EFFECT OF SALT ON BIODEGRADATION OF MODEL ALKANES AND CRUDE OIL SATURATES BY HYDROCARBON-DEGRADING BACTERIA

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Abstract

Crude oil leakages often give rise to *in situ* contamination with both oil and salt. In this study, the biodegradation of model alkanes and of saturated hydrocarbons in whole crude oil by hydrocarbon-degrading bacteria was investigated at different salt (NaCl, KCl, Na_2SO_4) concentrations. Changes in cell surface hydrophobicity at different NaCl concentrations were also investigated.

The results show that with increasing NaCl concentration, the lag phase for strain growth on hydrocarbons was prolonged; however, the total degradation efficiency was not influenced greatly. The formation of different sizes of cell aggregates at different salt concentrations indicated that salt could indirectly influence mass transfer of hydrocarbons from the medium to the interior of the cells. The results also showed that KCl had a less inhibitory effect on biodegradation than NaCl, and changes of Na_2SO_4 concentration did not greatly affect biodegradation. In addition, cell surface hydrophobicity increased with increasing NaCl concentration when the cultures were grown on hydrocarbons.

Résumé

La fuite de pétrole non-raffiné mène souvent à la contamination de l'environnement immédiat avec des sels et de différents hydrocarbure. Le travail présenté dans cette thèse étudie la biodégradation d'alcanes et de fractions pétrolières saturées à différentes concentrations de sels (NaCl, KCl, Na_2SO_4) à l'aide de bactéries capables de métaboliser ces hydrocarbures. L'hydrophobicité de la surface cellulaire de ces bactéries fut aussi étudiée à différentes concentrations de NaCl.

Les résultats démontrent que lorsque la concentration de NaCl est augmentée, la phase de latence et d'adaptation des bactéries cultivées sur hydrocarbure est allongée. L'efficacité de dégradation des hydrocarbures n'est toutefois pas affectée de façon significative. Le transfert des hydrocarbure du milieu de cultivation jusqu'à l'intérieur des cellules pourrait être indirectement affecté par la formation des agrégats de cellules de sels à différentes tailles observées. Les résultats démontrent également que l'effet inhibiteur du KCl est moins important que celui du NaCl, et que la concentration de Na_2SO_4 n'affecte pas le processus de biodégradation. L'hydrophobicité de la surface des cellules a augmenté avec une concentration de NaCl plus élevée lorsque les cellules sont cultivées en présence d'hydrocarbure.

Statement of Contributions and Collaborators

I hereby declare that I am the sole author of this thesis. I am responsible for all experiments and subsequent data analysis presented in this thesis.

Dr. Kathlyn Kirkwood supervised this thesis and project and provided continuous guidance and editorial contributions to this written work.

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List of Abbreviations

Analytical techniques

HPLC	High performance liquid chromatography
RID	Refractive index detector
GC	Gas chromatography
FID	Flame ionization detector
OD_{600}	Optical density at 600 nm

Organic substances

PAH	Polycyclic aromatic hydrocarbon(s)
TPH	Total petroleum hydrocarbons
SARA	Saturates, aromatics, resins and asphaltenes
BaP	Benzo[a]pyrene
DBT	Dibenzothiophene
DCM	Dichloromethane

Metabolites

$^{14}CO_2$	C-14 Carbon dioxide
ATP	Adenosine triphosphate
CoA	Coenzyme A

Growth Media

TSB	Trypticase soy broth
BHMV	Bushnell Häas medium with trace metals and vitamin solution
PCA	Plate count agar

Other

DD water	Distilled deionized water
PTFE	Polytetrafluoroethylene
BATH	Bacterial adherence to hydrocarbons
MATH	Microbial adherence to hydrocarbons
PBS	Phosphate buffer solution

Chapter 1 Introduction

1.1 Background

Crude oil or petroleum is a valuable resource and it is formed by anaerobic conversion of biomass under high pressure and temperature (Atlas and Bartha 1998). It is a viscous liquid and mainly includes saturated hydrocarbons (57% w/w), aromatic hydrocarbons (29%), resins and asphaltenes (14%) (Tissot and Welte 1984). By weight, the elemental composition of crude oil is 90% carbon and hydrogen; the other elements are primarily oxygen, nitrogen and sulphur and trace amounts of Fe, Si, Al and Ni (Salleh et al. 2003). Based on the number of carbon atoms in the hydrocarbons, the molecules in crude oil can be classified into fractions as a volatile fraction (C6-C10), a semi-volatile fraction (C10-C16), a nonvolatile fraction (C16-C33) and a recalcitrant fraction (C34-C50) (Greenberg et al. 2007).

With increasing exploitation, transportation and refinement of crude oil, oil spills in soil are also increasingly common and jeopardize the local ecological environment. Contaminant hydrocarbons not only affect soil physical structure through coating soil aggregates, but also block the movement of water and air in the soil matrix (Gawel 2006). Even though the soil has some capacity of self-cleaning, when the total petroleum hydrocarbons (TPH) concentration exceeds that capacity, they will accumulate in the soil (Ma et al. 2008). The TPH left in the soil may be absorbed by plants and move into human and animal bodies through the food chain (Fronca et al. 2006). Also, the polycyclic aromatic hydrocarbons (PAH) in crude oil could enter the human body by way of the respiratory tract and skin, and then affect human normal physiological function. Due to their toxicity and carcinogenic and mutagenic potentials, these components have already attracted public attention (Hidayat et al. 2013).

Because of their low solubility, high hydrophobicity and complex structure, crude oil compounds are hard to degrade under natural conditions (Lu et al. 2011). So far, there are three main methods to remediate contaminated soil: physical remediation, chemical remediation and biological remediation. The physical method is mainly using skimmers and absorbents to collect crude oil and the efficiency is only about 10-15%. The chemical method is using organic surfactants to disperse pollutants, which can endanger the local environment due to their toxicity (Thavasi et al. 2011). Compared with the physical and chemical methods, bioremediation can be a low-cost, high efficiency method which generates no secondary pollution. Bioremediation is typically using the local microorganisms to degrade the toxic pollutants as their food and transform them into innocuous substances such as H_2O, CO_2 and safe inorganic compounds (Joo and Kim 2013). It has been reported that about 25% of crude oil contaminated soil is being remediated with microorganisms (Holden et al. 2002). Abbasnezhad et al. (2011) described three parameters that affect bioremediation: microbial properties (e.g. adaptation to environmental conditions, microbial surface hydrophobicity, gene expression and regulation); contaminant properties (e.g., aqueous solubility, volatility, molecular weight); and environmental parameters (e.g., salinity, temperature, pH, nutrient availability, moisture level).

Numerous studies and reviews have investigated the above factors; however, few studies about the effect of salt on biodegradation have been reported so far. Crude oil leakages, inappropriate operations and refinery wastes give rise to *in situ* contamination with both petroleum hydrocarbons and salts. The produced water generated during oil and gas extraction (about 10 barrels produced water generated to produce one barrel oil) contains high salt concentrations from 1 to 250 g/L (Cuadros-Orellana et al. 2006, Fathepure 2014). In western Canada, salt-impacted sites are an unavoidable problem and brine water often contains various ions and salts

including SO_4^{2-}, HCO_3^- and chlorides of Na, K, Ca and Mg; among them, NaCl is the most prevalent salt (Greenberg et al. 2007). Generally, Cl^- and SO_4^{2-} are used as indicators of the degrees of a site's contaminant salinity and naturally salinity, respectively. A higher Cl^- : SO_4^{2-} ratio represents a higher level of contamination (Alberta Environment, 2001). Excess NaCl in the soil can affect ground water quality, destroy soil aggregation, inhibit plant ability to uptake water due to high osmotic potential and decrease the solubility of nutrients and oxygen in the soil matrix, which results in a low efficiency of pollution elimination (Gawel 2006). There is an inverse relationship between the solubility of hydrocarbons or oxygen and salinity; both the hydrocarbon and oxygen solubility is much lower in high salt conditions than in nonsaline conditions (Whitehouse 1984, McGenity et al. 2010). Unlike hydrocarbons, salts cannot be degraded by bacteria. Oren (1999) indicated that two primary mechanisms are utilized among all microorganisms to adapt to hypersaline conditions. The first one is to maintain a high intracellular salt concentration at least iso-osmotic with extracellular conditions; the other one is to balance osmotic pressure with organic solutes which can be synthesized by the cells or absorbed from the extracellular environment. The latter mechanism is always observed in halophilic and halotolerant microorganisms.

Present widely used methods to cope with salt and hydrocarbon co-contaminated soil are chemical amendments with gypsum or calcium nitrate and leaching before the biodegradation process; however, bioremediation will become time consuming and costly in this way (Alberta Environment, 2001). Several halotolerant bacterial strains have been selected from nature and applied for bioremediation, but the specific effects of salts on biodegradation are still not clear. Salt can be a crucial factor for the effective biodegradation of hydrocarbons.

1.2 Research objectives and thesis overview

The primary objective of this thesis was to determine the effects of different salts on cell growth, biodegradation of model hydrocarbons and crude oil by a pure cell culture and a defined consortium, and cell surface hydrophobicity. These results could give useful suggestions for enhancing the bioremediation of *in situ* oil-contaminated sites in hypersaline conditions. The research was mainly conducted in three phases: determine the halotolerance of *Rhodococcus erythropolis* EPWF with NaCl, KCl and Na_2SO_4; analyse the biodegradation of model saturated hydrocarbons and saturate fractions from crude oil; and measure the cell surface hydrophobicity with various salinities.

1.2.1 Halotolerance of *R. erythropolis* EPWF (Chapter 4.1)

The main purpose of this phase of research was to determine the ability of *R. erythropolis* EPWF to tolerate high salt concentrations. Glucose was used as the sole carbon source in the mineral salt medium over a range of salinities from 0-75 g/L NaCl, KCl and N_2SO_4.

Growth of EPWF was monitored through optical density measurement at 600 nm to provide the information for its halotolerance and to determine the impacts of potassium salt and sulfate. Glucose consumption was measured by high performance liquid chromatography (HPLC) in order to give a baseline for comparison with the pattern of the following biodegradation experiments.

1.2.2 Biodegradation of model saturated hydrocarbons by a pure culture (Chapter 4.2)

The aim of this section was to determine the effect of salt on biodegradation of saturated hydrocarbons. A pure bacterial culture, *R. erythropolis* strain EPWF, was applied for the biodegradation of model hydrocarbons and mixed hydrocarbons to establish a systematic understanding the influence of salt. Specific objectives included:

1. Quantify the biodegradation of pure hydrocarbon and mixed hydrocarbons by *R. erythropolis* EPWF at different NaCl concentrations.

2. Identify and quantify the biodegradation of a model hydrocarbon at different KCl and N_2SO_4 concentrations.

The straight-chain alkane hexadecane and the branched-chain alkane pristane were selected as model hydrocarbons. A mixture of these hydrocarbons was used to determine the effect of NaCl on biodegradation in a system with multiple carbon sources. Gas chromatography (GC) was used to analyze the samples based on the peak area of hydrocarbon.

The biodegradation of hexadecane experiments were also conducted by varying the concentration of potassium chloride and sodium sulfate, in order to compare the effects of different cations and anions on the biodegradation process.

1.2.3 Biodegradation of saturated alkanes in crude oil by a pure cell culture and a defined bacterial consortium (Chapter 4.3)

The primary purpose of this section was to investigate the biodegradation of the saturate fraction in crude oil at various salinities. Specific experimental objectives

included:

1. Identify any differences between the biodegradation of saturated alkanes extracted from crude oil by a single culture and a defined strain consortium at different NaCl concentrations.

2. Identify whether the results and conclusions obtained from the biodegradation of model hydrocarbons experiments (Chapter 4.2) have a wide applicability, which could account for the biodegradation of the saturate fraction in crude oil.

A pure *R. erythropolis* EPWF culture and a defined consortium (*R. erythropolis* Husky A, *R. erythropolis* S+14 He and *Gordonia* sp. Esso AGD) were used in this research. Due to the complex components in crude oil, the peak area of hydrocarbons could be interfered with by other components. Therefore, peak heights were measured in this case for approximate calculation. The results of this phase were compared with the results of section 4.2 to establish a systematic understanding of the effect of salt on alkane biodegradation.

1.2.4 Effect of NaCl on cell surface hydrophobicity (Chapter 4.4)

The purpose of this phase of research was to investigate the changes in cell surface hydrophobicity at various NaCl concentrations. The results could help give a better understanding of the mechanism of hydrocarbon uptake by *R. erythropolis* EPWF. Specific objectives included:

1. Identify the differences of cell surface hydrophobicity between glucose and hexadecane used as the carbon source.

2. Determine the changes in cell surface hydrophobicity during the incubation time at different salinities.

In this section, a new method was developed to disperse the cell aggregates formed in association with hydrocarbons, and cell surface hydrophobicity was analyzed by measuring the contact angle. Integration of the result with the previous results in chapters 4.1 and 4.2 provided a reasonable explanation for flocculation and the uptake of hydrocarbons at different NaCl concentrations.

Chapter 2 Literature Review

2.1 Mechanisms of biodegradation of hydrocarbons

Crude oil is a mixture of hydrocarbons; the major components are saturate and aromatic hydrocarbons (Tissot and Welte 1984). Depending on the size of hydrocarbon droplet, solubility and molecular structure of hydrocarbon, both uptake pathways and biodegradation pathways of saturates and aromatics are different. During biodegradation processes, two factors play important roles in the degradation of hydrocarbons. The first one is the activity of metabolic enzymes, which can be affected by many natural factors, such as temperature, pH, and salt concentration. The other challenge is that insoluble hydrocarbons are difficult for microorganisms to use (Kim et al. 2002). The solubility of longer alkanes (>C11) is less than 0.01 mg/L, while the solubilities of the polycyclic aromatic compounds phenanthrene (3 rings) and naphthalene (2 rings) are 1.29 and 31.6 mg/L at 25°C, respectively (Poeton et al. 1999). Bouchez et al. (1995) pointed out that hydrocarbons are more easily degraded from solution form than in crystalline form. Although the work in this thesis focused on saturated hydrocarbons, in this chapter the mechanisms of uptake and biodegradation of both saturated and aromatic hydrocarbons are reviewed in order to give a complete understanding, which would be helpful for future study.

2.1.1 Mechanisms of uptake of hydrocarbons

Hydrocarbon degrading microorganisms have developed several strategies to uptake hydrocarbons. Generally speaking, two main pathways have been widely proposed, depending on the aqueous solubility of the hydrocarbon and the size of hydrocarbon droplets relative to the size of cells. The first mechanism is through cells

directly contacting hydrocarbon droplets, which are larger than the cells (Beal and Betts 2000). It was reported that hydrophobic strains prefer to attach to hydrophobic hydrocarbons, while hydrophilic strains have a preference for solubilized substrates (Bos et al. 1999). The second mechanism is through excretion of surfactants or polysaccharides by microorganisms, which increases both the hydrophobicity of the cell membrane and the apparent solubility of hydrocarbons (Kaczorek and Olszanowski 2011; Bredholt et al. 2002). In this way, smaller sized hydrocarbon droplets can be surrounded by the surfactants and form pseudosolubilized hydrocarbon for cells to utilize (Goswami and Singh 1991, Beal and Betts 2000). This would be helpful for hydrophilic strains to uptake hydrophobic substrates; however, it has had no significant influence on the uptake of water-solubilized hydrocarbons (Kaczorek and Olszanowski 2011). Hua and Wang (2012) demonstrated that direct contact with big hydrocarbon drops always happened preferentially before cell contact with emulsified small hydrocarbon droplets. Also, some bacteria show a high specific affinity for a certain hydrocarbon and grow as a confluent biofilm with high uptake efficiency (Wick et al. 2002). Sometimes, the biodegradation process involves more than one mechanism.

When the hydrocarbons are in contact with the cell membrane, active or passive transport may take place depending on the strain and the substrate. Bateman et al. (1986) mentioned that a strain of *Pseudomonas putida* could transport naphthalene as an energy-independent process that did not require ATP or a protein carrier. Similarly, Bugg et al. (2000) reported the uptake of PAH by *Pseudomonas fluorescens* LP6a through passive diffusion, in contrast to the energy-driven efflux system in this organism, but efflux of naphthalene was still passive. In the case of active hydrocarbon uptake by cells, Whitman et al. (1998) and Beal and Betts (2000) demonstrated that naphthalene and hexadecane were transported by an energy-dependent system in

Pseudomonas fluorescens and *Pseudomonas aeruginosa*, respectively. Kim et al. (2002) reported that *Rhodococcus erythropolis* S+14He was able to transport and accumulate hexadecane by an active transport pathway. In addition, the strain could also distinguish and select the preferred substrate from mixed hydrocarbons with expenditure of metabolic energy.

2.1.2 Mechanism of degradation of alkanes, alicyclic hydrocarbons, and aromatic hydrocarbons

The biodegradation of hydrocarbons and crude oil varies with their molecular weight, chain length and structure. Atlas and Bartha (1998) reported that n-alkanes with a carbon number from 10 to 24 are degraded quickly; the short-chain-length n-alkanes normally evaporate from the contaminated soil. However, if the molecular weight of the hydrocarbons is over 500, they could not be used as a carbon source. They also demonstrated that branched alkanes are harder to degrade than n-alkanes due to the interference of tertiary and quaternary carbon atoms. Likewise, aromatic compounds and alicyclic compounds without long aliphatic side chains are both more difficult to degrade than linear hydrocarbons; more than two cooperating bacterial strains are needed in some cases (Atlas and Bartha 1998).

Degradation of alkanes: One or both terminal methyl groups of an alkane can be attacked by enzymes (monooxygenases or dioxygenases) and turned into alcohol (Atlas and Bartha 1998). Some *Rhodococcus* spp. can oxidize n-alkanes from both terminal groups (Whyte et al. 1998). The alcohol will be further oxidized, and finally become the carboxylic acid. Next, the fatty acid is converted to the fatty acyl–CoA (Equation 2.1) by fatty acyl–CoA synthetase and undergoes the repetitive process which is called β-oxidation (Equations 2.1-2.5) (Nelson and Cox 2008, Atlas and

Bartha 1998).

$$R\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}COOH \xrightarrow[+CoA\text{-}SH]{-H_2O} R\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}S\text{-}CoA \quad \text{(eq. 2.1)}$$

$$R\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}S\text{-}CoA \xrightarrow[-2H^+]{} R\text{-}CH_2\text{-}CH{=}CH\text{-}CO\text{-}S\text{-}CoA \quad \text{(eq. 2.2)}$$

$$R\text{-}CH_2\text{-}CH{=}CH\text{-}CO\text{-}S\text{-}CoA \xrightarrow[+H_2O]{} R\text{-}CH_2\text{-}CH(OH)\text{-}CH_2\text{-}CO\text{-}S\text{-}CoA \quad \text{(eq. 2.3)}$$

$$R\text{-}CH_2\text{-}CH(OH)\text{-}CH_2\text{-}CO\text{-}S\text{-}CoA \xrightarrow[-2H^+]{} R\text{-}CH_2\text{-}CO\text{-}CH_2\text{-}CO\text{-}S\text{-}CoA \quad \text{(eq. 2.4)}$$

$$R\text{-}CH_2\text{-}CO\text{-}CH_2\text{-}CO\text{-}S\text{-}CoA \xrightarrow[+CoA\text{-}SH]{} R\text{-}CH_2\text{-}CO\text{-}S\text{-}CoA + CH_3\text{-}CO\text{-}S\text{-}CoA \quad \text{(eq. 2.5)}$$

The fatty acid is shortened by a two-carbon unit after every cycle. The acetyl CoA will go through the tricarboxylic acid cycle and finally is converted to CO_2 (Atlas and Bartha 1998). The final products of hydrocarbon mineralization are therefore only water and carbon dioxide. Alkane biodegradation can also be achieved under anaerobic conditions. The hydrocarbons undergo a series of reactions leading through a 1-alkene, an alcohol, and an aldehyde to a fatty acid (Atlas and Bartha 1998).

Some microorganisms (like *Pseudomonas* spp. and *Acinetobacter* spp. (Whyte et al. 1998) can attack the subterminal methyl group of alkanes (Figure 2.1). In this way, a secondary alcohol will be formed and further oxidized to a ketone and finally to an ester (Atlas and Bartha 1998). The ester can then be hydrolyzed by adding water and become a primary alcohol and a fatty acid; the alcohol is oxidized and becomes a carboxylic acid (Atlas and Bartha 1998). Both the fatty acids will undergo β-oxidation as described before.

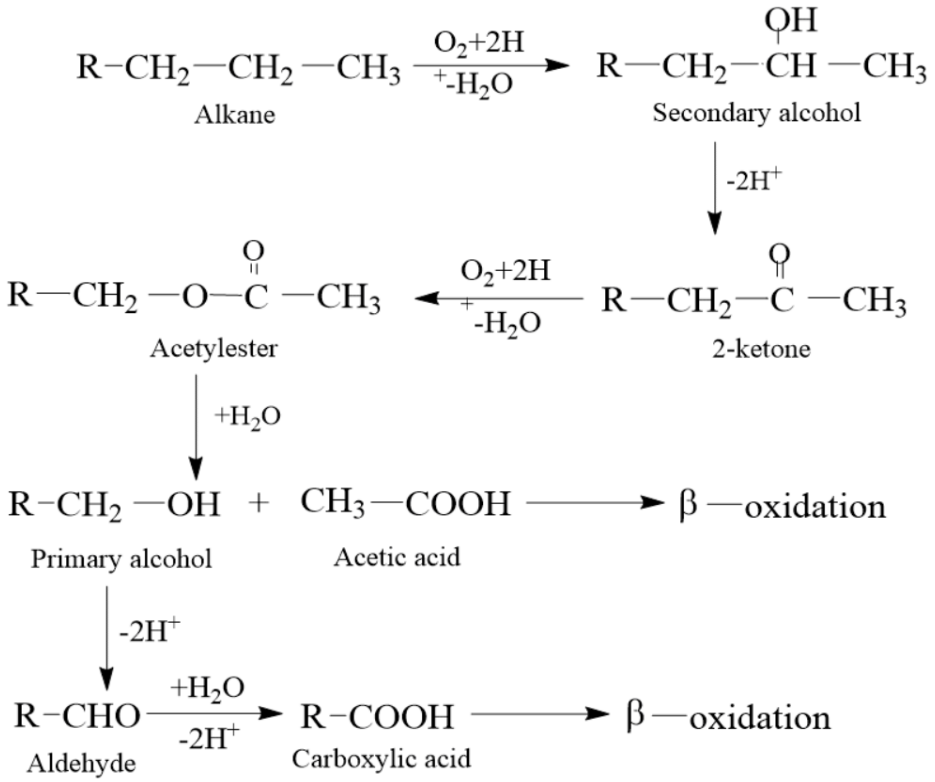

Figure 2.1 Metabolic pathway for sub-terminal n-alkane oxidation (adapted from Atlas and Bartha 1998).

Compared with n-alkanes, branched alkanes are more recalcitrant and hence accumulate in the environment. Schaeffer et al. (1979) have reported that oxidation processes can be severely impeded by iso-termini and prevented by anteiso-termini due to steric inhibition of terminal oxidizing enzymes. On this occasion where a branch is located at the β-position, the β-oxidation process will be prevented and the other oxidative pathways are needed (i.e., α-oxidation and ω-oxidation) (Schaeffer et al. 1979). In the case of the pathway of pristane (2,6,10,14-tetramethylpentadecane) degradation, Mikolasch et al. (2009) proposed that pristane can be catabolized through mono-, sub- and di-terminal oxidation depending on the species of microorganism. They reported that *Rhodococcus* spp. could degrade pristane through mono- or di-terminal oxidation with the production of mono- and dicarboxylic acids.

Degradation of alicyclic hydrocarbons: Since alicyclic hydrocarbons have no terminal methyl group, the mechanism of degradation is similar to subterminal oxidation as described above (Atlas and Bartha 1998) (Figure 2.2). For example, cyclohexane can be hydroxylated by monooxygenases and is converted to cyclohexanol, then cyclohexanone. The ketone will be further oxidized to ε-caprolactone and undergoes hydrolysis to form 6-hydroxyhexanoic acid. 6-Hydroxyhexanoic acid is subsequently dehydrogenated to adipic acid via 6-oxohexanoic acid. The fatty acid is then metabolized by β-oxidation and converted to H_2O and CO_2 (Atlas and Bartha 1998).

