potassium	5,60%
Galactinol	0,30%	GABA	0,75%	Sodium	1,10%
Inositol	0,14%	Glutamic acid	0,40%	Calcium	0,18%
Mannitol	0,10%	Aspartic acid	0,50%		
Glycerol	0,09%	Alanine	0,35%		
Choline	0,05%	Serine	0,25%		
		Valine	0,28%	Ammonium	0,50%
Uridine	0,10%	Iso-leucine	0,27%	Chloride	1,20%
Adenosine	0,12%	Leucine	0,28%	Sulfate	0,50%
Cytidine	0,08%	Glycine	0,11%	Nitrate	0,70%
Guanosine	0,10%	Thyrosine	0,30%	Phosphate	0,04%
Thymidine	0,01%	Proline	0,08%		
Adenine	0,05%	Threonine	0,06%		
Guanine	0,01%	Tryptophane	0,05%		
Uracil	0,003%	Fenyl-alanine	0,03%		
Thymine	0,00%	Methionine	0,05%		
Inosine	0,00%	Glutamine	0,02%		
Cytosine	0,01%	Arginine	0,02%		
		Histidine	0,02%		
		Cystine	0,01%		
		Lysine	0,01%		
		Asparagine	0,005%		

Nordzucker

Analyser for dansk melasse, %/TS

Sucrose	59,00%	Formic acid	0,40%
Raffinose	2,40%	Acetic acid	1,70%
1-Kestose	0,40%	Lactic acid	2,50%
Difructose	0,20%	Propionic acid	0,10%
6-Kestose	0,20%	Butyric acid	0,30%
Glucose	0,15%	Dihydroxy butyric acid	1,50%
Fructose	0,15%	Citric acid	0,30%
Melibiose	0,14%	Glycolic acid	0,20%
Rhamnose	0,10%	Gluconic acid	0,10%
Nystose	0,10%	Malic acid	0,40%
Galactose	0,10%	Oxalic acid	0,05%
Arabinose	0,10%	Maleic acid	0,15%
Neo-kestose	0,05%	Fumaric acid	0,15%
Mannose	0,05%	Succinic acid	0,12%
		Galacturonic acid	0,39%
		Aconitic acid	0,10%

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