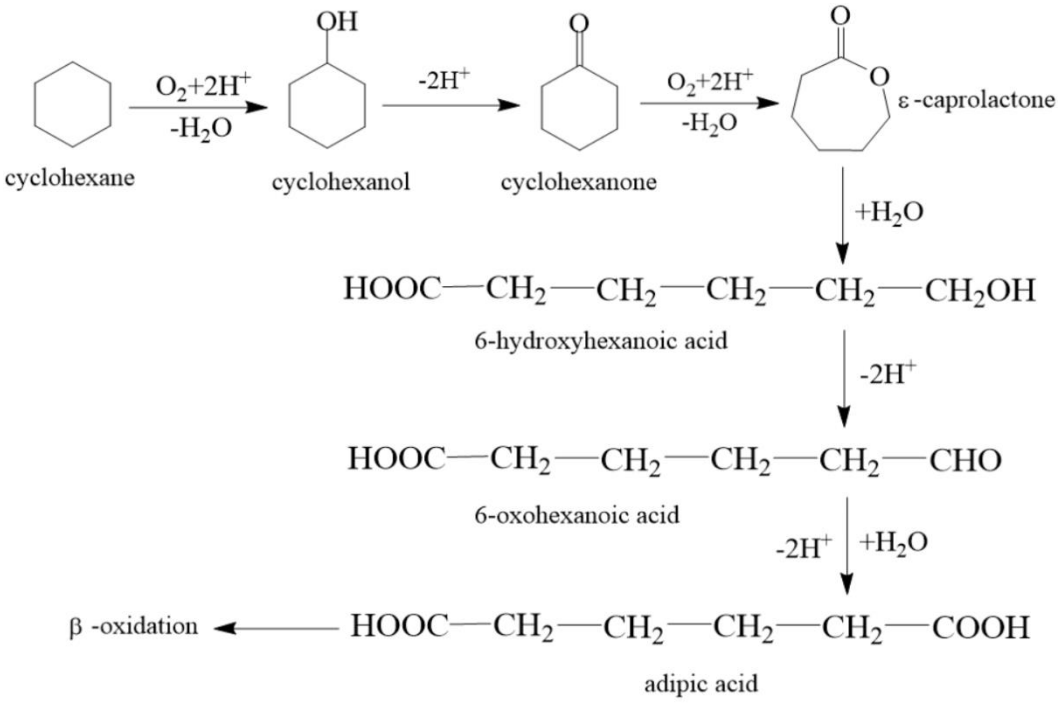

Figure 2.2 Metabolic pathway of cyclohexane biodegradation (adapted from Atlas and Bartha 1998).

Degradation of aromatic hydrocarbons: With an increasing number of fused benzene rings, the biodegradability of PAH decreases (Cerniglia and Heitcamp 1989). PAH can be metabolized through multienzyme systems and then converted into

dihydroxylated intermediates and finally become the intermediates in the tricarboxylic acid cycle (Van der Meer et a1. 1992).

Monoaromatic hydrocarbons such as benzene are oxidized by a three-enzyme system to a cis,cis-dihydrodiol, which is converted to catechol by dehydrogenation (Figure 2.3). Then the catechol can be further oxidized in two ways: ortho-cleavage and meta-cleavage; the cis,cis-muconic acid and the 2-hydroxy-cis,cis-muconic semialdehyde are formed, respectively. Both the hydrocarbons will be further metabolized which leads to acetaldehyde, pyruvic acid, succinic acid, and acetyl-CoA. Many reports also discussed toluene biodegradation and showed that different microorganisms have different pathways to degrade the hydrocarbons (Atlas and Bartha 1998).

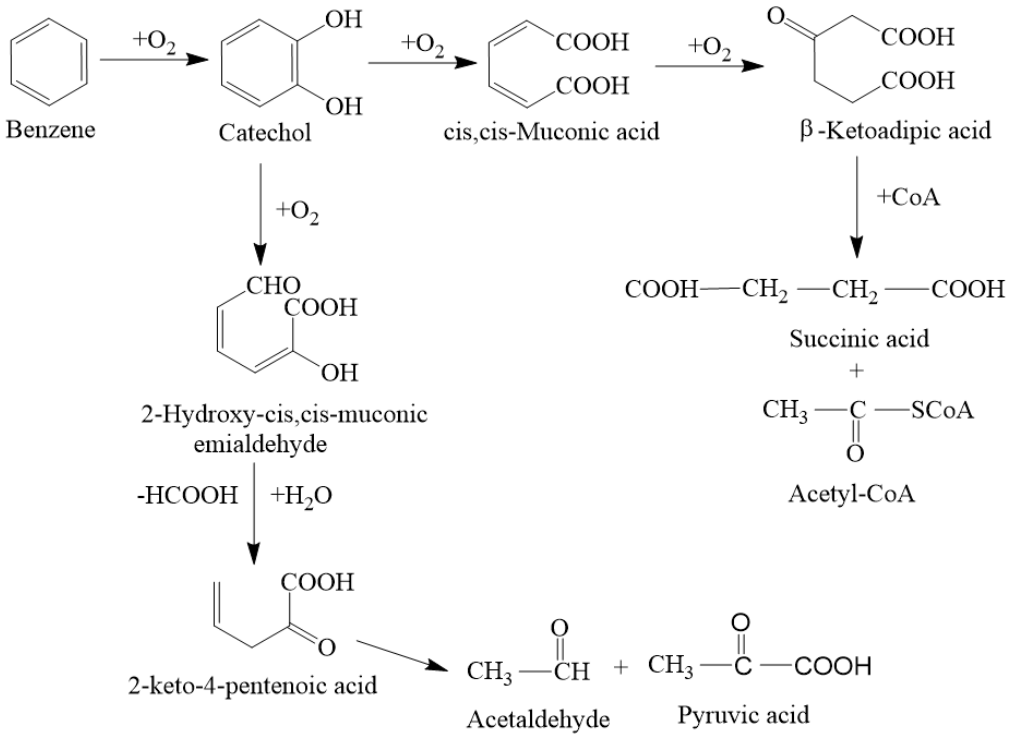

Figure 2.3 Metabolic pathway of benzene biodegradation by meta or ortho cleavage (adapted from Atlas and Bartha 1998).

PAH are also degraded by various microorganisms. Naphthalene, for example (Figure 2.4), is oxidized by the multicomponent enzyme naphthalene dioxygenase to form cis-1,2-dihydroxy-1,2-dihydronaphthalene, which is converted to 1,2-dihydroxynaphthalene through a cis-dihydrodiol dehydrogenase. This compound undergoes ring cleavage to produce salicylate. The salicylate will be decarboxylated to catechol, which is finally metabolized by ortho or meta cleavage as mentioned before. Biodegradation of other more complex PAH can also be achieved through different pathways of different microorganisms (Seo et al. 2009).

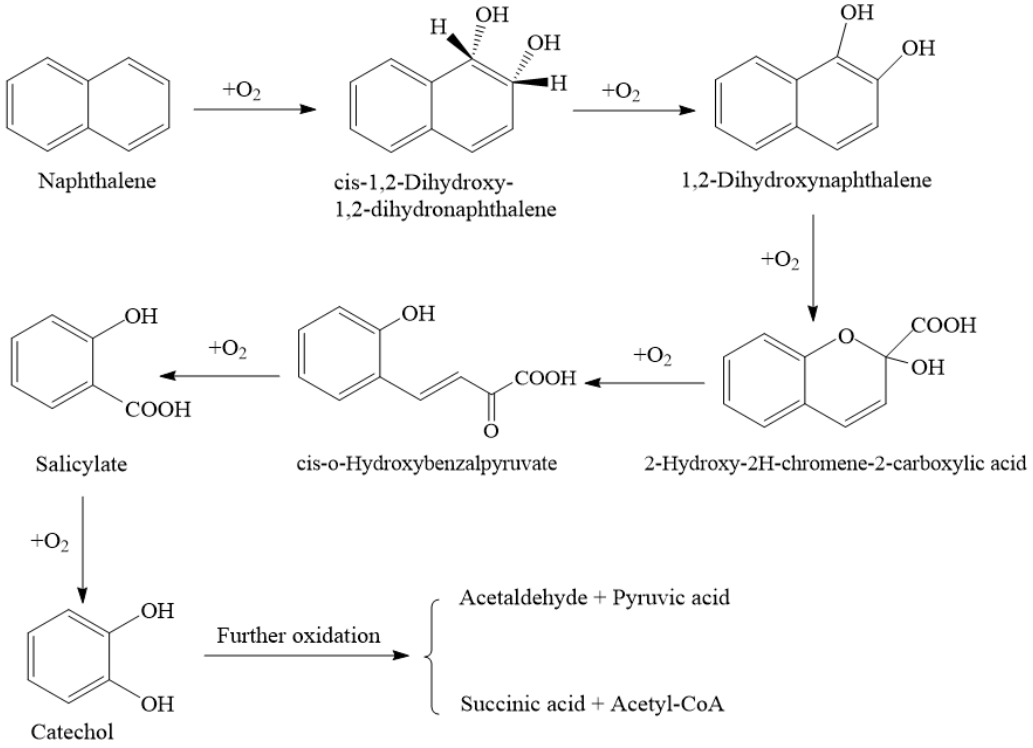

Figure 2.4 Metabolic pathway of naphthalene (adapted from Seo et al. 2009).

2.1.3 Pattern of degradation of crude oil

Crude oil is a complex mixture of primarily hydrocarbons, which can be classified as four groups, saturates, aromatics, resins and asphaltenes (SARA) (Gary 1994). Figure 2.5 presents the GC profile of saturated fractions in the light crude oil from the Strathcona refinery provided by Imperial Oil. The retention time of each compound is based on its boiling point. As the carbon chain elongates, the boiling point increases, which results in the longer retention time. In addition, alkanes with a branched chain usually have a lower boiling point than straight-chain alkanes when the carbon number is the same. Therefore, branched pristane (C19) and phytane (C20; 2,6,10,14-tetramethylhexadecane) elute immediately after n-heptadecane (C17) and n-octadecane (C18). Heteroatomic compounds are also common in crude oil, and include other elements such as sulfur, nitrogen and oxygen besides hydrogen and carbon (Jalilzadeh et al. 2014).

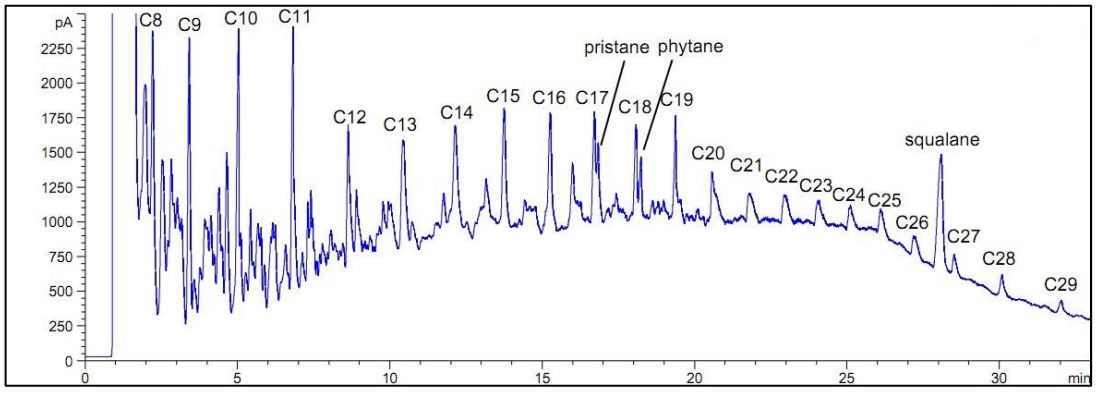

Figure 2.5 GC analysis of saturated fractions from crude oil in the control.

Many papers report that hydrocarbons in crude oil are degraded according to different susceptibility in the following order: n-alkanes > branched alkanes > low molecular weight aromatics and cyclic alkanes > polar compounds (resins and

asphaltenes) (Leahy and Colwell 1990, Chen et al. 2014). As the number of carbon atoms of an alkane increases, more time is needed for biodegradation. Vinas et al. (2002) found that with increasing the number of methyl groups in the carbon chain, the extent of biodegradation generally decreased. Lin et al. (2014) studied biodegradation of aromatic hydrocarbons and found that the degradation efficiency decreased with the increase in the number of rings of aromatic hydrocarbons. Degradability of resins and asphaltenes is quite low compared with that of saturate and aromatic hydrocarbons (Vinas et al. 2002).

2.2 Effect of salt on microbial growth

2.2.1 Effect of salt on cell growth

As is well known, inorganic salt is an indispensable element for organism growth. It could adjust the osmotic pressure of the cell's membrane, maintain enzyme activity, and has other important functions. Many studies have demonstrated that salt concentration is a key factor which has a prominent influence on microbial growth. High salt concentration (23.4 g/L NaCl) will inhibit over 90% of microorganisms in the soil (Rousk et al. 2011).

de Carvalho and Fonseca (2005a) found that higher salinity could prolong the lag phase of *R. erythropolis* strain DCL14. Similar results were also reported by other researchers. Wu et al. (2012) used crude oil as a carbon source to detect *Serratia* sp. BF40 strain reproductive capacity and found that the strain grew well at low NaCl concentration (<40 g/L); but when the salt concentration was up to 60 g/L, both the reproduction time and growth plateau decreased. However, bacterial growth was completely inhibited at a salt concentration above 80 g/L. Dastgheib et al. (2012) evaluated the effect of different NaCl concentrations on the growth of a bacterial

consortium (mixed culture) called Qphe with phenanthrene as sole carbon source. The results showed that Qphe has the highest growth rate between 50 g/L and 100 g/L NaCl, but once the salt concentration is above 100 g/L, the growth rate decreased sharply. Xia et al. (2012) measured the optical density (OD) of strain *Geobacillus pallidus* H9 at different NaCl concentrations when growing on crude oil and found that OD increased slowly at 0-30 g/L NaCl; the OD decreased above this range. Ulrich et al. (2009) studied the growth of hydrocarbon-adapted natural microorganisms in different stresses and salinities. They observed that higher salt concentrations can inhibit the activity of cells which were not adapted to salt and cause longer lag time, while physiological concentrations of salt could enhance the microbial activity. Qin et al. (2012) also reported in their paper that moderate salt concentration is needed to stimulate microbial strain growth. This light stimulation can be explained by the salt promoting the ionic balance or clay dispersion in the medium and thus enhancing the contact area for microbes to absorb trace nutrients.

Some previous studies gave the reasons for this inhibition. Pollard et al. (1994) reported that the salt could influence or change the tertiary protein structure of cells and denature enzymes in the cell. However, different bacteria have different sensitivity to salinity. Hartmans et al. (1991) confirmed that a high chloride ion concentration inhibits enzyme activity, through a kind of feedback inhibition. Lv et al. (2010) investigated the effect of NaCl on a core gene (psbA) expression in *Dunaliella salina.* They found that the expression level of the psbA gene reach the maximum value at 146.25 g/L NaCl when compared to the other concentrations and a high salt concentration (234 g/L NaCl) inhibited the gene expression significantly. Behnood et al. (2013) measured the activities of manganese peroxide enzyme (MnP) and lignin peroxide enzyme (LiP) in *Phanerochaete chrysosporium* with different NaCl concentrations. The activity of MnP enzyme was not affected within 20 g/L salt

concentration, but it decreased dramatically in 30 and 40 g/L salinities. The effect of salt on the activity of LiP enzyme was prominent, and nearly 100% of the enzyme was inhibited at 40 g/L salinity.

These papers illustrated that microorganism growth needs an optimum salt concentration. In most cases, physiological concentrations of NaCl will stimulate cell growth. However, once salt concentration is above this range, protein structure will be changed, affecting gene expression and causing non-reversible damage to cells. Consequently, the lag phase was prolonged and cell growth was affected.

2.2.2 Effect of salt on cell morphology

Besides inhibition of enzyme and gene expression, salt could also change the dimension of cells. Peyton et al. (2002) reported that high sodium concentrations may cause non-reversible damage to cells and affect their growth owing to plasmolysis. A similar result was also reported by Hua et al. (2010); they found cell disruption could occur once the salt concentration passed beyond a threshold due to increased membrane permeability. Rhykerd et al. (1995) showed that as salt concentration increased to a certain degree, the microorganism activity was decreased due to osmotic effects, but their viability will not be influenced. Therefore, this kind of bacterium should also be able to maintain autologous osmotic balance besides treating the pollutants (Zhuang et al. 2010). Zvyagintseva et al. (2001) reported that the oil-degrading strain *Dietzia maris* INMI 101 increased about 100% in size and decreased 25% in size when cells were grown at 5 and 100 g/L NaCl concentration, respectively, compared to the size of cell growth at 20-50 g/L NaCl concentration. de Carvalho and da Fonseca (2005a) studied *Rhodococcus erythropolis* DCL14 growth on hydrocarbons and found the cell size had decreased about 30% at the highest salt

concentration (25 g/L) as compared to the cells at 0 g/L NaCl. Cell shrinkage under hypertonic condition could result in the surface area between cells and substrates increasing. This could be helpful for transporting nutrition from the outer environment to the interior of the cells.

2.2.3 Mechanisms of halotolerance

Based on the microbial growth response to salt, bacteria can be divided into halotolerant and halophilic strains (Todar 2006). Halophiles have a specific requirement for high salt concentrations, which can be classified into mild (10-60 g/L NaCl), moderate (60-150 g/L NaCl) and extreme halophiles (15-300 g/L NaCl). Bacteria that can tolerate moderate salinities (60-150 g/L NaCl), but show the best cell growth in the absence of salt, are called halotolerant strains (Todar 2006).

Brown (1976) reported that salt could affect the transcription of halobacterial DNA and the activity of enzymes. At physiological conditions, the function of pyridine nucleotide-linked dehydrogenases is normal; however, the function will be inhibited at high salt concentrations. The extent of inhibition also depends on the salts; potassium salt showed a less severe effect on isocitrate dehydrogenase than sodium salt (Robinson & Stokes 2002).

Two mechanisms are usually adopted by cells to adapt to a hypersaline environment. The first one is the intracellular accumulation of ions (like K^+, Cl^-) in the cytoplasm, in order to maintain the osmotic pressure across the cell membrane (Roeßler and Müller 2001; Oren et al. 2002). The other one is through producing compatible solutes (like polyhydric alcohols, ectoine and hydroxyectoine) to minimize the salt stresses. These compatible solutes are highly soluble organic molecules, which accumulated intracellularly to partly protect enzyme activity and partly adjust osmotic

pressure (Brown 1976; Ofer et al. 2012).

2.3 Effect of salt on characteristics of the microbial membrane

2.3.1 Effect of salt on extracellular substances production

In order to adapt to new environmental conditions, strains could also modify the properties of their membrane through producing extracellular polymeric substances (EPS) or biosurfactants (Whyte et al. 1999). These excreted substances can emulsify the petroleum hydrocarbons, change the tension of the medium, and increase the apparent solubilities of hydrocarbons in the aqueous phase (Mnif et al. 2009, Darvishi et al. 2011). Ward et al. (2003) have already found that emulsification could promote the attachment between bacteria and hydrocarbons. Wu et al. (2012) measured the surface tension and emulsification activity of soil solutions with *Serratia* sp. BF40 under different salt concentrations of crude oil and found that the surface tension has been changed little with lower salt concentration (<20 g/L), but above 20 g/L NaCl a positive correlation between surface tension and salt concentration was observed. They also showed that salt concentration has a significant effect on emulsification activity, which decreased sharply above 10 g/L NaCl. This is in agreement with the results acquired in previous studies; Darvishi et al. (2011) demonstrated that increasing NaCl concentration led to biosurfactant production reduction and interfacial tension increase. Mishra and Jha (2009) also found the emulsifying activity of EPS produced by *Dunaliella salina* decreased from 85.76% retention to 66.37% retention with the salt concentration increasing from 29.25 to 292.5 g/L. However, Abbasnezhad et al. (2008) compared adhesion of hydrophilic strain *Pseudomonas fluorescens* LP6a to hexadecane with different ionic strengths in the medium and observed that higher ionic

strength promoted adhesion of LP6a cells both to oil and to each other. The authors also indicated that adhesion is dependent on the ionic strength of the medium but not on a certain cation.

2.3.2 Effect of salt on changes of cell membrane

Many papers report that some strains (*Rhodococcus* sp., *Halomonas* sp., *Pseudomonas* sp., *Ochrobactrum intermedium*) can regulate their cell-surface hydrophobicity when hydrophobic substrates are used as carbon sources, but do not show this characteristic during growth on water-soluble substrates (Bredholt et al. 2002, Mnif et al. 2009, Mishra et al. 2012). By forming a hydrophobic cell surface, the strains could establish an efficient way to uptake the hydrocarbons (Bredholt et al. 2002). The higher extent of hydrophobicity results in a higher level of adhesion between hydrophobic substrates and cells. de Carvalho et al. (2014) measured the unsaturated index (the sum of the percentage of each unsaturated fatty acid multiplied by the number of double bonds in the molecule) of *Rhodococcus erythropolis* DSM 1069 membrane under different salt concentrations with ethanol as the carbon source. They noticed that the index reached the lowest value when the cells were exposed to 25 and 35 g/L NaCl, which means the cell membrane showed the highest extent of hydrophobicity at these two salt concentrations. Both higher and lower salt concentration resulted in opposite behaviours.

From the above, higher salt concentration could increase the specific surface area of strains due to cell shrinkage and promote the transportation of nutrients. Also, with salt concentration increasing, surface tension will increase and emulsification activity will decrease which will go against the utilization of hydrocarbons. In addition, salt can affect the hydrophobicity of the cell membrane within a certain concentration. Consequently, the adhesion of strains to carbon sources can be affected by the salt from

many aspects, not just depending on a single factor.

2.4 Effect of salt on biodegradation of hydrocarbons and crude oil

2.4.1 Effect of NaCl on biodegradation of alkanes

Saturated hydrocarbons are the most prevalent components in crude oil, but few papers have evaluated the effect of salt on biodegradation of these hydrocarbons (Table 2.1). Ulrich et al. (2009) used indigenous microbes from a natural gas processing facility and a flare pit site to degrade radiolabeled n-decane and n-hexadecane, respectively, with 0-50 g/L NaCl. The $^{14}CO_2$ release rate and maximum cumulative $^{14}CO_2$ release were measured to evaluate the rate and extent of biodegradation. The best degradation result for hexadecane was observed at 10 g/L NaCl (34.3±3%) and for decane was only around 6% (50 g/L). Díaz et al. (2002) reported the isolation of a consortium named MPD-M from sediment which included several strains of *Marinobacter* sp., *Bacillus* sp. and *Erwinia* sp. The consortium could not only degrade n-heptadecane (n-C17) and n-octadecane (n-C18), but could also degrade pristane and phytane in the presence of up to 180 g/L NaCl. About 90% of n-C17 and n-C18 was degraded at 20 g/L NaCl, while only 4% was degraded at 180 g/L NaCl. The biodegradation efficiencies of pristane and phytane were lower (0.1-30% and 2-40%, respectively), and the optimal salt concentration for biodegradation of these two branched alkanes was 40 g/L salinity. Once the salt concentration was higher than 100 g/L, the biodegradation decreased significantly. The use of halotolerant and halophilic strains to degrade alkanes in hypersaline environments has also been reported. Tapilatu et al. (2010) studied halophilic *Haloferax* sp. MSNC 16 to degrade n-C17 and n-C20 (n-eicosane) with 225 g/L NaCl and obtained degradation efficiencies of 95% and 67%,

respectively. Al-Mailem et al. (2010) isolated halophilic *Halobacterium* sp. HA-3 from hypersaline coasts of the Arabian Gulf and found that this strain could degrade n-C18 at salinities ranging from 58.5 to 234 g/L NaCl, while the best biodegradation result occurred at 175.5 g/L (65%). Dastgheib et al. (2011) have reported the degradation of n-tetracosane (n-C24) by halotolerant *Alcanivorax* sp. Qtet3 under 0-200 g/L NaCl. They found that the optimal salt concentration was 50 g/L with a biodegradation efficiency of 90%; however, the efficiency reached a minimum at a salinity of 200 g/L (approximately 2%).

These reports demonstrated that increased salt concentration generally resulted in decreased biodegradation. However, due to the different types of microorganisms studied, the optimal salt concentrations were also different. Once the salinity exceeded an optimal value, the degradation efficiency always decreased sharply. In addition, as the number of carbon atoms in the carbon chain increased, the extent of degradation decreased. Compared with branched alkanes, straight-chain alkanes were easier for degraders to utilize.

Table 2.1 Biodegradation of alkanes under different NaCl concentrations

Hydrocarbon	Degrader	NaCl (g/L)	Optimal NaCl concentration (g/L) (removal efficiency (%))	Reference
n-Decane	Indigenous microbes from a natural gas processing facility	0-50	50 (6)	Ulrich et al. (2009)
n-Hexadecane	Indigenous microbes from a flare pit site	0-50	10 (34.3±3)	Ulrich et al. (2009)
n-Heptadecane	Consortium MPD-M (*Marinobacter* sp., *Bacillus* sp. *Erwinia* sp.)	0-180	20 (90)	Díaz et al. (2002)
	Haloferax sp. MSNC 16	225	225 (95)	Tapilatu et al. (2010)
n-Octadecane	Consortium MPD-M	0-180	20 (85)	Díaz et al. (2002)
	Halobacterium sp. HA-3	58.5-234	175.5 (65)	Al-Mailem et al. (2010)
Pristane	Consortium MPD-M	0-180	40 (30)	Díaz et al. (2002)
Phytane	Consortium MPD-M	0-180	40 (40)	Díaz et al. (2002)
n- Eicosane	*Haloferax* sp. MSNC 16	225	225 (67)	Tapilatu et al. (2010)
n-Tetracosane	*Alcanivorax* sp. Qtet3	0-200	50 (90)	Dastgheib et al. (2011)

2.4.2 Effect of NaCl on biodegradation of aromatic hydrocarbons

The effect of polycyclic aromatic hydrocarbons (PAHs) on the environment cannot be neglected, due to their carcinogenicity and toxicity. PAHs in crude oil usually contain two to four or five fused rings per molecule, and the number of rings usually affects the persistence of PAHs in the environment (Fathepure 2014). Some work has been done to demonstrate the effect of salt on bioremediation of aromatic hydrocarbons (Table 2.2). Naphthalene is the simplest aromatic hydrocarbon with a low boiling point. Wang et al. (2009) isolated *Microbacterium* sp. 3-28 from PAH contaminated soil and determined its ability to degrade naphthalene in the presence of 10-50 g/L NaCl. At salinities ranging from 10-30 g/L, naphthalene biodegradation efficiencies were 70-85%, and decreased to 28% at 50 g/L NaCl. Vaidya and Kadam (2011) have isolated *Burkholderia glathei*, *Alcaligenes denitrificans* and *Pseudomonas putida* from petroleum contaminated soils in Mumbai, India. Their results showed that these strains could efficiently degrade about 35-43% of naphthalene at 5-30 g/L NaCl, and the optimal salt concentration for biodegradation was 15 g/L. Anthracene and phenanthrene are both three-ring aromatic hydrocarbons studied by several researchers. Wang et al. (2009) utilized *Microbacterium* sp. 3-28 to degrade anthracene and phenanthrene at 10-50 g/L NaCl. The results showed that about 72% of anthracene and 82% of phenanthrene were degraded at 20 g/L NaCl; however, at high salt concentration only about 30% of each hydrocarbon was degraded by the strain. Kumar et al. (2007) evaluated the ability of *Bacillus* sp. DHT to utilize phenanthrene and dibenzothiophene (DBT) in a medium with 0-100 g/L NaCl and observed that the efficiency did not change obviously (67-69% and 72-74%, respectively) over the full range of salt concentrations. Ulrich et al. (2009) collected indigenous microbes from a flare pit site and reported the microbes could degrade

phenanthrene in the presence of 0-50 g/L NaCl. The most effective degradation observed in the medium was at a salinity of 5 g/L (55±5%).

Studies of the degradation of phenanthrene by halophilic strains and consortia were also reported in recent years. Another halophilic strain *Martelella* sp. AD-3 was collected by Feng et al. (2012) from petroleum-contaminated soil with high salinity. It was observed that *Martelella* sp. AD-3 could degrade 28% of phenanthrene at 150 g/L, while the best degradation occurred at 30 g/L NaCl with an efficiency of 83%. In another work by Lin et al. (2014), *Pseudomonas* sp. BZ-3 was isolated and reported to degrade phenanthrene over a range of salinities from 20-120 g/L NaCl. They found that the increased NaCl concentration led to the reduction of biodegradation efficiency; the biodegradation of phenanthrene decreased from about 72% at 20 g/L NaCl to 51% at 120 g/L NaCl.

Degradation of four- and five-ring PAHs at different salt concentrations has also been reported. Wang et al. (2009) investigated the ability of *Microbacterium* sp. 3-28 to degrade pyrene in the presence of 10-50 g/L NaCl, about 70% of the initial pyrene was degraded at its optimal salinity (20 g/L). Badejo et al. (2013) measured the amount of residual pyrene in the culture over a range of salinities from 0-58.5 g/L NaCl using *Mycobacterium gilvum* PYR-GCK. The biodegradation efficiency reached nearly 100% from 0 to 10 g/L NaCl, and more than 75% of the pyrene was degraded at 58.5 g/L NaCl. Hadibarata et al. (2014) have isolated *Pleurotus eryngii* F032 from decayed wood in Indonesia's tropical rain forest and determined the ability of the strain to degrade fluoranthene at 5.85-29.25 g/L NaCl. The results showed that the fluoranthene degradation was 87% at 5.85 g/L, around 35% at 11.7 g/L, and 10% with 29.25 g/L. Liang et al. (2014) assessed benzo[a]pyrene (BaP) biodegradation capacity of *Pseudomonas* sp. JP1 with 0-40 g/L NaCl. They noticed that when the salt

concentration was 10-20 g/L, the strain showed the strongest ability to degrade BaP (19%), however, the efficiency decreased to 2% at a salinity of 40 g/L. Overall, studies reveal that an increased number of rings in aromatic hydrocarbons resulted in a reduction in the extent of biodegradation. Many strains could degrade hydrocarbons over a wide range of salinities, but the best degradation result always occurred at the physiological salt concentration.

Table 2.2 Biodegradation of aromatic hydrocarbons under different NaCl concentrations

Hydrocarbon	Degrader	NaCl (g/L)	Optimal NaCl concentration (g/L) (removal efficiency (%))	Reference
Naphthalene	*Microbacterium* sp. 3-28	10-50	30 (85)	Wang et al. (2009)
	Burkholderia glathei	5-30	15 (41)	Vaidya and Kadam (2011)
	Alcaligenes denitrificans		15 (43)	
	Pseudomonas putida		15 (45)	
Anthracene	*Microbacterium* sp. 3-28	10-50	20 (72)	Wang et al. (2009)
Phenanthrene	*Bacillus* sp. DHT	0-100	100 (69±3)	Kumar et al. (2007)
	Indigenous microbes from a flare pit site	0-50	5 (55±5)	Ulrich et al. (2009)
	Microbacterium sp. 3-28	10-50	20 (82)	Wang et al. (2009)
	Martelella sp. AD-3	5-150	30 (83)	Feng et al. (2012)
	Pseudomonas sp. BZ-3	20-120	20 (72)	Lin et al. (2014)
Dibenzothiophene (DBT)	*Bacillus* sp. DHT	0-100	0 (74±4)	Kumar et al. (2007)
Pyrene	*Microbacterium* sp. 3-28	10-50	20 (70)	Wang et al. (2009)
	Mycobacterium gilvum PYR-GCK	0-58.5	0-10 (100)	Badejo et al. (2013)
Fluoranthene	*Pleurotus eryngii* F032	5.85-29.25	5.85 (87)	Hadibarata et al. (2014)
Benzo[a]pyrene (BaP)	*Pseudomonas* sp. JP1	0-40	20 (19)	Liang et al. (2014)

2.4.3 Effect of NaCl on biodegradation of crude oil

Several studies about biodegradation of crude oil and TPH under different salinities have been reported and are summarized in Table 2.3. Minai-Tehrani et al. (2009) isolated microbes from soil near a refinery and found that the microorganisms degraded heavy crude oil in the presence of 0-50 g/L NaCl. Degradation of heavy crude oil was higher at 0 g/L salt concentration (40%), and lower at 50 g/L NaCl (13%). Similarly, Zhang et al. (2011) investigated the indigenous microorganisms from an unpolluted soil in Beijing to utilize crude oil as the sole carbon source in the presence of 0-29.35 g/L NaCl. They found that the degradation efficiency reached the highest and the lowest values at 0 and 23.4 g/L NaCl (85.39 and 50%, respectively). Darvishi et al. (2011) isolated a strain of *Enterobacter cloacea* ERCPPI-1 from heavy crude oil in the south of Iran and investigated its capacity for heavy crude oil degradation. ERCPPI-1 could degrade 61% of total hydrocarbons at 0 g/L NaCl, and 17% at 150 g/L NaCl. Wu et al. (2012) also found a negative correlation between salt concentration and extent of biodegradation. They tested the ability of *Serratia* sp. BF40 to degrade crude oil at 2.2-12 g/L NaCl and found the biodegradation decreased from 38% at 2.2 g/L NaCl to 29.8% at 12 g/L. These papers showed that increased salt concentrations could decrease the efficiency of the biodegradation.

Several studies have demonstrated that a suitable salt concentration can promote the biodegradation of hydrocarbons or crude oil. Olajide and Ogbeifun (2010) isolated a *Proteus vulgaris* strain from a fish sample in the Niger Delta region. They found that *P. vulgaris* could tolerate 20 g/L NaCl and showed the best degradation efficiency of light crude oil at 10 g/L NaCl. *Rhodococcus* sp. JZX-01, isolated from contaminated soil, was studied by Li et al. (2011). This bacterium degraded crude oil as the carbon source over a range of salinities from 5 to 30 g/L NaCl. The biodegradation efficiency

decreased from 65% at 5 g/L NaCl to 50% at 30 g/L. Another halotolerant strain, *Alcanivorax* sp. Qtet3, was isolated by Dastgheib et al. (2011) from saline soil to utilize TPH at a wide range of salt concentrations. They observed the degradation was 24.8% in a medium without added salt and around 10% at 125 g/L NaCl, reaching the highest biodegradation (26.1%) at 25 g/L. Chen et al. (2012) isolated a strain of *Virgibacillus* sp. from drilling wastewater of Jidong Oilfield (China) and studied the effect of salt on biodegradation of crude oil at salinities from 5 to 200 g/L NaCl. The results showed that the optimal degradation occurred at 50 g/L NaCl; when the salinity was lower than 50 g/L, the degradation rate increased with increasing salt concentration. However, the degradation rate decreased dramatically once the salinity was higher than this value. Wang et al. (2014) investigated the ability of *Acinetobacter* sp. 2 to degrade crude oil at 10-50 g/L NaCl. The best degradation result occurred at 10 g/L NaCl (58%), however, it decreased to 38% degradation at 50 g/L NaCl. These results demonstrate that the best biodegradation result was usually not achieved at the no salt condition, but probably at a physiological salt concentration due to the activity of some enzymes being reached at the optimal salt concentration.

Studies also have reported the ability of halophilic strains to degrade crude oil in high salinity conditions. Díaz et al. (2002) immobilized the consortium MPD-M (*Marinobacter* sp., *Bacillus* sp. and *Erwinia* sp.) on polypropylene fiber and found that the culture could degrade crude oil over 0-180 g/L NaCl concentrations. Total crude oil degradation by the consortium ranged from 4-49%, and the best degradation result was achieved with 20-40 g/L NaCl. Riis et al. (2003) isolated a consortium designated CR1 (*Cellulomonas* sp., *Bacillus* sp. *Dietzia* sp. and *Halomonas* sp.) from Argentinean saline soil and tested it for the ability to degrade crude oil at high salt concentration. The consortium showed a broad range of salt tolerance (0-175 g/L NaCl), and biodegradation efficiency decreased very little with increasing the salt concentration

(65% at 0 g/L and 58% at 175 g/L). Al-Mailem et al. (2010) have shown the degradation of crude oil by a halophilic strain *Haloferax* sp. HA-2 from a hypersaline coastal area of the Arabian Gulf. The data suggested that the *Haloferax* sp. HA-2 could degrade 46% of crude oil at 175.5 g/L salt concentration and still had a considerable efficiency (35%) at 234 g/L NaCl. They also reported another two strains of *Marinobacter sedimentarum* and *Marinobacter flavimaris* that could degrade crude oil at up to 292.5 g/L NaCl with a biodegradation efficiency of 23%. The medium without added salt showed the best degradation result (46%) (Al-Mailem et al. 2013). Mnif et al. (2011) evaluated the abilities of *Halomonas* sp. C2SS100, *Pseudomonas* sp. C450R and *Lysinibacillus fusiformis* C250R to degrade crude oil under 50 or 100 g/L salt concentration. The results showed that *Halomonas* sp. C2SS100 could degrade about 93.3% of oil (at 100 g/L) and *Pseudomonas* sp. C450R and *Lysinibacillus fusiformis* C250R had degradation efficiencies of 96.2 and 65.6%, respectively, with 50 g/L NaCl. These halophilic strains can withstand extremely high salt concentrations and could be used to degrade hydrocarbons and crude oil in hypersaline contaminated sites.

The bioremediation of crude oil by fungi under saline condition has also been reported. Obuekwe et al. (2005) collected *Fusarium lateritium* and *Drechslera* sp. from a salt marsh in the Kuwaiti desert and determined their abilities to utilize crude oil at salinities of 0-100 g/L. They found that by increasing the salt concentration, the degradation of crude oil increased somewhat from 18.3-24.9% and 26-30.7%, respectively. However, the efficiency of degradation by fungi was much lower compared with the biodegradation by bacteria.

Table 2.3 Biodegradation of crude oil under different NaCl concentrations

Hydrocarbon	Degrader	NaCl (g/L)	Optimal NaCl concentration (g/L) and removal efficiency (%)	Reference
Total Alwyn crude Oil	Consortium MPD-M (*Marinobacter* sp., *Bacillus* sp. *Erwinia* sp.)	0-180	20-40 (49)	Díaz et al. (2002)
Diesel fuel	Consortium CR1 (*Cellulomonas* sp., *Bacillus* sp. *Dietzia* sp. and *Halomonas* sp.)	0-175	0 (65)	Riis et al. (2003)
Weathered Burgan crude oil	*Fusarium lateritium* *Drechslera* sp.	0-100 0-100	100 (24.9) 100 (30.7)	Obuekwe et al. (2005)
Heavy crude oil from Soroush oil field in the north of Persian Gulf	Indigenous microbes near refinery	0-50	0 (40)	Minai-Tehrani et al. (2009)
Bonny light crude oil	*Proteus vulgaris*	0-20	10 (78.1)	Olajide and Ogbeifun (2010)
Crude oil	*Haloferax* sp. HA-2	58.5-234	175.5 (46)	Al-Mailem et al. (2010)
25 % crude oil (Boxi Offshore Oil Field) and 75 % diesel fuel	*Rhodococcus* sp. JZX-01	5-30	5 (65)	Li et al. (2011)

Hydrocarbon	Degrader	NaCl (g/L)	Optimal NaCl concentration (g/L) and removal efficiency (%)	Reference
Crude oil from Tehran refinery	*Alcanivorax* sp. Qtet3	0-125	25 (26.1)	Dastgheib et al. (2011)
Crude oil from Thyna Petroleum Services	*Halomonas* sp. C2SS100	100	100 (93.3)	Mnif et al. (2011)
	Pseudomonas sp. C450R	50	50 (96.2)	
	Lysinibacillus fusiformis C250R	50	50 (65.6)	
Crude oil from Daqing Oil Field in China	Indigenous microorganisms	0-29.25	0 (85.93)	Zhang et al. (2011)
Crude oil	*Virgibacillus* sp.	5-200	50 (68)	Chen et al. (2012)
Crude oil from the Shengli Oilfield in China	*Serratia* sp. BF40	2.2-12	2.2 (38)	Wu et al. (2012)
Light Kuwait Crude	*Marinobacter sedimentarum* *Marinobacter flavimaris*	0-292.5	0 (46)	Al-Mailem et al. (2013)
Crude oil from the Changqing Oilfield in China	*Acinetobacter* sp. 2	10-50	10 (58)	Wang et al. (2014)

Chapter 3 Materials and Methods

3.1 Chemicals

Hydrocarbons and solvents used are listed in Table 3.1

Table 3.1 Sources of hydrocarbons and solvents used

Chemical	Purity	Company
D-Glucose	99%	Fisher Scientific Co.
Dichloromethane	99.9%	Fisher Scientific Co.
n-Hexadecane	99%	Acros Oganics (NJ, USA)
Pentane	99.6%	Fisher Scientific Co.
Pristane	98%	Sigma-Aldrich Chemical Co.
Squalane	99%	Acros Oganics (NJ, USA)

Light crude oil from the Strathcona refinery was provided by Imperial Oil.

3.2 Growth media, stock solutions, and amendments

Plate Count Agar (PCA) and Trypticase Soy Broth (TSB) were from BD (Franklin Lakes, NJ) and were prepared using distilled deionized (DD) water according to the manufacturer's directions. Modified Bushnell Häas Medium (BHMV) was prepared using the formulations in Tables 3.2-3.4

Table 3.2 List of materials and quantities required for the Modified Bushnell Häas medium. (Rosenburg et al. 1980)

Component	Mass/Volume Added per Liter of Solution
$CaCl_2$	0.02 g
$MgSO_4$	0.2 g
KH_2PO_4	1.0 g
K_2HPO_4	1.0 g
$(NH_4)_2SO_4$	1.0 g
$FeCl_3 \cdot 6H_2O$	0.0833 g
Trace metal solution	1.0 mL
DI Water	1.0 L
Pfennig's vitamins solution*	1.0 mL

* Filter-sterilized and added after autoclaving

Table 3.3 List of materials and quantities required for the Pfennig's vitamin solution. (Macpherson et al. 1998)

Component	Mass/Volume Added per Liter of Solution
p-Amino benzoic acid	50 mg
Vitamin B-12	50 mg
Biotin	10 mg
Thiamine	100 mg
DD Water	1.0 L

Table 3.4 List of materials and quantities required for trace metal solution. (Fedorak & Grbić-Galić 1991)

Component	Mass/Volume Added per Liter of Solution
$CaCl_2 \cdot 2H_2O$	3.7 g
H_3BO_3	2.5 g
$FeCl_3$	0.65 g
$CoCl_2$	0.01g
$ZnCl_2$	0.44 g
$MnCl_2$	0.87 g
$Na_2MoO_4 \cdot 2H_2O$	0.29 g

3.3 Strains

The bacterial strains used in the experiments are listed in Table 3.6 and were provided by Dr. Julia Foght (University of Alberta).

Table 3.5 List of strains used for biodegradation experiments

Strain	Type of degradation	Source	Identification
EPWF	Saturate	Oily sand	*Rhodococcus erythropolis*
S+14 He	Saturate	Freshwater pond	*Rhodococcus erythropolis*
Husky A	Saturate	Oil-contaminated soil	*Rhodococcus erythropolis*
Esso AGD	Saturate	Refinery wastewater	*Gordonia* sp.

Strains were transferred from the preservative tube to Plate Count Agar (PCA) at 22°C and were maintained by transferring to fresh plates every week. One day before the experiment, strains were transferred from PCA plates to pre-culture media with a sterilized inoculation loop. Each pre-culture tube was a capped test tube with 10 mL of sterilized Trypticase Soy Broth (TSB). The tubes were cultivated on a tube roller overnight at 22°C.

3.4 Experimental conditions

On the day of inoculation, hydrocarbon/crude oil (Table 3.6) and vitamin solution was added into each 250-mL Erlenmeyer flask with sterile BHMV at different salt concentrations (0, 25, 50, 75 g/L) and then 500 μL of pre-culture broth was added. Glucose was autoclaved and pure alkanes and light crude oil were filter-sterilized with a sterile syringe filter (0.2 μm). All the flasks were put into a shaking incubator at 22°C, 150 rpm for up to 11 d. All the cultures were prepared in triplicate for each condition tested, to quantitatively assess the consistency and repeatability of culture behaviour.

Triplicate culture flasks were also prepared for each time point in experiments with hydrocarbons. The sterile control flasks were treated under the same conditions to control for the abiotic factors' influences such as evaporation of hydrocarbons.

Table 3.6 List of amount of carbon sources and type of stopper

Carbon source	Amount	Type of stopper
Glucose	0.25 g	Foam
Hexadecane	150 μL	Foam
Pristane	150 μL	Foam
Hexadecane and Pristane	75 μL (each)	Foam
Crude oil (EPWF)	250 μL	Neoprene rubber
Crude oil (Mixed strains)	0.5 g	Neoprene rubber

3.5 Analytical methods

3.5.1 Growth measurement with optical density

Cell growth on glucose was monitored by measuring optical density at a wavelength of 600 nm (OD_{600}). Sterile BHMV medium without carbon source was used as a blank for necessary dilution when OD_{600} was above 0.5.

3.5.2 Glucose measurement with HPLC

Culture samples (1 mL) were taken aseptically from a flask and then added into a 2-mL centrifuge tube. The vials were centrifuged at room temperature and 12,000 rpm for 3 min. 0.8 mL of supernatant was collected in a glass vial and sealed with a PTFE cap (Agilent Technologies Co. Ltd., USA) for high performance liquid chromatograph (HPLC) analysis.

The HPLC used was an Agilent 1200 (Agilent Technologies Co. Ltd., USA)

system with Refractive Index Detector (RID) and Aminex HPX-87H Column (BIO-RAD Laboratories, Inc., Hercules, CA). Filter-sterilized 0.005 M H_2SO_4 was used as the carrier liquid with a flow rate of 0.4 mL/min. Temperature of column and RID was set at 55°C and 50°C, respectively. 2 μL of the sample was injected into the column and run for 25 min. The amount of residual glucose in the sample was calculated using the peak area with a glucose standard curve.

3.5.3 Liquid-liquid extraction of cultures

For pure hydrocarbon (hexadecane, pristane) and a mixture of alkanes (hexadecane and pristane) biodegradation experiments, cultures were acidified with 1 mL of 4 N HCl after 2, 5, 8 and 11 d and transferred to a separatory funnel. Squalane (60 mg) was added as an internal standard, and the cultures were extracted three times with 25 mL dichloromethane (DCM). The organic phase was collected in a round-bottomed flask and dehydrated with anhydrous sodium sulfate (Na_2SO_4). About 1.5 ml of extract solution was added into a glass vial and sealed with a PTFE cap for gas chromatography (GC) analysis.

3.5.4 Analysis of culture extracts with GC

Sterile control samples and samples of residual alkanes after biodegradation were quantified by gas chromatography using an Agilent 6890 or 7890 (Agilent Technologies Co. Ltd. USA) system with a flame ionization detector (FID) and HP-1 capillary column (Crosslinked Methyl Siloxane, maximum temperature 325°C, column dimensions: 30 m × 0.320 mm × 0.25 μm). Helium was used as the carrier gas with a flow rate of 22 mL/min; hydrogen, air and nitrogen were used at the detector with 55, 220 and 25 mL/min, respectively. The temperatures of the injection port and FID detector were set at 250 and 300°C. The initial temperature of the oven was set at 90°C

for 2 min, then heated up to 250°C at a rate of 10°C / min, and held at 250°C for 10 min. 2 μL of the sample was injected into the injection port and the total run time was 28 min.

3.5.5 Extraction of residual crude oil

For crude oil biodegradation by EPWF, cultures were acidified with 1 mL of 4 N HCl at day 2, 5, 8 and 11. Then 5 mL of pentane containing 8 mg (approximately) of squalane was added into each flask, and the flask was stoppered and inverted several times gently to remove oil from the sides of the flask. The stoppers were secured with tape and the flasks were placed in a refrigerated shaking incubator for 10 min (10°C, 150 rpm). After that, a piece of glass tubing was inserted into the bottom of the flask. The glass tubing was connected to a cold water tap with latex tubing and water was added slowly to raise the pentane layer to the top of the flask. A Pasteur pipette was used to transfer 1.5 mL (approximately) of supernatant into a vial containing 0.2 g of Na_2SO_4 to remove water in the sample. The vial was sealed with a PTFE-lined cap and stored for GC analysis.

3.5.6 Fractionation of residual crude oil

For crude oil biodegradation by the microbial consortium, 1 mL of 4 N HCl was added into the culture at day 2, 5, 8 and 11 after the start of the experiments. The hydrocarbons in the culture were extracted with 25 mL DCM once with squalane (approximately 8 mg) as internal standard. The samples were left open in the fume hood to remove the organic solvent. Partial fractionation to recover the saturates fraction was based on the procedure described by Fedorak and Westlake (1981) as described below.

A) Preparation of SiO_2 column

8.0 g of silica was weighed and added into a small flask and activated in a 125°C oven overnight. Then the flasks were removed from the oven and cooled for 10 min. The cooled silica gel was poured into a small beaker. Enough DCM was added to cover the silica and the beaker was swirled gently in order to remove the bubbles. The slurry was poured into a 1.1 cm ID (inner diameter) × 30 cm long chromatography column, which was plugged with a small amount of glass wool and had been rinsed and filled to the reservoir with DCM. The beaker was rinsed two or three times with DCM and the slurry was added into the column. About 45 min later, all the silica gel was settled in the column and then 0.5 cm of sea sand was added to the top of the silica. After that the DCM was drained to a level about 2 mm from the top of the sand. Then 60 mL of n-pentane was added and the column was drained until the pentane level was at the top of the sand.

B) Oil fractionation

2 mL of n-pentane was added into the extracted oil sample. The mixture was added to the top of the column and then drained until the level of sample in pentane was level to the top of the sand. The column was developed with 10 mL of n-pentane and 20 mL of 20% DCM in n-pentane. The first 10 mL solvent was collected as the void volume and the second 20 mL was the saturate fraction which was placed open in the fume hood in order to remove the organic solvent. After that, 2 mL of DCM was added to dissolve the saturate fraction which was transferred into a glass vial for GC analysis.

3.5.7 Quantitation of hydrocarbons

Degradation of the hydrocarbons was measured as the differences between the initial hydrocarbon amount of sterile control samples and the remaining hydrocarbon amount in the cultures after biodegradation. The amount of hydrocarbon in the sample was calculated based on the corresponding hydrocarbon standard curves and the peak area ratio of extracted hydrocarbon and internal standard (shown in Appendix A). The calculated concentrations of triplicate cultures were used to determine the average and standard deviation values reported in the Results.

Degradation of crude oil was calculated by comparing the heights of each alkane's peak before and after biodegradation.

3.5.8 Contact angle measurement

Contact angle measurements were conducted in both glucose consumption and hexadecane degradation experiments on day 2, 5, 8 and 11. The cultures were transferred to 50-mL centrifuge tubes and centrifuged at room temperature and 6000 rpm for 23 min. Due to the flocculation occurring in hexadecane medium, 3% pentane was added to enhance dispersion of the cells before centrifuging. The pellets on the inner wall of the centrifuge tubes were resuspended with 5 mL PBS buffer solution using a vortex mixer to make the suspension homogeneous. The cell suspension was filtered through a membrane filter (Millipore, Fisher Scientific Co.) leaving a cell lawn on the 0.45 μm filter paper. The cell lawn was left at room temperature for about 30 min to dry. The hydrophobicity of the cell lawn surface was measured with a contact angle system VCA-Optima^{TM} (AST Products, Inc., Billerica, USA). A droplet of 1μL of DD water was placed on the dried cell lawn. The contact angle α was measured by the equipment as shown in Figure 3.1. A larger value of contact angle α represents a

higher level of hydrophobicity of the cell membrane. Measurements were repeated at least five times at different locations.

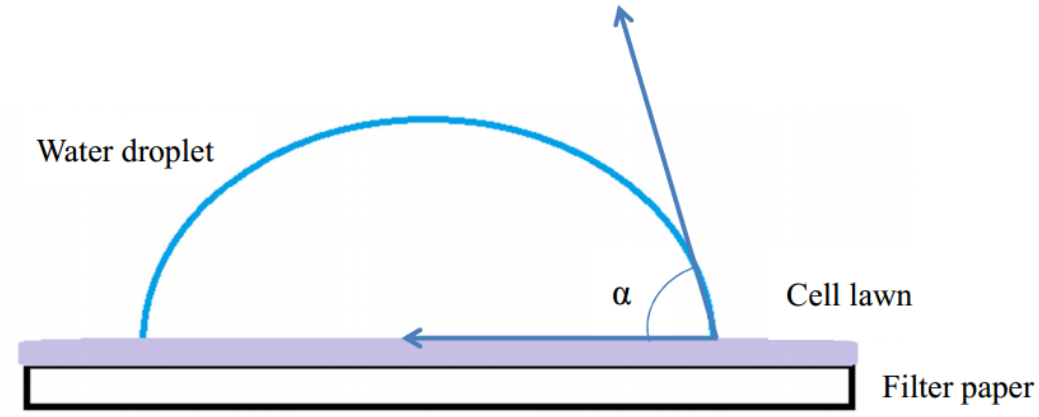

Figure 3.1 An illustration of the contact angle measurement where α represents contact angle.

Chapter 4 Results and Discussion

4.1 Effect of salts on consumption of glucose by EPWF

Rhodococcus erythropolis is a Gram-positive bacterium, well known for its biotransformation abilities (de Carvalho and da Fonseca 2005b), and some studies reported that *R. erythropolis* could degrade saturate alkanes (e.g. Kim et al. 2002; Pacheco et al. 2010). The strain used in this project, *R. erythropolis* strain EPWF, was originally isolated from oily sand (Kirkwood et al. 2005) and was recently shown to be halotolerant[1].

In this project, the experiments testing glucose consumption were conducted prior to the biodegradation of model hydrocarbons and crude oil in order to establish a baseline for the effect of salts (NaCl, KCl, and Na_2SO_4) on cell growth and to enable a comparison with biodegradation of model hydrocarbons at different salt concentrations. In this section, OD_{600} value was measured to evaluate the cell growth and residual glucose in the medium was determined by HPLC to calculate the consumption of carbon source. Triplicate cultures at each salt concentration tested were sampled daily for OD_{600} and HPLC analysis.

4.1.1 Effect of NaCl on EPWF growth and glucose consumption

The OD_{600} values and amount of remaining glucose in the medium at different NaCl concentrations (0, 25, 50 and 75 g/L) are shown in Figure 4.1 (a) and (b).

It was observed that the 0 g/L NaCl group showed the best growth result in total 12 d

[1] Longang, A. (2012). *Halotolerance and hydrophobicity in hydrocarbon-degrading bacteria*. BASc. Thesis. University of Ottawa: Canada.

(as shown in Figure 4.1 (a)), the OD_{600} reached the maximum value (2.9) on day 10. For the 25 g/L group, the growth trend showed the similar result as 0 g/L NaCl in the first two days; however, it began to slow down from day 3 and achieved the maximum OD value of 1.9 on day 12. Both 50 and 75 g/L NaCl groups showed a longer lag time compared to the other two groups. In the presence of 50 g/L NaCl, the OD began to increase after day 1 and no obvious exponential phase was observed during the whole experiment. The maximum OD value detected was only 36% of that in the no salt group. However, 75 g/L NaCl did not show obvious cell growth in 12 d, its maximum OD value was just a little bit higher than the negative control. Despite the lack of growth, live cells were recovered on the PCA plate streaked from the cultures in 75 g/L NaCl medium.

The glucose consumption trend of each NaCl group corresponded to each cell growth curve (Figure 4.1 (b)). The group without salt showed the best consumption result; about 71% of initial glucose was consumed in 11 d. The medium with 25 g/L NaCl showed a slower consumption rate, and the amount of glucose consumed was only half as much as that in 0 g/L NaCl group. No glucose was measurably utilized in the 50 and 75 g/L groups, this may be due to the cells using the remaining nutrient in TSB inoculum first to support growth and then to use glucose in the medium. A similar result was also reported by Wu et al. (2012) for a halotolerant strain of *Serratia*. They observed that the final OD_{630} value was the highest for cultures with no added salt, followed by 20, 40, and 60 g/L NaCl. The cells almost stopped growing when the salt concentration was over 80 g/L NaCl

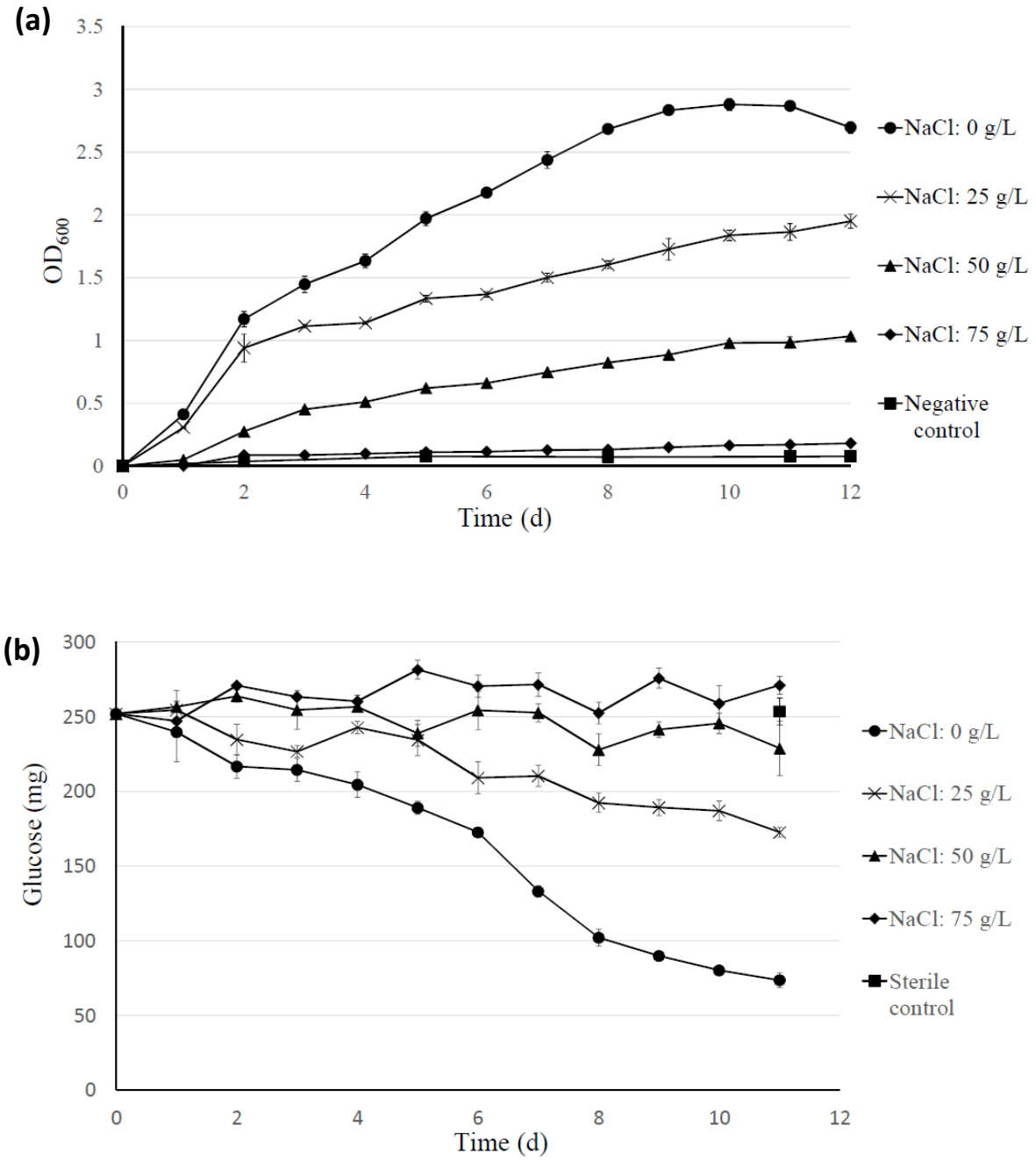

Figure 4.1 (a) Growth of *R. erythropolis* EPWF on glucose with different NaCl concentrations (0, 25, 50 and 75 g/L) in 12 d. (b) The amount of remaining glucose in the medium with different NaCl concentrations (0, 25, 50 and 75 g/L) in 11 d. (average ± standard deviation, n=3).

4.1.2 Effect of KCl on EPWF growth and glucose consumption

In general, both the OD_{600} value and the amount of glucose consumed in the cultures with added KCl showed better results (Figure 4.2 a and b) compared with those in the cultures with the same mass concentration of NaCl.

In the first five days, each KCl concentration showed a similar growth trend compared to that at the corresponding NaCl concentration. After day 5, cell growth in the medium with 75 g/L KCl increased, which indicated that the cells had adapted to the high salt concentration, even though a longer time was taken. From day 5 to the end of the experiment, the 0 and 25 g/L KCl groups showed faster growth rates than those of the 50 and 75 g/L KCl groups. At the end of this experiment, the cultures with 25 g/L KCl achieved a higher OD value than the cultures with 25 g/L NaCl; however, the maximum OD_{600} value of the 50 g/L groups was the same. The maximum OD_{600} at 75 g/L KCl was more than three times higher than that at 75 g/L NaCl. Oren (1999) reported that most microorganisms exclude Na^+ and sequester K^+ across the cell membrane. K^+ accumulation could occur via passive diffusion or active transport with ATP consumption to keep osmotic pressure in the high K^+ concentration environment (Oren 1999; Jensen et al. 2014). Therefore, in the case of 75 g/L KCl, the cells required more energy to accumulate the potassium and maintain the osmotic potential, resulted in less energy available for executing other cellular functions (Al-Mailem et al. 2013).

The glucose consumption in 0 and 50 g/L KCl groups did not change obviously (Figure 4.2 (b)) compared to the cultures with NaCl. In the presence of 25 g/L KCl, the amount of glucose consumption was doubled compared with that at the same mass concentration of NaCl; about 63% of initial glucose was utilized in 11 d. No glucose

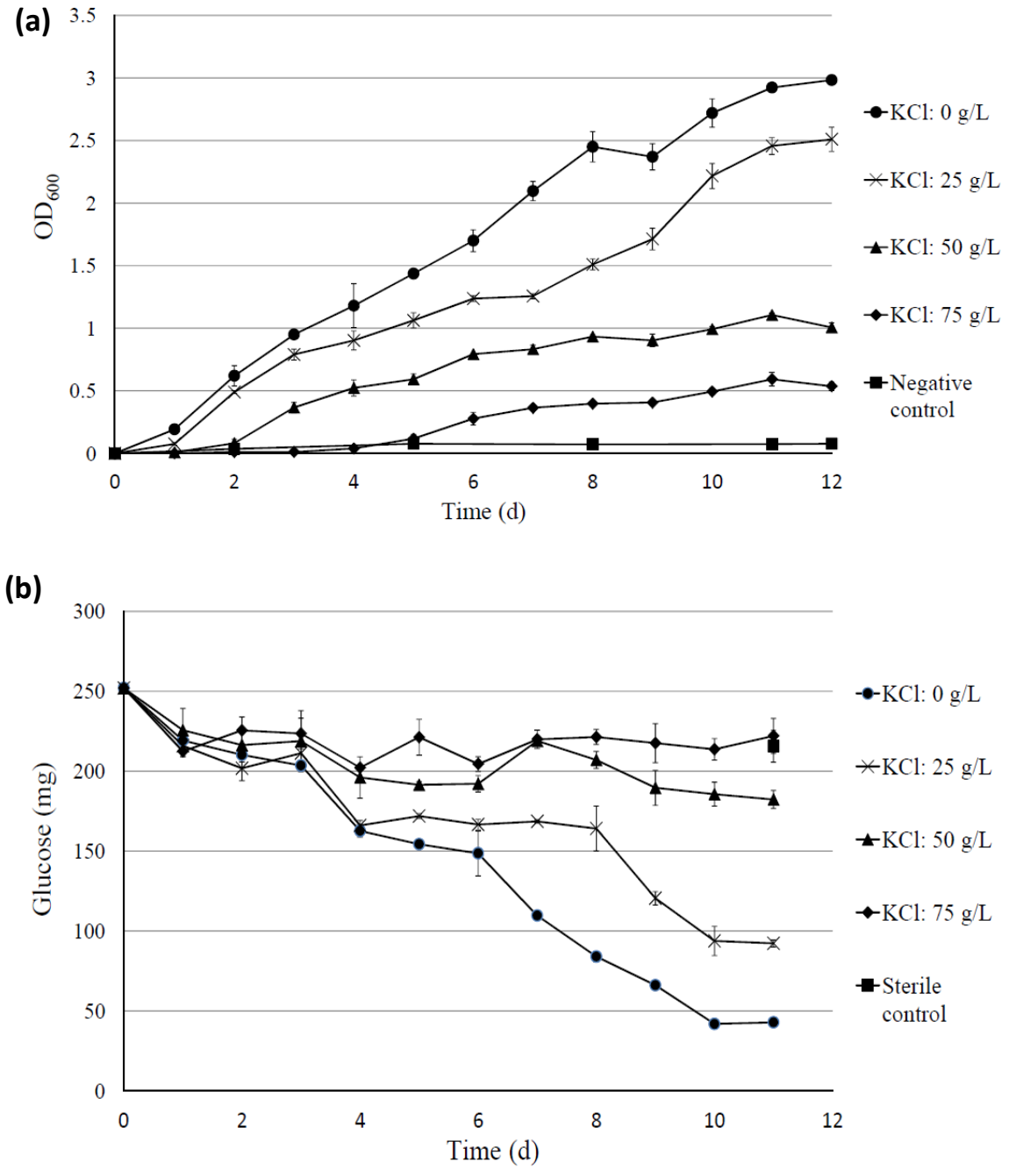

Figure 4.2 (a) Growth of *R. erythropolis* EPWF on glucose with different KCl concentrations (0, 25, 50 and 75 g/L) in 12 d. (b) The amount of remaining glucose in the medium with different KCl concentrations (0, 25, 50 and 75 g/L) in 11 d. (average ± standard deviation, n=3).

consumption was detected in the medium with 75 g/L KCl, which was probably due to the signaling pathway being affected by salt stress or the excessive intracellular potassium leading to inhibition of cell growth and carbon source consumption (Oren 1999; Al-Mailem et al. 2013).

4.1.3 Effect of Na_2SO_4 on EPWF growth and glucose consumption

The profiles of cell growth and glucose consumption in the medium at 0, 25, 50 and 75 g/L Na_2SO_4 are shown in Figure 4.3 (a) and (b), respectively, and are very different from the profiles with the other two kinds of salt.

Cultures with 0, 25 and 50 g/L Na_2SO_4 showed a quite similar cell growth trend in 12 d. Even though the cell concentration at 50 g/L Na_2SO_4 increased more slowly compared to the 0 and 25 g/L groups in the later stage of this experiment (after day 5), the maximum OD_{600} value was still two times higher than that at 50 g/L NaCl. The effect of 75 g/L Na_2SO_4 on cell growth was significant, no lag phase was observed and the growth rate was much faster than that at the same amount of NaCl and KCl. The highest OD_{600} value (1.6) of 75 g/L Na_2SO_4 group was achieved on day 11, which was about 3 times and 8 times the maximum OD_{600} value with KCl and NaCl, respectively.

To some degree, glucose consumption could indirectly reflect the conditions of cell growth in this experiment. In the first 5 d, all the groups over a range of salinities from 0-75 g/L Na_2SO_4 showed a similar consumption result. After that, both 50 and 75 g/L Na_2SO_4 groups displayed slower consumption rates compared with the other two Na_2SO_4 concentrations, but they still showed considerable consumption results (42 and 29%, respectively) at the end of the experiment.

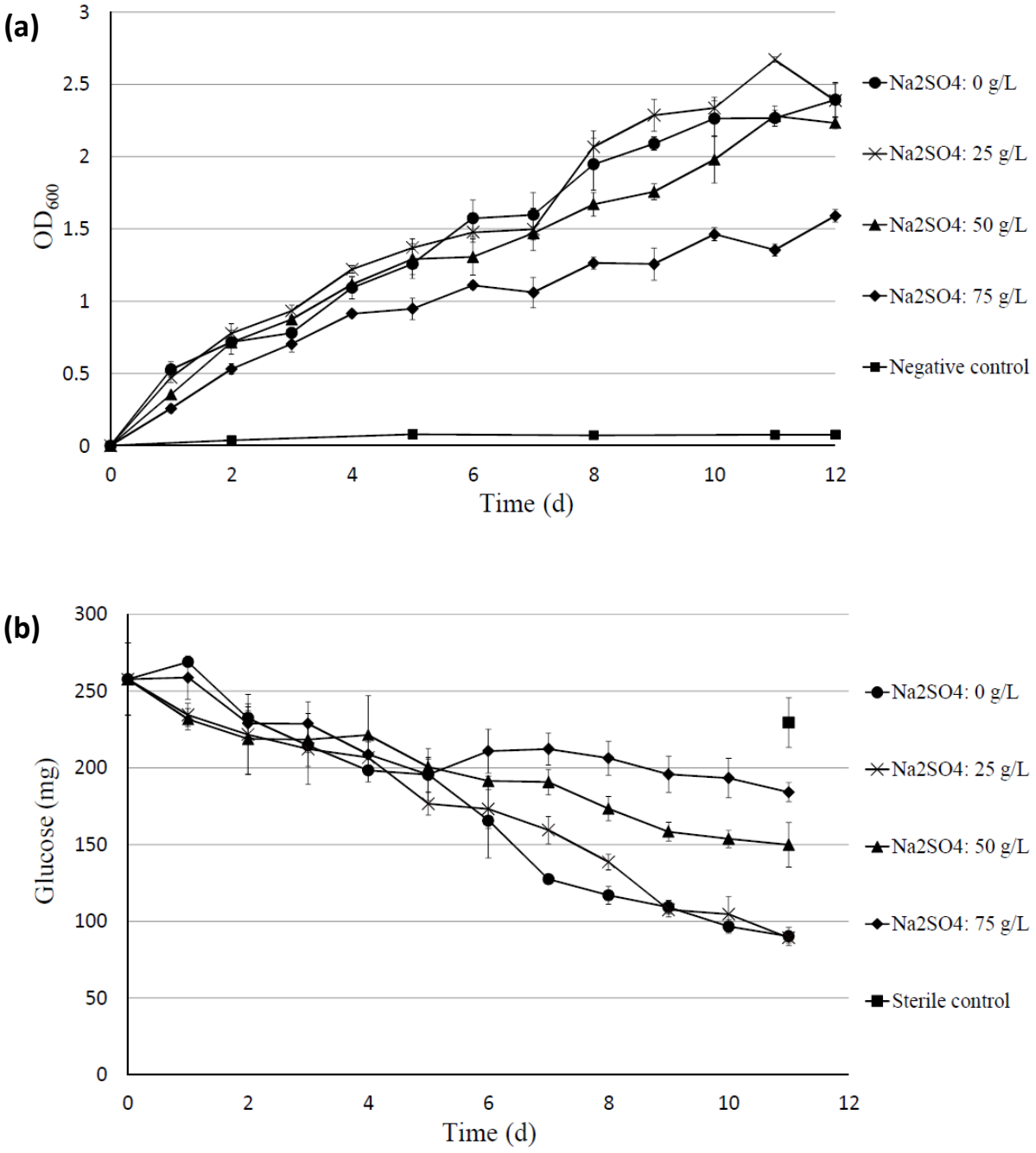

Figure 4.3 (a) Growth of *R. erythropolis* EPWF on glucose with different Na_2SO_4 concentrations (0, 25, 50 and 75 g/L) in 12 d. (b) The amount of remaining glucose in the medium with different Na_2SO_4 concentrations (0, 25, 50 and 75 g/L) in 11 d. (average ± standard deviation, n=3).

4.2 Degradation of model hydrocarbons by *R. erythropolis* EPWF

In this section, the amount of model hydrocarbons used was calculated to give the same molar carbon concentration as 2.5 g/L glucose. In the case of mixed hydrocarbon degradation, half the amount of hexadecane and half the amount of pristane was used to keep the same total carbon concentration. Hexadecane and pristane are ubiquitous hydrocarbons in crude oil and commonly used as model straight and branched hydrocarbons in research.

Hydrocarbon consumption was determined by GC after liquid-liquid extraction of the cultures. Triplicate flasks were sacrificed and extracted for analysis at each selected time point for each salt concentration tested.

4.2.1 Degradation of hexadecane with NaCl (0, 25, 50, 75 g/L) by EPWF

Figure 4.4 shows the changes in the amount of hexadecane in the medium at 0, 25, 50 and 75 g/L NaCl over 11 d. In the medium without added NaCl, the amount of hexadecane decreased very quickly, and more than 50% of the hexadecane was degraded in the first two days. After that, the degradation rate became slower, and the final degradation efficiency was 79%. This is similar to the results of glucose consumption without NaCl (Figure 4.1); both the glucose consumption and hexadecane degradation trends changed very little after 8 d, which indicates the cells were in the stationary phase.

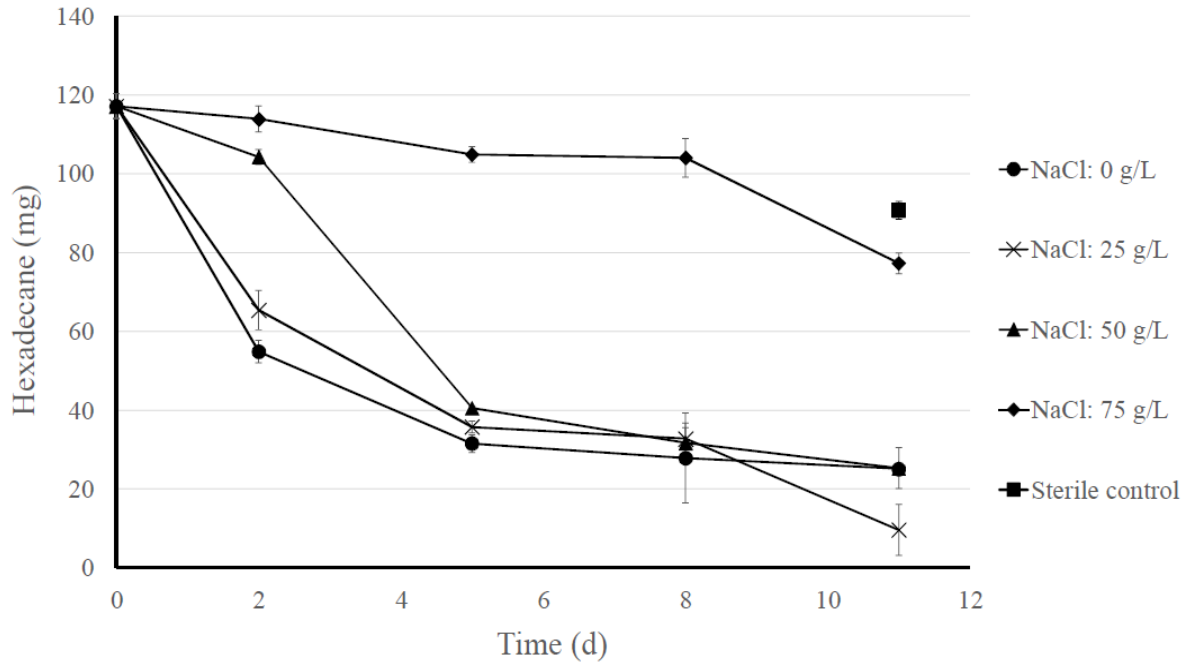

Figure 4.4 The effect of salt (NaCl) concentration on the amount of hexadecane remaining in 100 mL cultures of *R. erythropolis* strain EPWF grown in BHMV medium. (average ± standard deviation, n=3).

In the presence of 25 g/L NaCl, the degradation trend was similar to that of the 0 g/L group in the first 8 d. However, the degradation rate increased after day eight and achieved a better final result (89%) than in the salt free medium.

Only a small amount of hexadecane degradation was detected in the medium with 50 g/L NaCl on day 2, which was maybe due to the higher NaCl concentration inhibiting the activity of the cells and prolonging the lag phase (de Carvalho and da Fonseca, 2005a; Ulrich et al., 2009). But the degradation efficiency caught up with the 0 and 25 g/L NaCl groups on day 5 and showed a similar degradation result as the other two groups on day 11. This result indicated that even though NaCl could affect the initial cell growth, the final extent of degradation of hexadecane was not changed greatly once the strains were adapted to the new environment.

In the presence of 75 g/L NaCl, nearly no hexadecane degradation was observed until day 8, after which an obvious reduction of hexadecane concentration was detected at the end of this experiment; this indicates the EPWF cells could tolerate the high salt concentration, but a long time was needed to adapt to the hypersaline environment. Jones and Jennings (1964) reported that low sodium concentration could stimulate cell dry weight production; however, the higher concentration inhibited the production greatly. Børresen and Rike (2007) reported that changes of environment always have a negative effect on the activity of microorganisms. Microorganisms need to utilize more energy to adapt to adverse conditions.

Different patterns of macroscopic cell growth were observed at the different salt concentrations. In the first two days of growth, both 0 and 25 g/L NaCl groups developed a cell layer associated with the hexadecane on the top surface of the medium. On day 5, large (about 1 cm) cellular aggregates were observed at the surface of medium in both the 0 and 25 g/L NaCl groups. de Carvalho and da Fonseca (2005a) also observed flocculation and found through fluorescence microscopy that the flocculation were mainly cells associated with hydrocarbon droplets. After day 8, the cellular flocculation in the medium with 0 g/L NaCl decomposed into very tiny particles (less than 1 mm); however, the aggregates in the 25 g/L cultures were still the same large size. At the end of the experiment, the salt free medium was turbid and no flocculation was observed. The flocculation in the 25 g/L NaCl group began to decompose into smaller pieces between day 8 and day 11. This phenomenon could explain why the hexadecane decreased again in these cultures after day 8. The further decomposition of cellular flocculation resulted in the increase of the cells' specific surface area, thus promoting the hydrocarbon uptake.

In the 50 g/L cultures, a cell layer was observed on day 5, suggesting that the

microorganisms adapted to the new environment at this time. From day 5 to day 8, a similar size of cell aggregate formed (approximately 1 cm) and was suspended in the medium. From day 8 to 11 the particles settled to the bottom of the medium. This is the same time as consumption of hexadecane occurred, presumably increasing the density of the particles leading to a reduction in buoyancy. Cell growth at 75 g/L NaCl was observed on the top of the medium on day 11; the loss of hexadecane from these cultures also confirmed that the cells had adapted to the adverse conditions and started growing.

The above results indicate that NaCl not only affects the cell growth, but also affects the formation and the dimension of cell aggregates in the medium with hexadecane as carbon source. Ugochukwu et al. (2014) found the efficiency of biodegradation of saturates in crude oil is proportional to cellular surface area. Bouchez-Naïtali et al. (2001) suggested that contact with the hydrocarbons and oxygen is rate-limiting for flocculated cells. The smaller the size of flocculation in the medium, the better the biodegradation results that can be achieved.

4.2.2 Degradation of pristane with NaCl (0, 25, 50 g/L) by EPWF

Pristane (2,6,10,14-tetramethylpentadecane) is a multiply branched alkane which is commonly present in soils, sediments and crude oil (Volkman and Maxwell 1986). Due to its recalcitrant properties, pristane is commonly used as biomarker in environmental studies and crude oil analysis (Mikolasch et al. 2009). The pristane degradation experiment was conducted with 0, 25 and 50 g/L NaCl, due to 75 g/L NaCl being too high for *R. erythropolis* EPWF to grow without a long adaptation time (based on the results with hexadecane). The result of this experiment is shown in Figure 4.5.

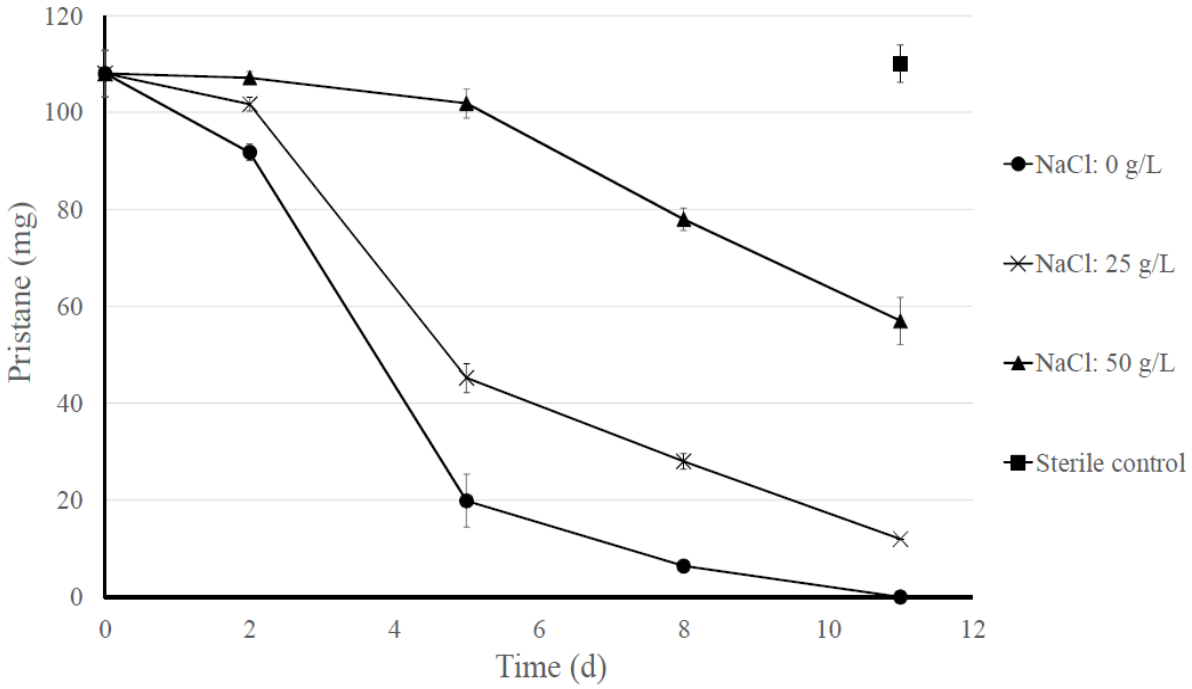

Figure 4.5 The effect of salt (NaCl) concentration on the amount of pristane remaining in 100 mL cultures of *R. erythropolis* strain EPWF grown in BHMV medium. (average ± standard deviation, n=3).

In the medium without NaCl, the initial degradation rate of pristane was lower than when hexadecane was the carbon source, but it was still higher than for the other two salt concentrations. The degradation increased dramatically from day 2 to day 5 and slowed down from day 5 to the end of this experiment. In contrast to the result of hexadecane degradation at 0 g/L NaCl, all the pristane was degraded by EPWF completely in 11 d.

In the presence of 25 g/L NaCl, the degradation trend was quite similar to that of 0 g/L NaCl, with the best degradation rate shown from day 5 to day 8. The final degradation result was also considerable, about 89% of the initial pristane was degraded during the whole process.

No obvious pristane degradation was observed in the medium with 50 g/L NaCl in the first five days, showing the longest lag time among all the salt concentrations. Besides the reason of a high salt concentration prolonging the lag phase, branched alkanes are also harder than n-alkanes for microorganisms to use. However, the degradation rate increased after day 5 and was constant until the end of the experiment, with a total degradation efficiency of 47%.

Unlike the large yellow aggregates formed in the medium with hexadecane as the sole carbon source, both the dimension and the colour of flocculation formed with pristane as the carbon source were totally different. Cell growth in the 0 and 25 g/L NaCl cultures was observed on day 2; and many tiny (less than 1 mm) white particles formed and were observed on the surface of the medium on day 5. From day 5 to day 11, the medium became more turbid and the number of particles kept increasing.

In the presence of 50 g/L NaCl, the medium was clear and nearly no cell growth or flocculation was observed in the first five days. On day 8, the medium became a little bit turbid and similar suspended particles could be seen. From day 8 to day 11, the turbidity increased and more flocculation was observed.

Compared with the efficiency of hexadecane degradation at 0 and 25 g/L NaCl, the final biodegradation efficiency of pristane at the corresponding salinities was higher. This may be attributed to the dimension of the flocculation formed in the medium. The size of the flocculation formed with pristane was much smaller than those formed with hexadecane, which leads to a higher specific surface area and probably enhanced the efficiency of hydrocarbon contact and uptake. Therefore, the negative effect of low bioavailability of branched alkanes could be overcome by the high efficiency of uptake.

4.2.3 Degradation of mixed hexadecane and pristane with NaCl (0, 25, 50 g/L) by EPWF

Many studies either focused on the degradation of pure hydrocarbons or on the degradation of crude oil; however, few reported the degradation of mixed defined hydrocarbons. In this section, a mixture of hexadecane and pristane was provided as the sole carbon source for EPWF at 0, 25 and 50 g/L NaCl (Figure 4.6). The goal was to use a defined system, rather than a complex crude oil, to understand the impact of salt on degradation of mixed hydrocarbons.

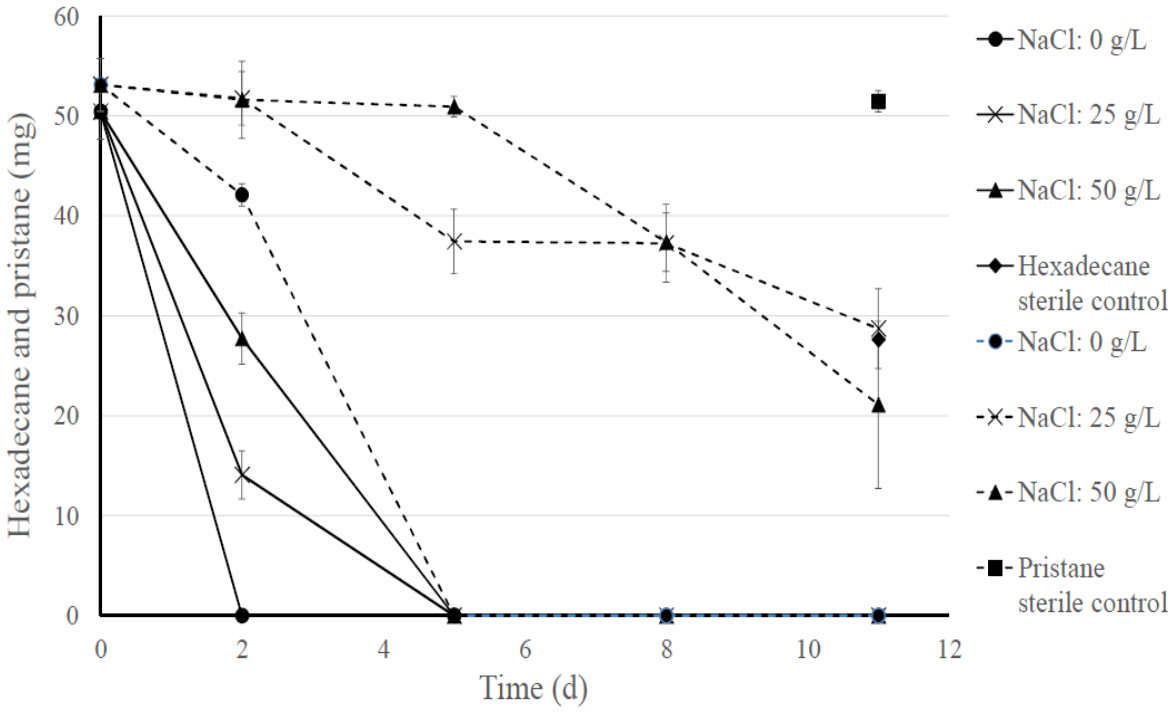

Figure 4.6 The effect of salt (NaCl) concentration on the amount of hexadecane and pristane remaining in 100 mL cultures of *R. erythropolis* strain EPWF grown on both carbon sources together in BHMV medium (solid lines represent hexadecane and dashed lines represent pristane).

The medium with 0 g/L NaCl showed the highest efficiency, with all the hexadecane and pristane degraded completely on day 2 and day 5, respectively. In the presence of 25 g/L NaCl, the initial degradation rate of hexadecane was similar to that of the salt free group. Most of the hexadecane (72%) was degraded, and no pristane degradation was detected on day 2. The hexadecane was completely consumed by day 5, and then the amount of pristane began to decrease. However, nearly no pristane was degraded from day 5 to day 8. From day 8 to the end of the experiment, pristane degradation resumed and the total degradation efficiency reached 46%. The 50 g/L NaCl group showed that the amount of hexadecane degraded in the first two days was much more than the amount of hexadecane degraded when used as the sole carbon source. On day 5, nearly 100% of hexadecane was gone and very little pristane degradation was detected. From day 5 to the end of this experiment, the degradation rate increased and achieved a considerable degradation efficiency of 60%. Compared to the amount of pristane degraded in the medium without hexadecane at both 25 and 50 g/L NaCl from day 5 to day 11, the amount of pristane degraded in the medium with hexadecane was much lower at the same salinity.

Cell growth was very fast at 0 g/L NaCl, and a lot of small cell aggregates (1-2 mm) were observed at the bottom of the medium on day 2. These small particles kept decomposing into tiny ones (less than 1 mm) until the last day of the experiment. In the presence of 25 g/L NaCl, a cell layer formation was observed on day 2, and the cells gradually associated and formed one or two large aggregates at the bottom of the medium (Figure 4.7). The precipitated flocculation lasted from day 5 to day 8 and then decomposed into small pieces, which accounts for the lack of degradation detected during that period and the increase afterwards. In the presence of 50 g/L NaCl, small particles (about 2-3 mm) were observed at both top and bottom of the medium on day 5. As the amount of hexadecane decreased, more particles precipitated to the bottom of

the medium.

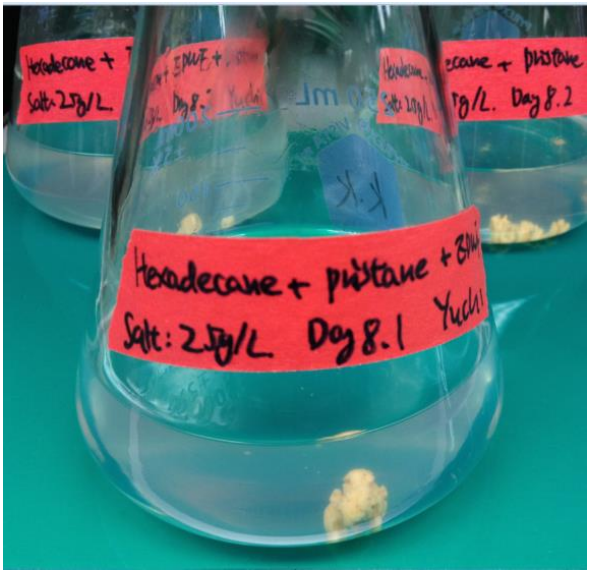

Figure 4.7 Flocculation in the medium with hexadecane at 25 g/L NaCl on day 8.

Compared with the degradation of pristane as the sole carbon source, it is suggested that the presence of hexadecane in the medium is unfavourable for the degradation of pristane. *R. erythropolis* EPWF could uptake hexadecane first and form adhesive cellular flocculation, which may prevent the subsequent uptake and biodegradation of pristane. A similar result was also reported by Kunihiro et al. (2005). They used *Rhodococcus* sp. strains TMP2 and T12 to degrade pristane, pentadecane and a mixture of pristane and pentadecane and found that the biodegradation efficiency of pristane in the medium with the mixed carbon sources was lower than the efficiency for pristane as the sole carbon source. They hypothesized that gene regulation (e. g., catabolite repression) or competitive inhibition of enzymes may lead to this result.

4.2.4 Effect of Na_2SO_4 on degradation of hexadecane by EPWF

In the presence of Na_2SO_4, the degradation trends (shown in Figure 4.8) were different from the trends with NaCl (Figure 4.4) over a range of salinities from 25-75 g/L. All the groups with Na_2SO_4 showed high initial degradation rates without any observable lag time in the first 2 d, after which the rates decreased significantly. In the presence of 25 g/L Na_2SO_4, a general lower degradation efficiency was achieved (66%) during the whole biodegradation experiment compared to the efficiency with the same amount of NaCl (88%). This is contrary to the result of glucose consumption at 25 g/L NaCl and Na_2SO_4, more glucose was consumed by EPWF with Na_2SO_4 rather than with NaCl.

In the cases of 50 and 75 g/L Na_2SO_4, not only did the lag phase observed with NaCl disappear, but the initial degradation rates were also higher than at the same NaCl concentrations during the first 5 d. After that, these two groups showed a quite similar degradation trend as the 25 g/L Na_2SO_4 group and all of them reached a total degradation efficiency of more than 60%. Compared with the result of the glucose consumption experiment with Na_2SO_4 (Figure 4.3b), the medium with 50 and 75 g/L Na_2SO_4 showed a better bioavailability of hexadecane.

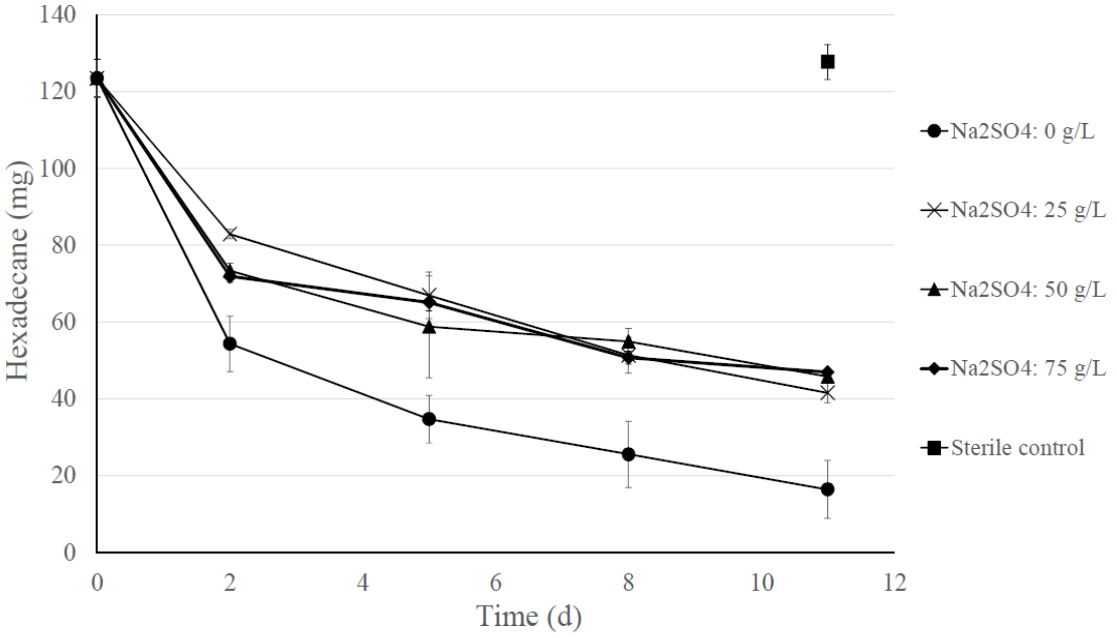

Figure 4.8 The effect of salt (Na_2SO_4) concentration on the amount of hexadecane remaining in 100 mL cultures of *R. erythropolis* strain EPWF grown in BHMV medium. (average ± standard deviation, n=3).

As in the NaCl experiments, flocculation was also observed with Na_2SO_4 in the medium. A big yellow cell aggregate (approximately 1 cm) was formed in the medium with 25 g/L Na_2SO_4 on day 5, and then it decomposed gradually into a smaller size pieces (about 5-6 mm) with numerous powder-like particles. From day 5 to the end of the experiment, the smaller particles gradually settled to the bottom of the medium. In the presence of 50 g/L Na_2SO_4, aggregates with a diameter of 1-2 mm formed on the top of the medium in the first five days. Afterwards, they combined to form one or two particles (about 1 cm), which lasted until day 11. A cell layer formation was observed in the 75 g/L Na_2SO_4 group on day 2, and a similar size of flocculation (1 cm) appeared in the 50 g/L group on day 5. The flocculation did not show any changes until

the last day of the experiment, which corresponds to the constant degradation rate seen in Figure 4.8.

It is noteworthy that the fastest degradation rate was reached when a cell layer formed on the surface of the medium, and the rate began to decrease after cell flocculation. Also, the cell flocculation in the medium with Na_2SO_4 did not decompose into as many small pieces as were seen in the medium with NaCl; in particular, at 50 and 75 g/L Na_2SO_4, nearly no decomposition was observed during the whole incubation time. This accounts for the degradation rate being higher at the beginning and slowing down subsequently, as the specific surface area of cell material was changed, limiting mass transfer.

Lippard and Berg (1994) reported that SO_4^{2-} is well known as an inorganic growth factor and is involved in many enzyme reactions and in protein synthesis. Gawel et al. (2006) reported that nutrient sulfur is needed for bioremediation of crude oil contaminated soil. In this study, the 50 and 75 g/L Na_2SO_4 groups demonstrated a totally different initial degradation rate compared to the groups with the same amount of NaCl, which indicated that SO_4^{2-} has less impact than Cl^- on cell growth. But Yuan et al. (2001) reported that phenanthrene biodegradation could be affected at higher sulfate concentrations due to inhibition of enzyme synthesis. Børresen and Rike (2007) also pointed out that the advantages of increased nutrient salt concentration could be overcome by the negative effect of depressed cell activity due to high ionic strength in the medium.

4.2.5 Effect of KCl on degradation of hexadecane by EPWF

The results for biodegradation of hexadecane at 0, 25, 50 and 75 g/L KCl are shown in Figure 4.9. The 0 and 25 g/L KCl groups showed a quite similar degradation

trend during the whole process. In contrast to the glucose consumption with 25 g/L KCl, which slowed down after day 5, the hexadecane kept decreasing until the last day of the experiment. About 96 and 85% of the initial hexadecane were degraded at 0 and 25 g/L KCl, respectively.

Unlike the lack of glucose consumption with 50 and 75 g/L KCl, the amount of hexadecane at these two salinities decreased dramatically during the whole incubation time. Furthermore, the 50 g/L KCl group did not exhibit an obvious lag time and showed a considerable initial degradation rate compared to the cultures at 50 g/L NaCl. At the end of day 5, the 50 g/L KCl group caught up with the other two groups and achieved a final degradation efficiency of 94%. The group with the highest KCl concentration (75 g/L) showed a short lag time (about two days), and the amount of hexadecane decreased dramatically from day 2 to day 5. The degradation rate slowed down gradually, and still a high efficiency of 78% was achieved (compared to 15% with NaCl).

In the cultures with 25 g/L KCl, a big cell aggregate (about 1 cm) was observed on the upper surface of the medium on day 2. The flocculation decomposed into several smaller pieces (1-2 mm) on day 5 and remained the same until the last day of the experiment. In the presence of 50 g/L KCl, a cell layer was seen in the first two days; then it turned into small particles (1-2 mm) that precipitated at the bottom of the medium on day 5. After that, the small particles did not show any obvious changes. A thick cell layer was observed at 75 g/L KCl on day 2, and a big aggregate (about 1 cm) was formed on day 5. Then the big aggregate decomposed into one big particle with several small particles (about 1-2 mm), some of the them stuck on the inner wall of the flask. On day 11, the big particle was observed at the bottom of the medium, which was probably caused by the decrease in the amount of hexadecane in the particle leading to a higher average density.

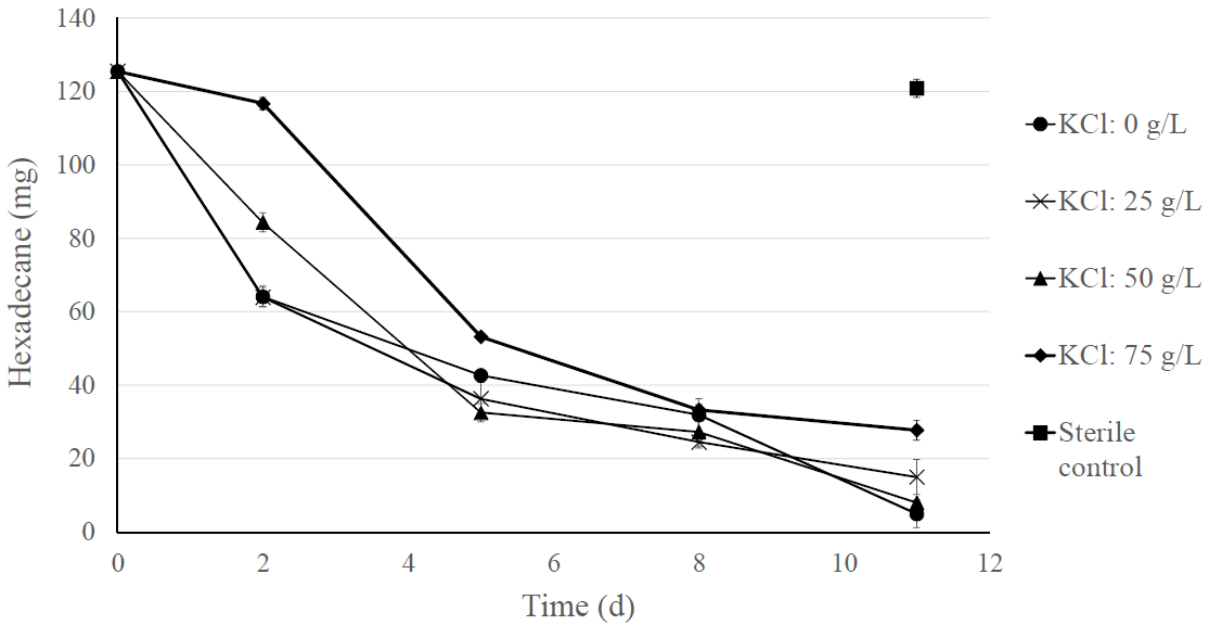

Figure 4.9 The effect of salt (KCl) concentration on the amount of hexadecane remaining in 100 mL cultures of *R. erythropolis* strain EPWF grown in BHMV medium. (average ± standard deviation, n=3).

It is well known that K^+ contributes to the activity of several enzymes and plays an important role in protein synthesis (Al-Mailem et al. 2013). Halotolerant and halophilic strains are reported to rapidly accumulate K^+ in the cell as an osmoregulator to maintain the osmotic pressure in a hypersaline environment (Hua et al. 2010; Al-Mailem et al. 2013). With the extracellular potassium concentration increasing, the intracellular K^+ concentration also increased (Jensen et al. 2014). Al-Mailem et al. (2013) also found that divalent cations like Ca^{2+} and Mg^{2+} show a similar function to K^+. Thus, in the experiment with EPWF, even though lag time increased somewhat at a high KCl concentration (75 g/L), the final biodegradation efficiency was not affected greatly.

4.3 Effect of NaCl of biodegradation of light crude oil by *R. erythropolis* EPWF and a defined bacterial consortium

4.3.1 Effect of NaCl on biodegradation of the saturate fraction in crude oil by EPWF

A light crude oil from the Strathcona refinery was provided by Imperial Oil. Since EPWF is a saturate-degrading microorganism, the saturate fraction of the light crude oil was extracted with pentane, and the peak height of each component was analyzed with gas chromatography (GC) as a semi-quantitative measure of hydrocarbon consumption. The degradation efficiency was calculated based on the peak height. As in the pure hydrocarbon experiments, triplicate flasks were sacrificed and extracted for analysis at each selected time point for each salt concentration tested. Single representative chromatograms are shown here, and average values are reported in the text.

Figure 4.10 shows the changes of saturate alkanes in the sterile controls over 11 d， Compare to sterile control on day 0 (Figure 2.5), it can be observed that the light fractions (C8-C12) evaporated very fast, but the amount of heavier fractions (C13-C29) and the branched alkanes (pristane and phytane) were still quite high in the medium after 11 d.

The medium with 0 g/L NaCl showed a considerable degradation result, where almost 100% of the initial n-alkanes and more than half of the pristane and phytane (57% and 61%) were degraded by EPWF in two days (Figure 4.11). According to the profiles on day 5 and day 8, pristane and phytane were further degraded, and the total degradation efficiency reached 83% and 81%, respectively. However, no further biodegradation of pristane and phytane was detected on day 11.

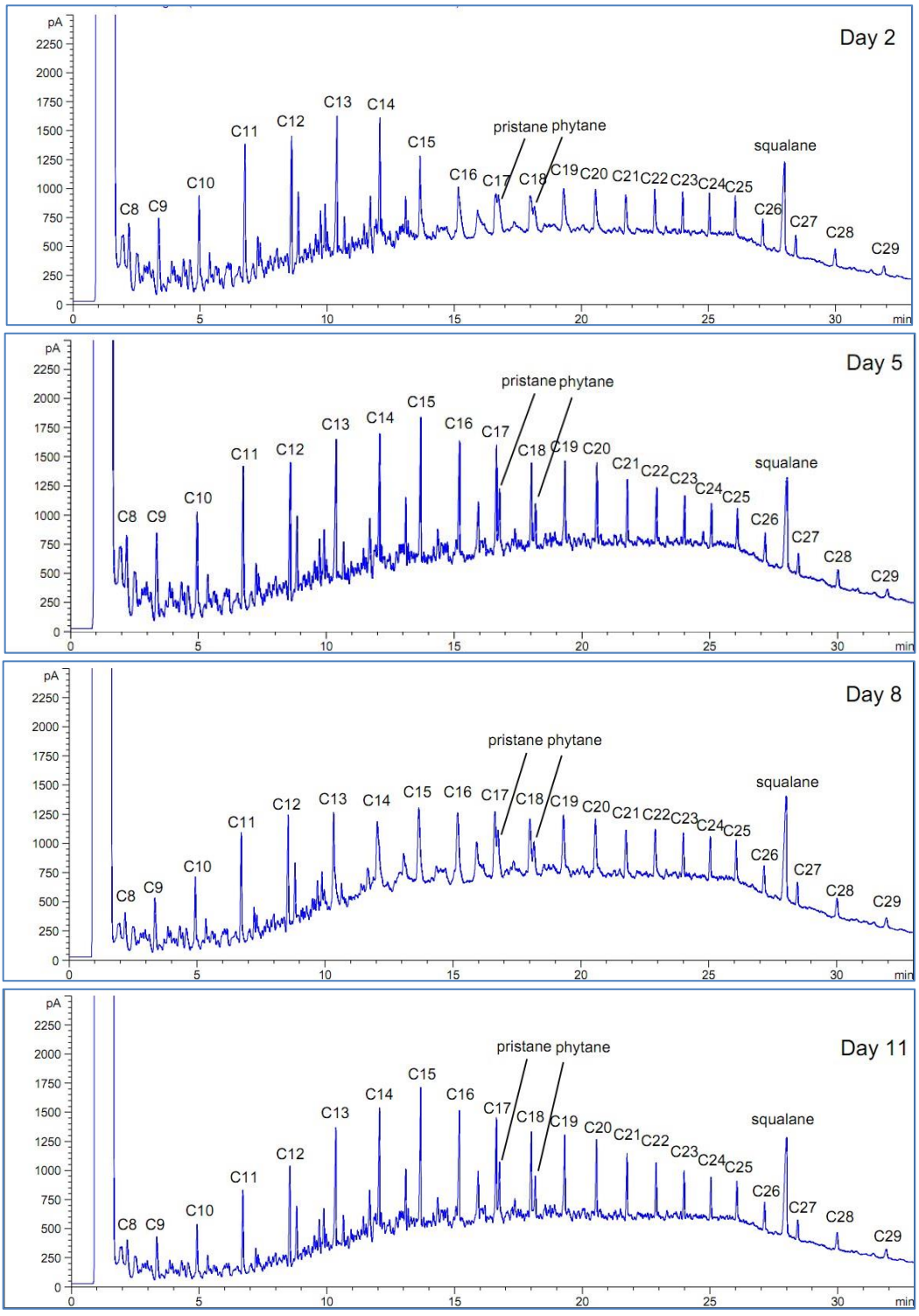

Figure 4.10 GC analysis of saturate fractions in the sterile controls over 11 d.

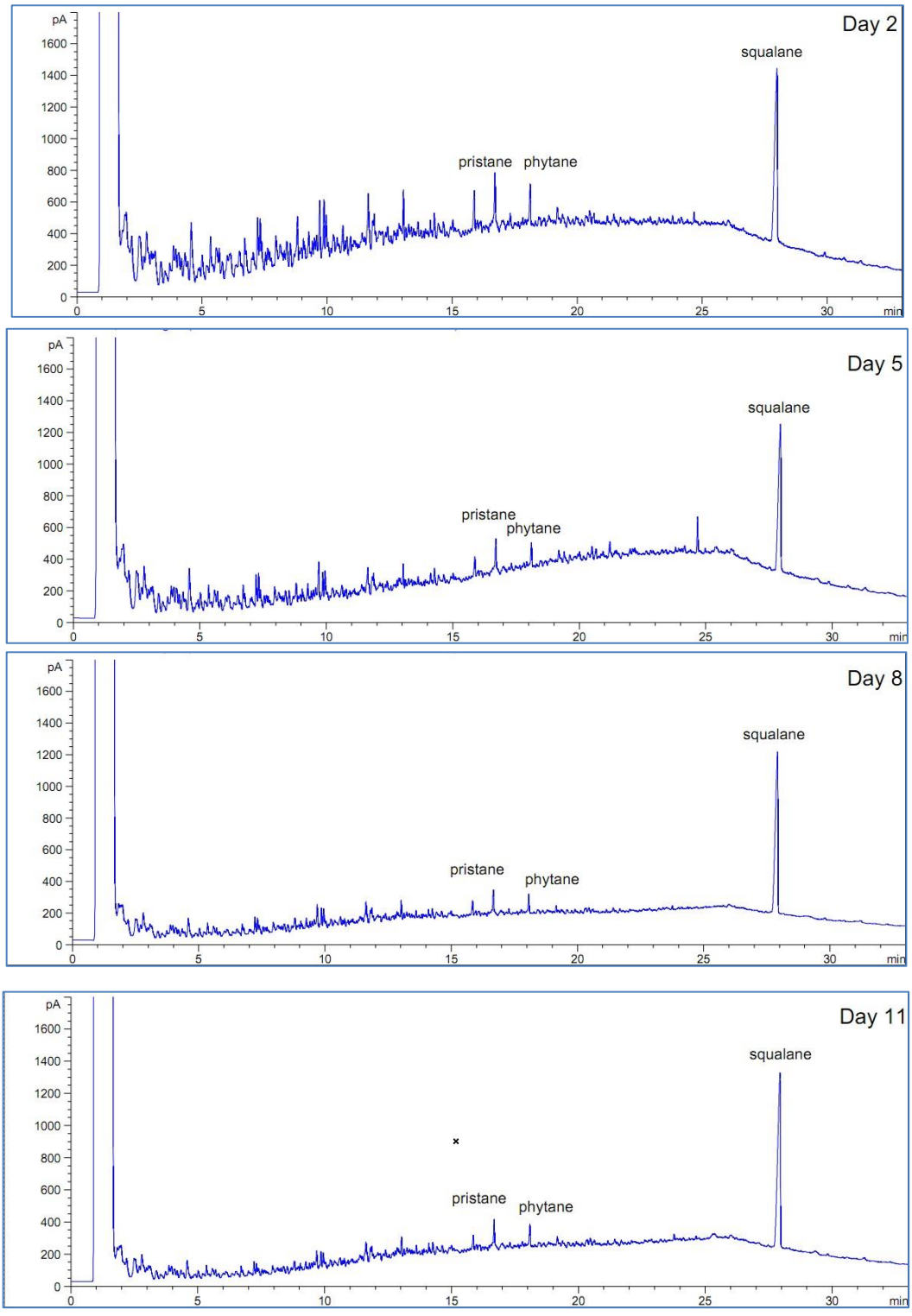

Figure 4.11 GC analysis of saturate fractions degraded by EPWF at 0 g/L NaCl in 11 d.

In the presence of 25 g/L NaCl, the degradation results were quite similar to those in the 0 g/L NaCl group, and almost all the n-alkanes were degraded except pentadecane (C15) on day 2 (Figure 4.12). But no degradation of pristane and phytane was detected at the end of day 2. The GC profile on day 5 shows that the residual pentadecane was gone completely and the amount of branched alkanes had decreased. On day 8, about 80% of the pristane and 81% of the phytane were degraded, which is a similar result to the 0 g/L NaCl group. No obvious changes were observed from day 8 until the end of this experiment. Zhang et al. (2011) used the local microorganisms to degrade total petroleum hydrocarbon (TPH), and reported that neither pristane nor phytane was degraded in the later time of the experiment (day 13 to day 30). These results were probably due to flocculation early in the experiment affecting the subsequent uptake of hydrocarbons by EPWF (Figure 4.13).

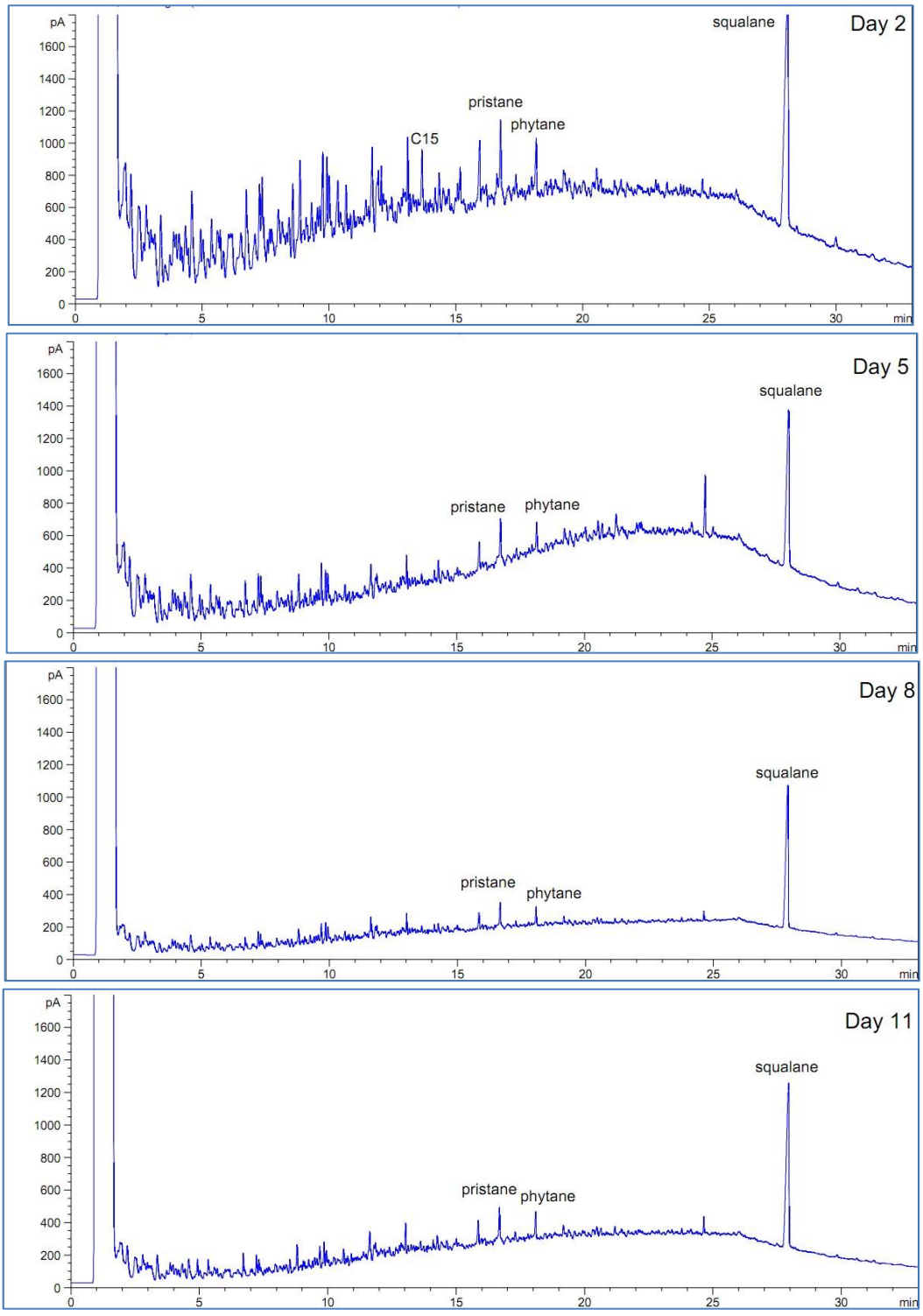

Figure 4.12 GC analysis of saturate fractions degraded by EPWF at 25 g/L NaCl on day 2, 5, 8 and 11.

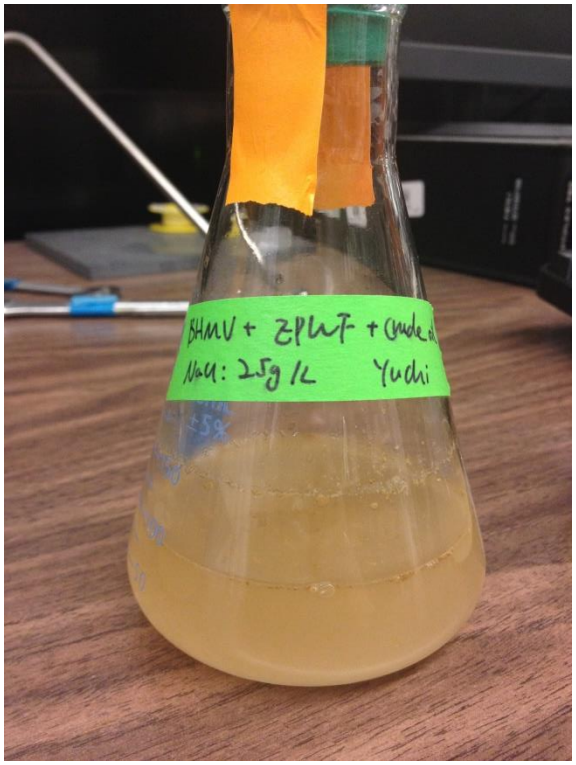

Figure 4.13 Flocculation in the medium with crude oil at 25 g/L NaCl on day 8.

Comparing the GC profile at 50 g/L NaCl on day 2 with that of the control, the height of each peak only changed slightly (Figure 4.14). Very little of the n-alkanes were degraded, and no branched alkanes were degraded. It is noticeable that the result on day 5 shows a remarkable reduction of n-alkanes and pristane. The average degradation efficiency of the lighter fractions (C8-C15) was 71%; while for the heavier fractions (C16-C29) it was close to 100%. This is probably due to the high initial concentration of light hydrocarbons in this light crude oil. In addition, de Carvalho and da Fonseca (2005a) observed *R. erythropolis* DCL14 showed a higher growth rate with longer chain hydrocarbons than the shorter ones with motor oil as carbon source.

The profiles of the 50 g/L NaCl cultures on day 8 and day 11 were identical to those at the lower salt concentrations, and no more changes were observed. This confirmed that NaCl could affect initial cell growth and biodegradation rate, but not change the overall extent of biodegradation. Zvyagintseva et al. (2001) studied the ability of *R. erythropolis* INMI 100 to degrade hydrocarbons in the presence of 50 g/L NaCl. They found that even though the initial degradation rates of turbine oil and paraffins were lower at 50 g/L NaCl than those at 5 g/L NaCl, both of the final biodegradation results were quite similar. The above results suggest that *R. erythropolis* EPWF is suitable for *in situ* bioremediation of hydrocarbons and crude oil with high NaCl concentrations.

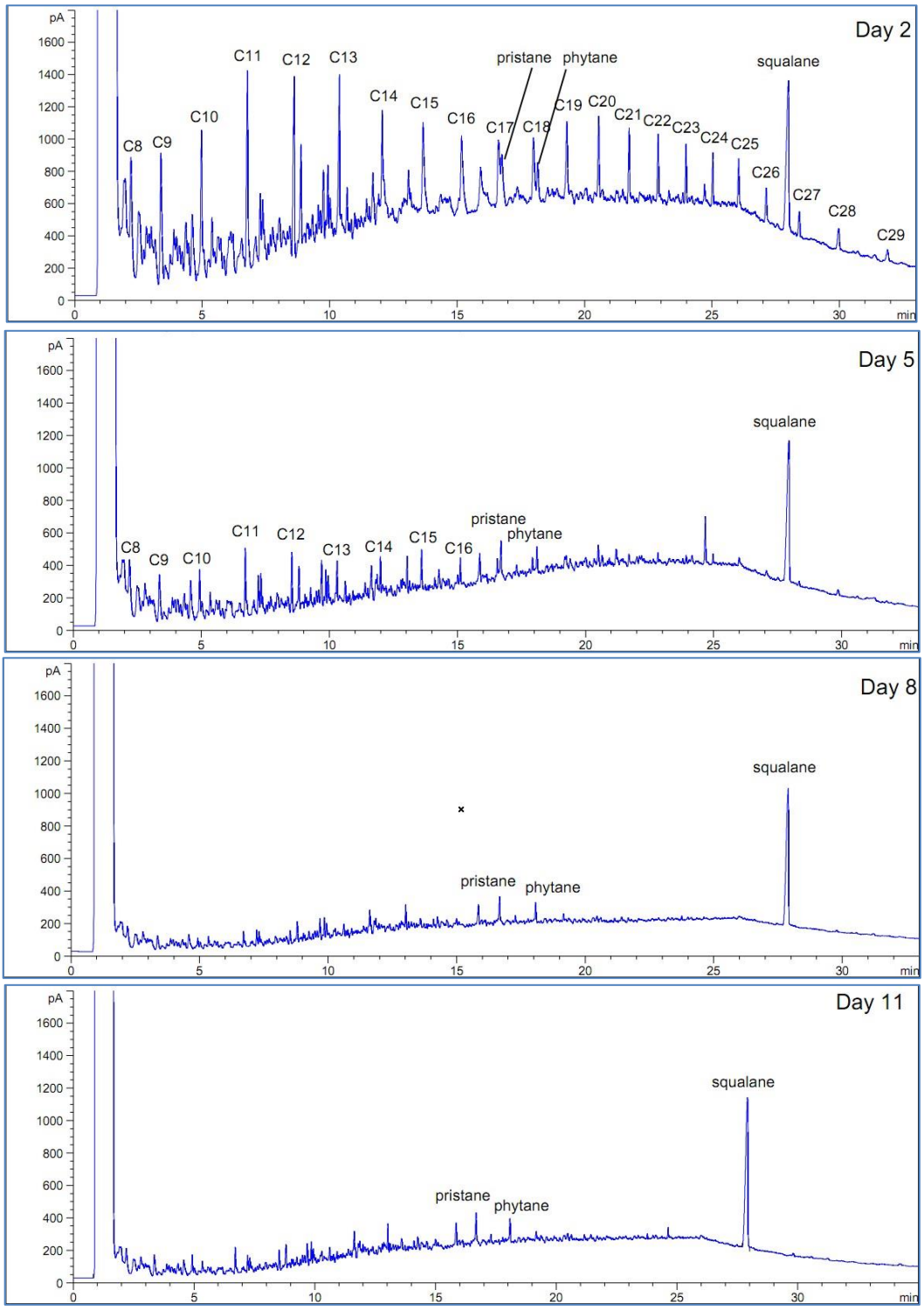

Figure 4.14 GC analysis of saturate fractions degraded by EPWF at 50 g/L NaCl on day 2, 5, 8 and 11.

4.3.2 Effect of NaCl on biodegradation of the saturate fraction in crude oil by a defined bacterial consortium

Many papers report the biodegradation of hydrocarbons and crude oil by pure cultures; however, the effect of NaCl on degradation by consortia is rarely reported. In this section, the biodegradation of saturate fractions in light crude oil by a defined consortium of saturate-degrading strains (*R. erythropolis* Husky A, *R. erythropolis* S+14 He and *Gordonia* sp. Esso AGD) under different NaCl concentrations is reported. These strains were originally isolated from heavy crude oil contaminated soil or refinery wastewater and have been used as a standard consortium for testing of hydrocarbon biodegradation (Foght et al. 1998). In order to avoid the interference of volatile hydrocarbons (C8-C12) on the calculation of the biodegradation, the crude oil used as the carbon source in this experiment was placed in the fume hood overnight to remove the lightest fractions. The oil was extracted from the cultures with DCM and partially fractionated to recover the saturate fraction for analysis. As in the experiment with pure strain EPWF, triplicate flasks were sacrificed and extracted for analysis at each selected time point for each salt concentration tested. Single representative chromatograms are shown here, and qualitative comparison of the results is presented in the text.

The GC profiles of the sterile controls on day 0 and 11 are shown in Figure 4.15. There was no obvious change in the saturate fraction of the crude oil after 11 d, and all the hydrocarbons were still present.

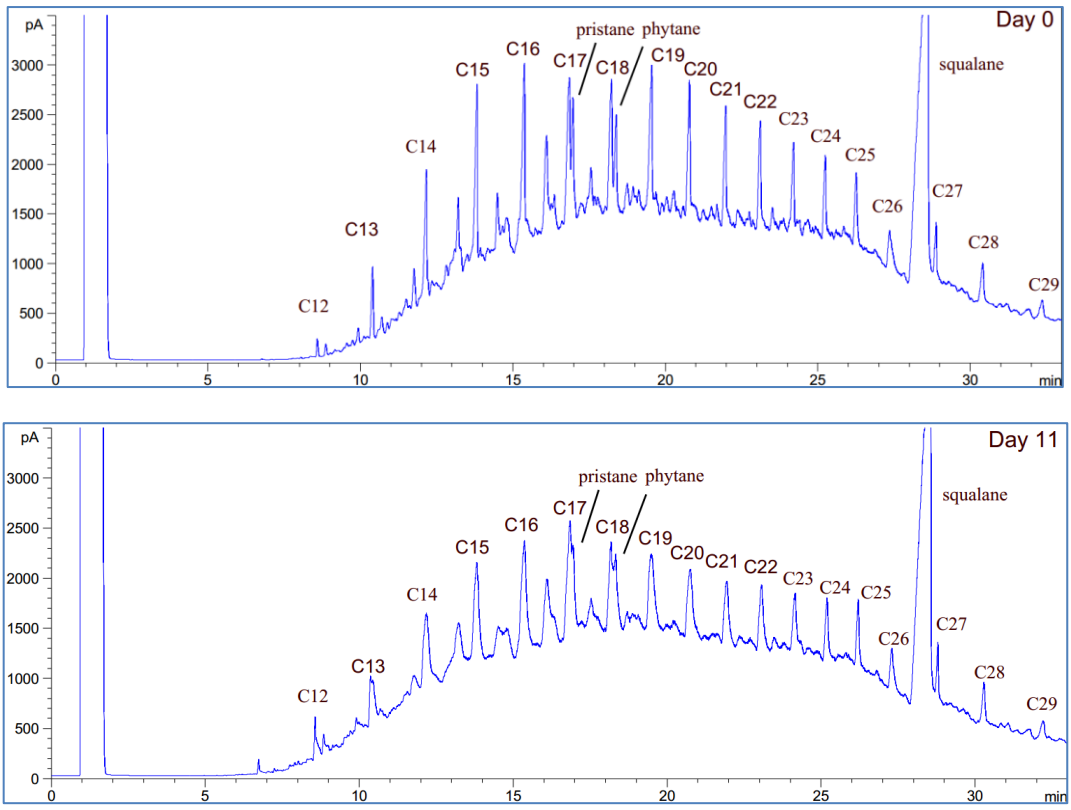

Figure 4.15 GC analysis of pre-treatment saturate fractions in the sterile controls on day 0 and 11.

The result of biodegradation by the mixed strains at 0 g/L NaCl (Figure 4.16) was similar to the result of the biodegradation by EPWF. All the straight-chain alkanes were degraded very quickly in the first two days, and only branched alkanes (pristane and phytane) were left in the medium on day 2. Li et al. (2013) observed that the branched alkanes can be degraded by *Rhodococcus* sp. JZX-01, but their degradation rates were not very high.

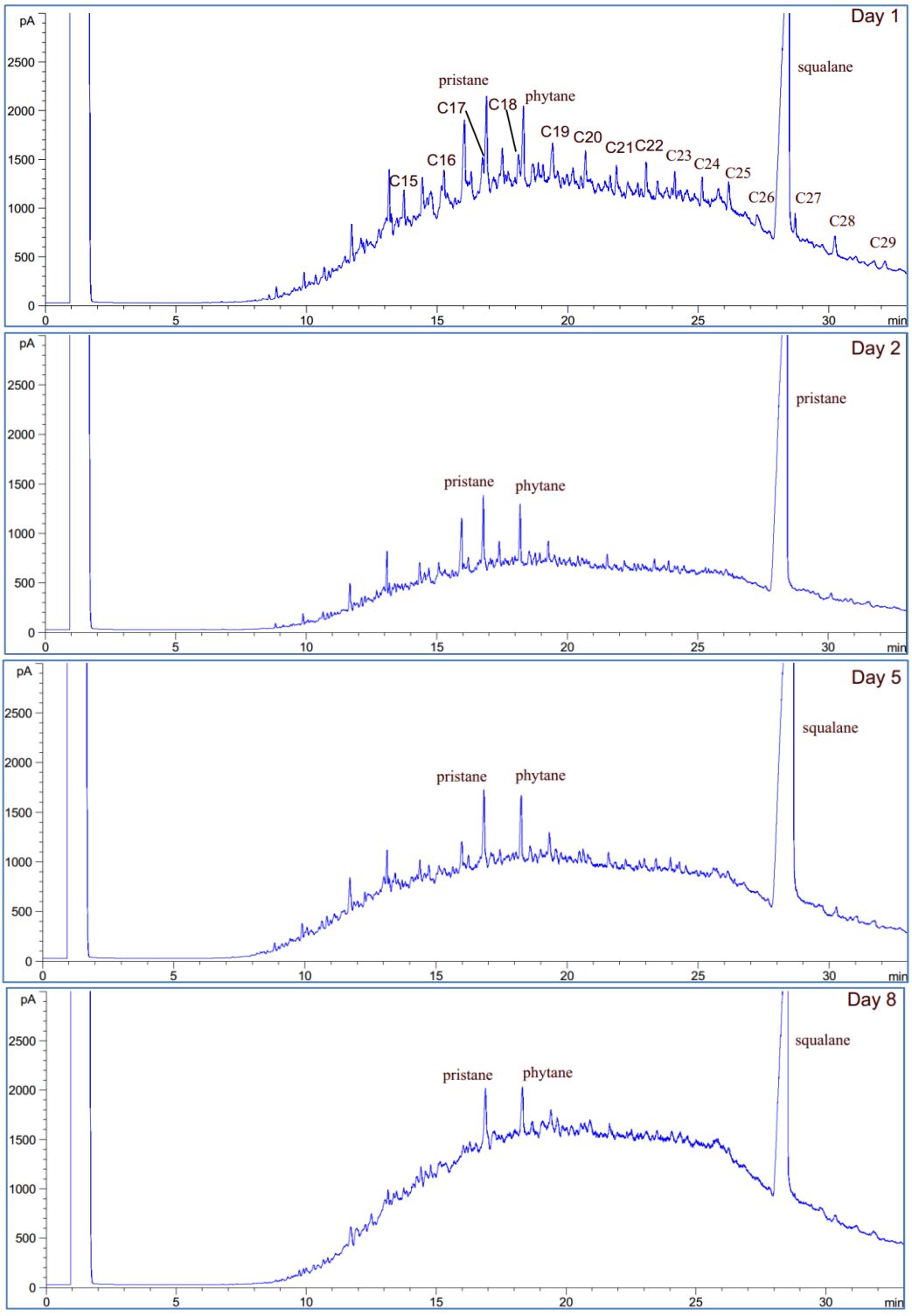

Figure 4.16 GC analysis of saturate fractions degraded by the consortium at 0 g/L NaCl on day 1, 2, 5 and 8.

Compared with the biodegradation at 0 g/L NaCl, the final biodegradation result at 25 g/L NaCl was quite similar (Figure 4.17). Unlike the 0 g/L NaCl group which had a high initial degradation rate, the 25 g/L NaCl group did not show obvious degradation on day 1. But according to the GC profile on day 2, a similar degradation extent was achieved as at 0 g/L NaCl. In addition, a better degradation efficiency was achieved by the consortium compared to the efficiency achieved with a single strain, and all the straight chain alkanes were degraded completely. The profiles at later time points did not show any apparent changes, the recalcitrant branched alkanes remained in the cultures until the last day of the experiment (day 11, not shown).

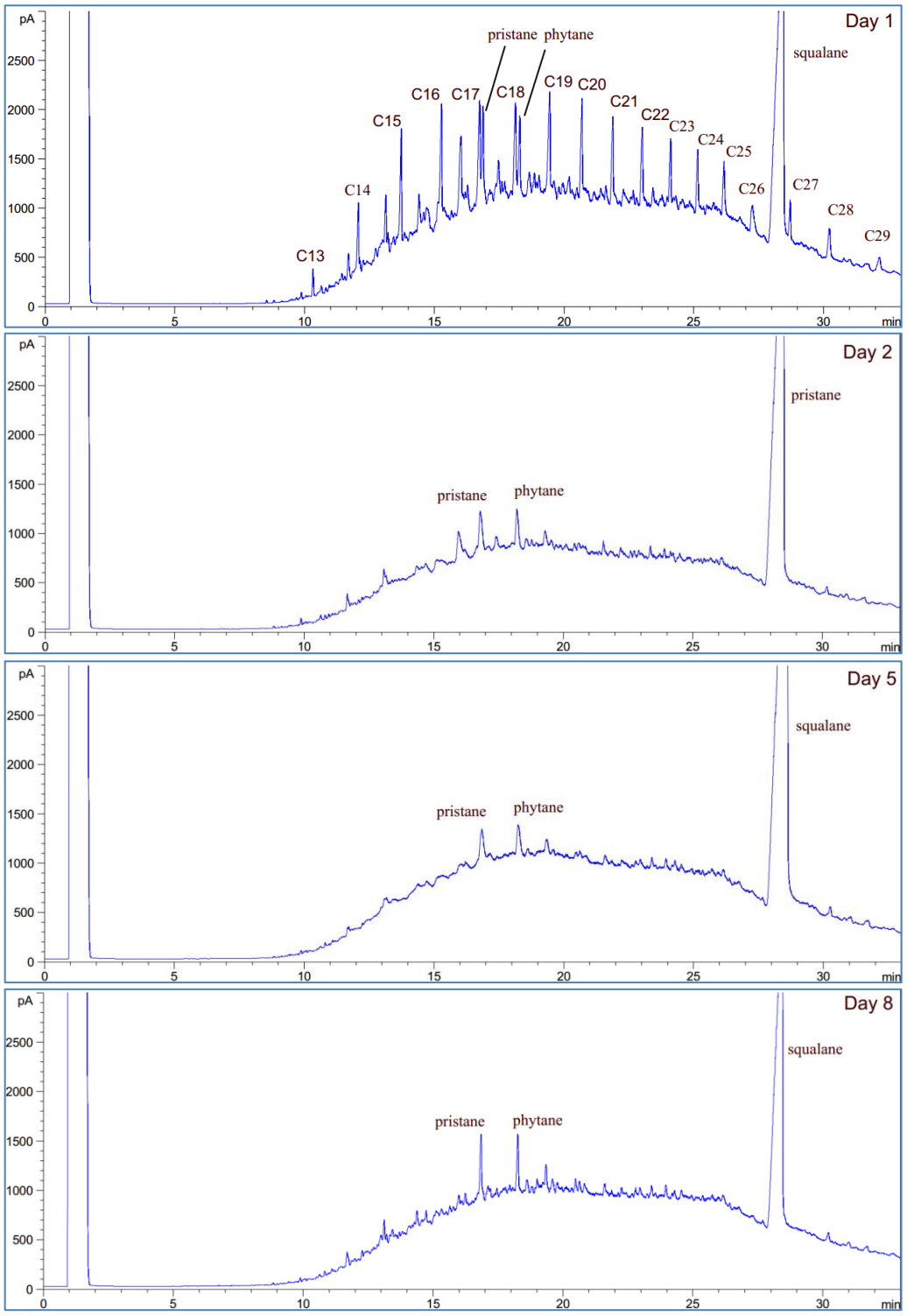

Figure 4.17 GC analysis of saturate fractions degraded by the consortium at 25 g/L NaCl on day 1, 2, 5 and 8.

Biodegradation by the consortium at 50 g/L NaCl is shown in Figure 4.18. As with the 25 g/L NaCl cultures, no degradation was observed on the first day. A remarkable reduction of the saturated hydrocarbons was shown on day 2, which indicates the cells gradually adapted to the high salt concentrations. Compared with the height ratios of C17 to pristane and C18 to phytane on day 2 shown in Figure 4.14 for EPWF, the ratios in Figure 4.18 for the consortium were notably lower. These results indicate that the high NaCl concentration indeed prolonged the lag time, and that the consortium shows a better adaptive capacity and a higher degradation rate. The profile on day 5 confirmed the above results; the C13 to C16 alkanes were not detected with the mixed strains, which was unlike the result on day 5 for EPWF. The results obtained on day 8 and on day 11 (not shown) did not show any obvious changes, with only pristane and phytane left in the cultures.

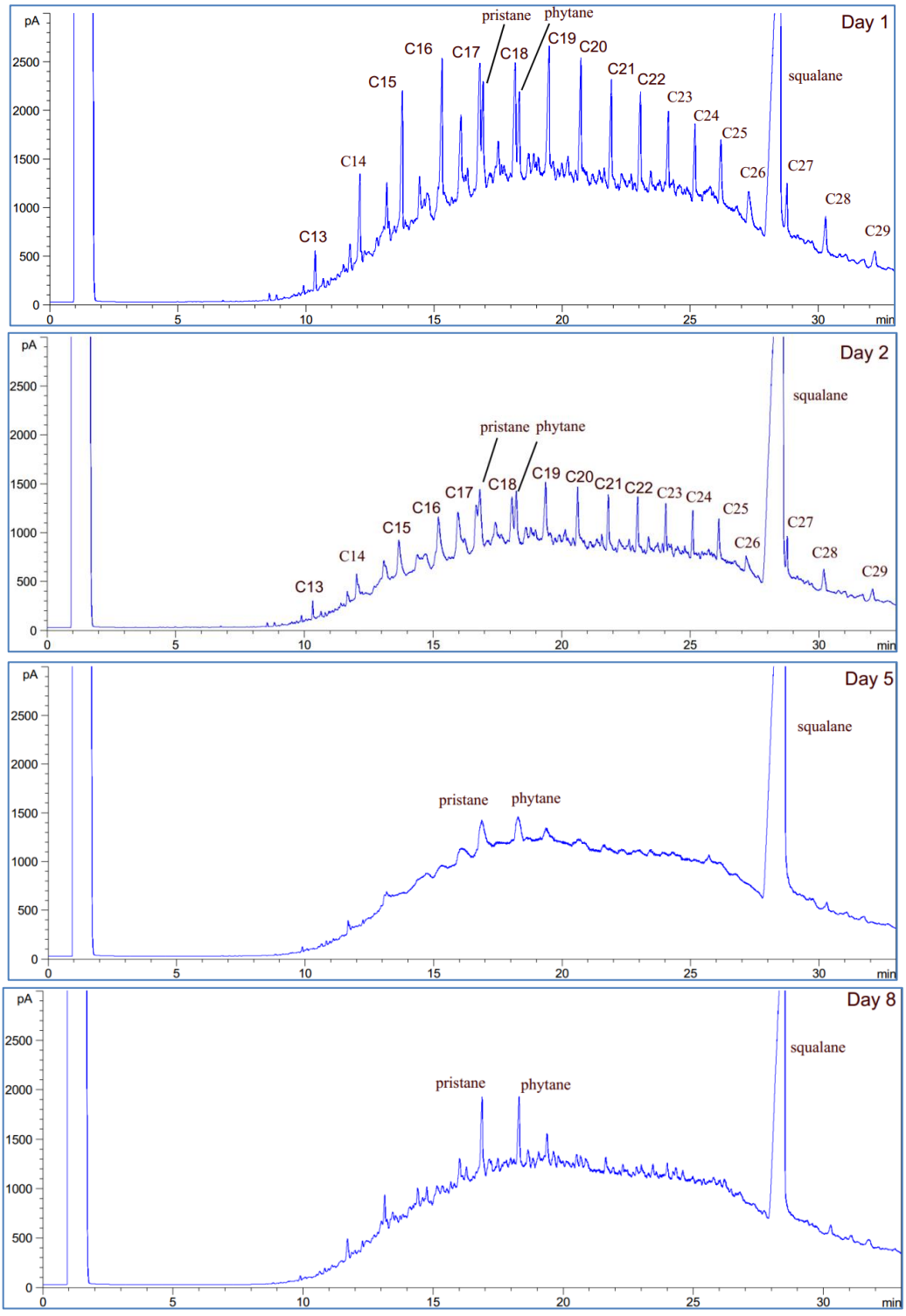

Figure 4.18 GC analysis of saturate fractions degraded by the consortium at 50 g/L NaCl on day 1, 2, 5 and 8.

According to the results of biodegradation of saturated alkanes in crude oil by both a single pure strain *R. erythropolis* EPWF and by the consortium, it is noticeable that NaCl could affect the strain growth through increasing the lag time; however, the final biodegradation extents were not obviously affected. Furthermore, the mixed strains showed a better degradation efficiency than a single strain. Barin et al. (2014) and Chen et al. (2014) studied a bacterial community to degrade TPH and crude oil, respectively, and found that mixed strains showed a higher degradation rate than pure strains. The reason for the incomplete branched alkanes degradation was probably that the experiment was conducted in sealed flasks and may have been oxygen-limited. Moreover, flocculation formation, nutrient depletion and toxic metabolites may also lead to a low biodegradation efficiency.

4.4 Effect of salt on *R. erythropolis* EPWF cell surface hydrophobicity

As mentioned before, some strains can develop a hydrophobic cell membrane when using hydrophobic substances as carbon sources. Bouchez-Naïtali et al. (2001) reported that higher cell hydrophobicity could lead to significant aggregation among alkane-degrading microorganisms. Based on the observations of EPWF cell growth in the medium with alkanes, flocculation formation and transformation were observed during incubation. When hexadecane was used as the carbon source in the presence of high NaCl concentration (50 g/L), some cellular aggregates formed and stuck to the inner wall of the flask, and similar adhesive flocculation was observed at 75 g/L KCl. Determining the effect of salt on cell surface hydrophobicity may help give a better understanding of the effect of salt on biodegradation of hydrocarbons and crude oil. Very few papers have reported the changes of cell surface hydrophobicity with different salt concentrations.

Several researchers have studied the hydrophobicity of the cell membrane with the method of Bacterial Adherence to Hydrocarbons (BATH) Assay or Microbial Adherence to Hydrocarbons (MATH) Assay, first proposed by Rosenberg et al. (1980). However, it does not always give an accurate quantitative measurement of the hydrophobicity of the cell membrane, and is not appropriate when using hydrophobic substrates as a carbon source due to cellular flocculation, which prevents optical density measurements. In this project, a new method was developed to disperse the cell flocculation using pentane[2], and contact angle measurement was used to evaluate the cell surface hydrophobicity (Chapter 3.5.8). The experiments were conducted with both glucose and hexadecane as carbon sources at 0, 25 and 50 g/L NaCl over 11 d.

4.4.1 Effect of NaCl on *R. erythropolis* EPWF cell surface hydrophobicity with glucose as the carbon source

When glucose was used as the sole carbon source, the cell surface hydrophobicity was low (contact angles less than 35°) over a range of salinities from 0-50 g/L NaCl over 11 d of growth (Figure 4.19). Since there were not enough cells harvested on day 2 in the 50 g/L group, the data for this group were recorded from day 5.

[2] * Hamameh, R. (2014). *The change in* Rhodococcus erythropolis *strain EPWF cells hydrophobicity during the growth on hydrophilic and hydrophobic hydrocarbon substrates*. BASc. Thesis. University of Ottawa: Canada.

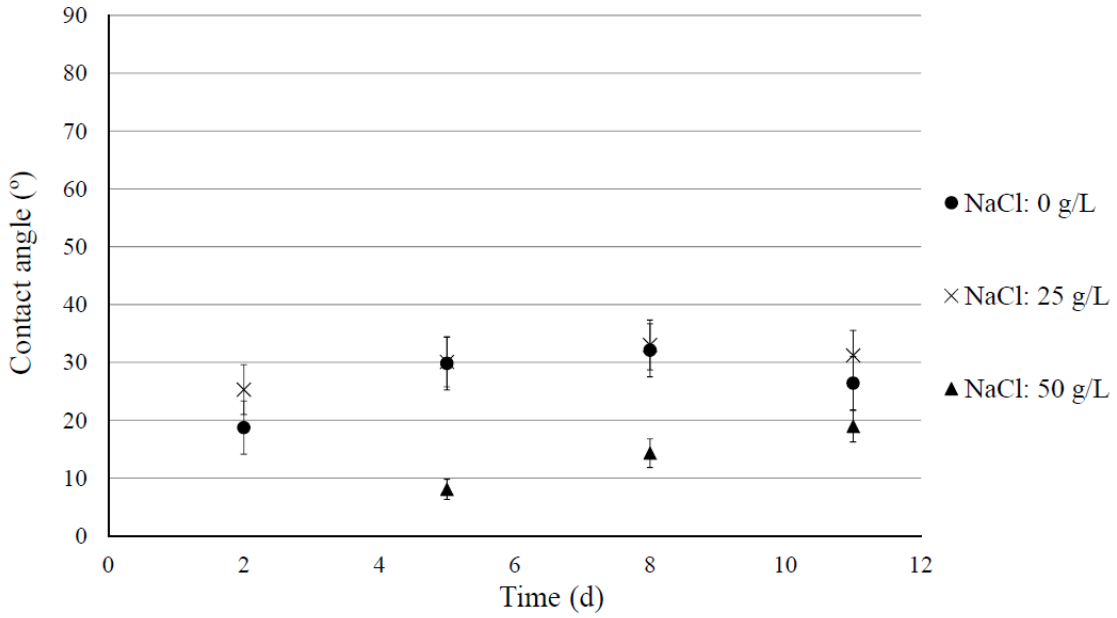

Figure 4.19 Contact angle of *R. erythropolis* EPWF grown on glucose at different NaCl concentrations (0, 25 and 50 g/L) for 11 d (average ± standard deviation, n=5).

In the presence of 0 and 25 g/L NaCl, it was observed that the contact angles increased gradually in the first 8 d and declined afterwards. Bredholt et al. (2002) reported that the cell surface hydrophobicity increased during the exponential phase and began to decrease when the cells entered stationary phase. In the 50 g/L NaCl group, the contact angles kept increasing from day five to the last day of the experiment, which corresponds to the delayed exponential phase in the EPWF growth curve at the same salt concentration (Figure 4.4).

In addition, the 0 and 25 g/L NaCl groups showed similar contact angles during the whole incubation period. However, the 50 g/L group displayed a less hydrophobic surface than the other two groups. Earlier studies by Hart et al. (1987) showed that when increasing the NaCl concentration, the surface hydrophobicity of *Halomonas elongata* decreased when grown on water-soluble carbon sources. This was probably due to the loss of cell-wall-associated water at high salt concentration resulting in a

progressive tightening of the cell wall, which shielded hydrophobic groups and produced a more hydrophilic surface (Vreeland et al. 1984). de Carvalho et al. (2014) measured the percentage of unsaturated fatty acids from *R. erythropolis* DSM 1069 cell membranes using ethanol as a single carbon source. They found that the percentage reached the lowest value at 25-35 g/L NaCl, however, the percentage of unsaturated fatty acids increased dramatically once the salt concentration was over 40 g/L, which indicated that the cell surface was less hydrophobic at higher salt concentrations (Whyte et al. 1999).

4.4.2 Effect of NaCl on *R. erythropolis* EPWF cell surface hydrophobicity with hexadecane as the carbon source

While EPWF cells showed a relatively hydrophilic surface during growth on glucose, a more hydrophobic cell surface was observed with hexadecane as the substrate. Sample images from contact angle measurements on day eight are shown in Figure 4.20, which presents the contact angles for cell grown in the medium with glucose and hexadecane in the presence of 50 g/L NaCl. Differences in the cell surface hydrophobicity between water-soluble and water-insoluble substrates were reported by Angelova and Schmauder (1999), Bredholt et al. (2002) and Mnif et al. (2009), who showed that strains could modify their lipid composition and membrane structure to form a hydrophobic surface in order to grow on hydrophobic substrates. de Carvalho et al. (2014) also reported that salt stress affected polyunsaturated fatty acid production by *R. erythropolis*.

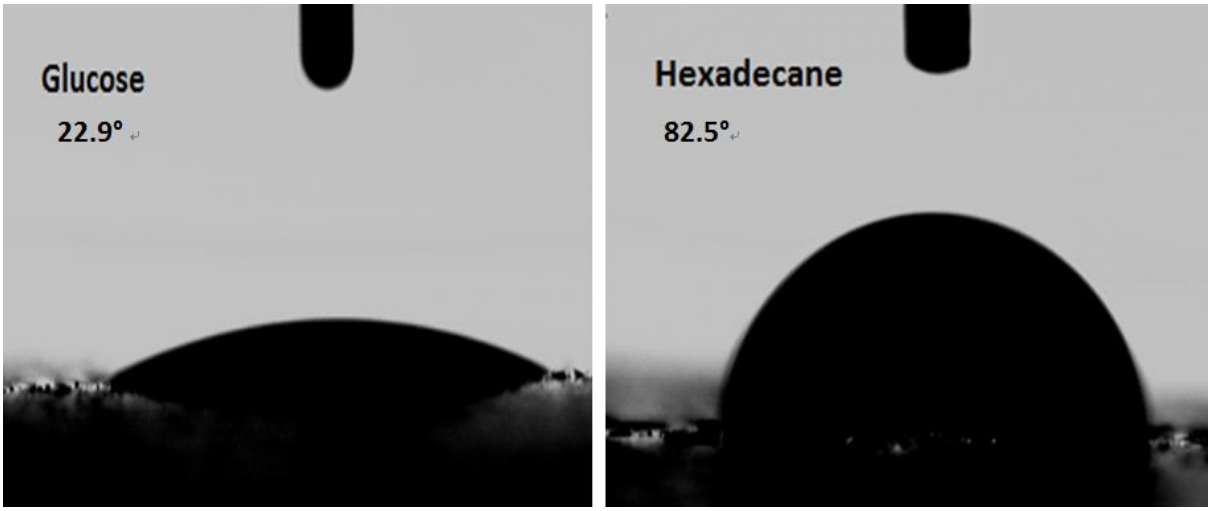

Figure 4.20 Changes in contact angle of *R. erythropolis* EPWF after 8 d growth in BHMV medium with 50 g/L NaCl and either glucose or hexadecane as the carbon source.

The changes in contact angles during 11 d growth with hexadecane as the carbon source at different NaCl concentrations are shown in Figure 4.21. Both the 0 g/L and 25 g/L groups presented higher contact angles at the beginning of the experiment, and both of them showed a declining trend afterwards. The contact angles of the 0 and 25 g/L NaCl groups were around 50-60°, which were higher than those in glucose medium. In the presence of 50 g/L NaCl, the cells developed higher hydrophobic surfaces than at the other two salt concentrations. The highest contact angle (82.5°) was measured on day eight and it decreased sharply later, corresponding to the delayed exponential phase. It is noticeable that all the contact angles were quite close at the end of this experiment, which is a similar result to Figure 4.19. The cell surface hydrophobicity at different growth phases could affect the ability to interact with hydrophobic substrates, and then influence the biodegradation rate.

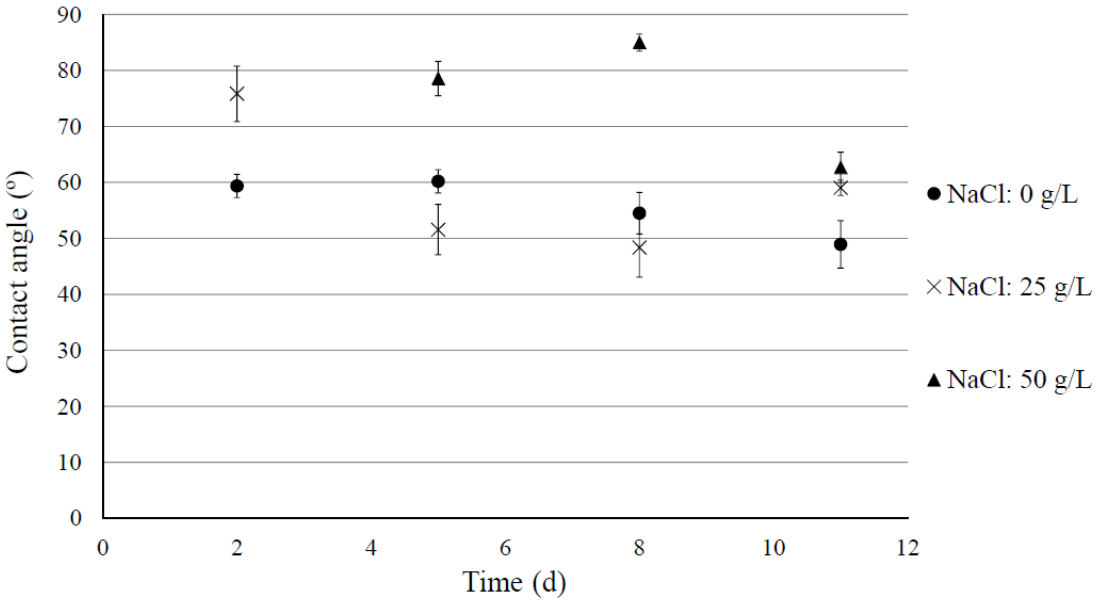

Figure 4.21 Contact angle of *R. erythropolis* EPWF grown on hexadecane at different NaCl concentrations (0, 25 and 50 g/L) in 11 d (average ± standard deviation, n=5).

In contrast to the results with glucose, the 50 g/L NaCl cultures showed higher average contact angles than the lower salt concentrations with hexadecane as the substrate, which corresponds to the results of some previous studies. Brown (1976) reported that high salt concentration increases the strength of hydrophobic interactions and stiffens the cell membrane. Angelova and Schmauder (1999) reported that hydrophobic substrates could induce biosurfactant production or release, which could facilitate uptake of hydrocarbons by hydrophobic strains as mentioned before. However, a medium with high salt concentration not only inhibits cell growth, but also decreases the biosurfactant production and increases the interfacial tension between aqueous phase and hydrocarbon, which is not helpful for uptake of hydrocarbons. de Carvalho et al. (2014) showed that cells could change the lipid composition of their membrane at different salt concentrations. In the case of *R. erythropolis* EPWF, development of a more hydrophobic surface under the adverse condition of elevated

salt concentration may serve to improve the bioavailability of hexadecane. This suggests that halotolerant strains not only can withstand high salt concentrations, but also can change their cell surface hydrophobicity depending on the salt concentration to improve uptake of carbon sources.

Chapter 5 Overall Discussion and Conclusions

In this thesis, the following work was accomplished in three phases:

1. Determining the effect of different kinds of salt at different concentrations on the growth of the alkane-degrading bacterium *R. erythropolis* EPWF in order to define its halotolerance.

2. Establishing a systematic understanding of the effect of salt on biodegradation of saturated hydrocarbons by a pure culture and a consortium.

3. Investigating the changes in cell surface hydrophobicity at different salinities to develop a better understanding of the physiological responses to salinity affecting uptake of saturated hydrocarbons.

5.1 Halotolerance of *R. erythropolis* EPWF with different kinds of salt

NaCl is the most prevalent salt in oil-contaminated sites, originating from the produced water from the oil formation. *R. erythropolis* EPWF used in this study tolerated up to 75 g/L NaCl, and kept essential activity with 50 g/L NaCl in a mineral salt medium with either glucose or hydrocarbons as carbon sources. Based on the results of glucose consumption and growth curves, the lag time increased with increasing NaCl concentration. However, the cell growth rate increased quickly after the cells adapted to the new environment. The results indicate that *R. erythropolis* EPWF would be suitable for application in an environment with high NaCl concentrations.

Besides Na^+ and Cl^-, another two ions (K^+ and SO_4^{2-}) are also common in the environment and were studied to evaluate their impact on cell growth. Both KCl and Na_2SO_4 showed less effect than NaCl on cell growth over a range of salinities from 25 to 75 g/L. K^+ is well known as an osmoregulator and SO_4^{2-} is involved in synthesis of multiple enzymes and other proteins (Al-Mailem et al. 2013; Lippard and Berg 1994). These physiological roles may explain the higher tolerance to these ions. Just as the lag time increased in the presence of high NaCl concentrations, a similar phenomenon also occurred with high KCl concentrations (Figure 4.2a), but not with the same amount of Na_2SO_4 (Figure 4.3a).

5.2 Effect of salt on biodegradation of model alkanes and light crude oil

The results of biodegradation of pure saturated hydrocarbons confirmed that high NaCl concentrations prolonged the lag phase, but did not greatly affect the total biodegradation efficiency. The branched alkane pristane was more resistant to degradation than the straight alkane hexadecane at the same salinity; this is known to be due to steric inhibition of the terminal-oxidizing enzymes (Schaeffer et al. 1979). Flocculation was observed in the medium with hydrocarbons as the carbon sources, and the flocculation dimension and colour changed when changing the NaCl concentration or the carbon source. Bouchez-Naïtali et al. (2001) reported that strains that do not produce biosurfactant usually contact hydrocarbons directly and form cell aggregates, allowing direct uptake from the hydrocarbon phase. Changes in flocculation behaviour at different salt concentrations could therefore affect uptake and biodegradation efficiency.

The results of mixed hydrocarbon degradation (Figure 4.7) indicated that flocculation not only affected the initial hexadecane biodegradation, but also had an influence on the subsequent pristane uptake and degradation. The smaller the size of the particles formed, the higher the degradation rate that was observed. This is presumed to be caused by the higher specific surface area leading to an increase in the contact of cells with hydrocarbons and oxygen.

Degradation of the saturate fraction in light crude oil by both EPWF and by a defined saturate-degrading consortium was investigated at different salinities. The strains preferred to consume the short straight-chain alkanes and then to consume the longer ones, followed by the branched alkanes (pristane and phytane). The lag phase observed at high NaCl concentration and the similarity in the final biodegradation profiles regardless of NaCl concentration corresponded to the earlier results for biodegradation of model hydrocarbons. In this case, the consortium contained strains of *Rhodococcus erythropolis* and the closely related *Gordonia* sp.; it is not evident whether the observed effect of NaCl on biodegradation of hydrocarbons can be extended to different microorganisms.

As with NaCl, KCl had a clear effect on cell growth, but the final results of biodegradation of hexadecane (Figure 4.9) were quite similar at all KCl concentrations. Flocculation formation and disruption was observed at all the KCl concentrations tested. The cultures with Na_2SO_4 at all concentrations showed a similar high initial degradation rate, while the degradation results were not as good as those with the other two salts (Figure 4.8). No changes in the size of flocculation were observed over a range of Na_2SO_4 from 25-75 g/L during biodegradation. It can be concluded that besides the effect of salt on cell growth, the impact of salt on cell flocculation formation and dimensions also plays an important role in hydrocarbon uptake and

biodegradation, through changing the surface area for contact between cells and substrates (Ugochukwu et al. 2014).

5.3 Effect of salt on cell surface hydrophobicity

R. erythropolis EPWF developed a higher cell surface hydrophobicity in the medium with hexadecane compared with that in glucose medium, which is in agreement with the study of Mnif et al. (2009). In addition, cells in the different growth phases also demonstrated changes in surface hydrophobicity; the highest surface hydrophobicity was always seen during the exponential phase. When grown on hexadecane, EPWF showed a higher surface hydrophobicity when NaCl was added to the medium, which can be interpreted as a mechanism to enhance hydrocarbon uptake. The cell surface hydrophobicity also increased with increasing NaCl concentration, when hydrocarbons were used as carbon sources.

5.4 Future work

R. erythropolis studied in this research are recognized for their tolerance of high NaCl concentration and excellent performance of saturated hydrocarbon degradation in liquid medium. Therefore, investigation of the performance of EPWF to degrade alkanes in a soil matrix could be the next step.

K^+ is known as an osmoregulator, which could be accumulated intracellularly and maintain osmotic pressure; and sulfate is widely reported to be involved in multiple cellular functions. Therefore, the addition of potassium salt during the biodegradation process may be helpful for cells to adapt to the hypertonic environment. Other cations (Mg^{2+}, Ca^{2+}) are also reported to demonstrate a similar function (Al-Mailem et al. 2013). Gypsum ($CaSO_4$) is usually used during the pre-treatment phase (leaching) of

the remediation process to maintain soil physical properties (Finlayson & Reid 2007). Therefore, these salts and other nutrient amendments could be further studied to improve the degradation efficiency under hypersaline conditions.

Chapter 6 References

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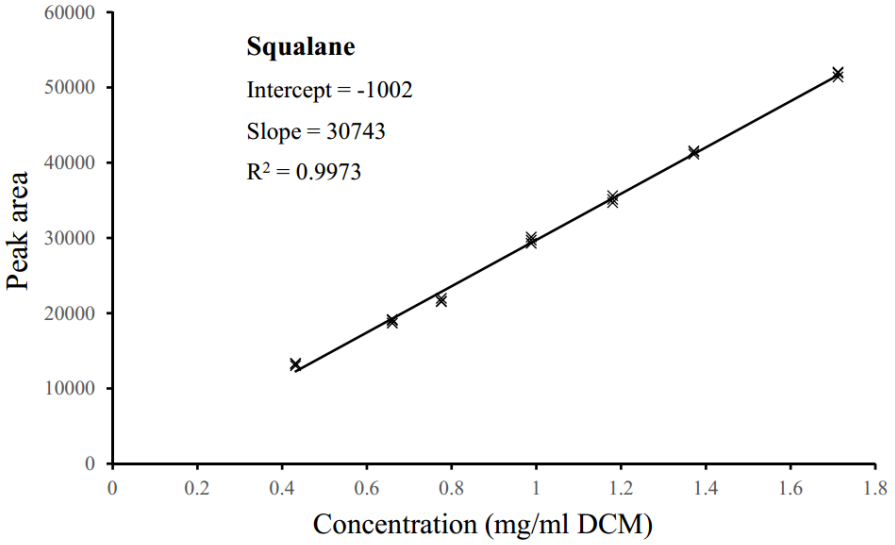
Appendix A Standard curves and calculations for GC-FID analyses

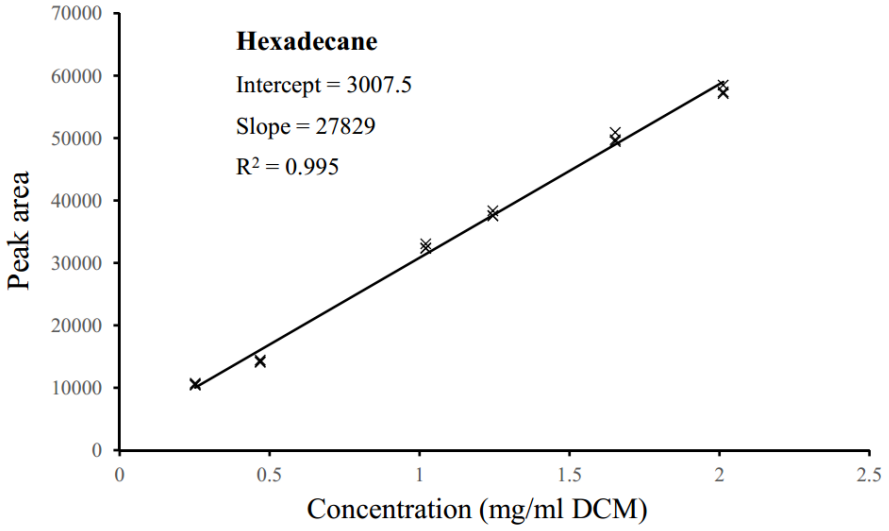
A.1 Standard curves for hydrocarbons with Agilent 7890 series GC

This appendix presents the standard curves of organic compounds from GC-FID quantitation, showing the linear relationship between the samples' mass concentration and the corresponding peak area obtained from GC profile.

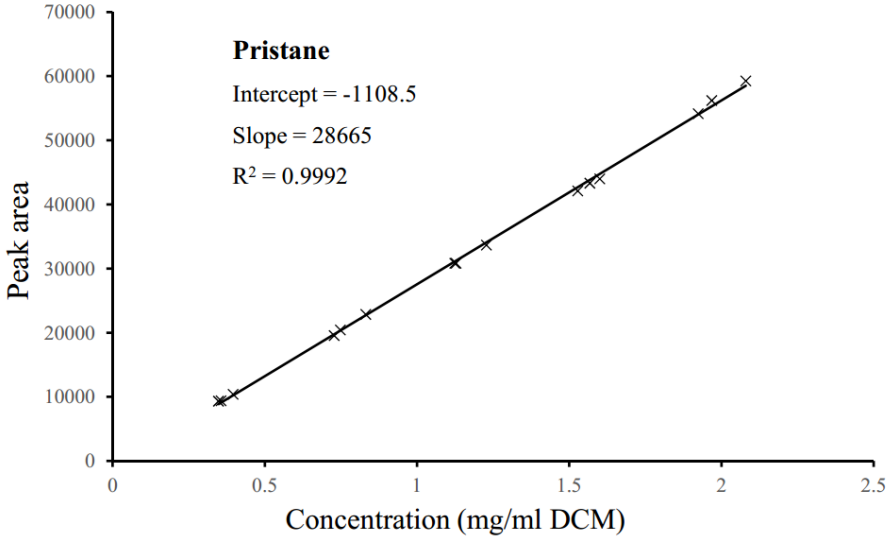
In the experiments of "Effect of NaCl on biodegradation of hexadecane, pristane, mixed hydrocarbons and crude oil", the standard curves were obtained by using an Agilent 7890 system with a flame ionization detector (FID) and a 30-m HP-1 capillary column (Agilent Technologies Co. Ltd. USA).



Sample standard curve for squalane.



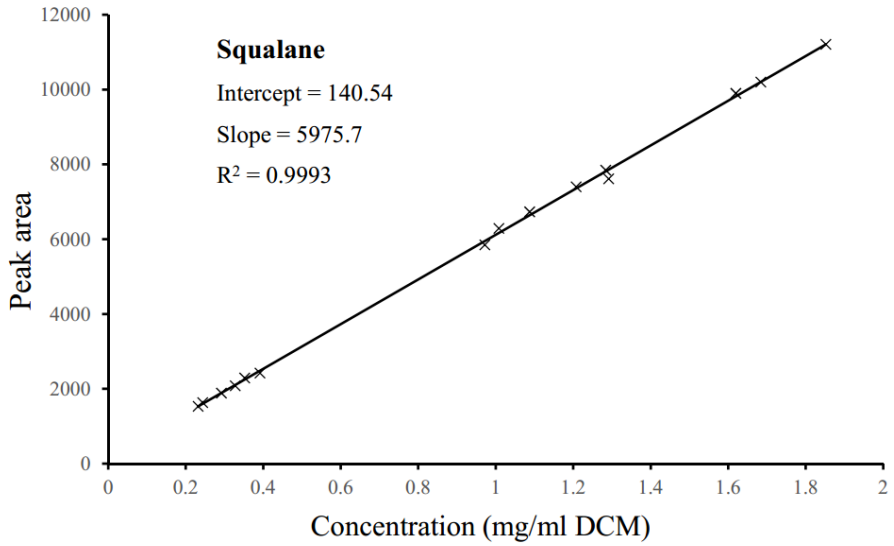
Sample standard curve for hexadecane.



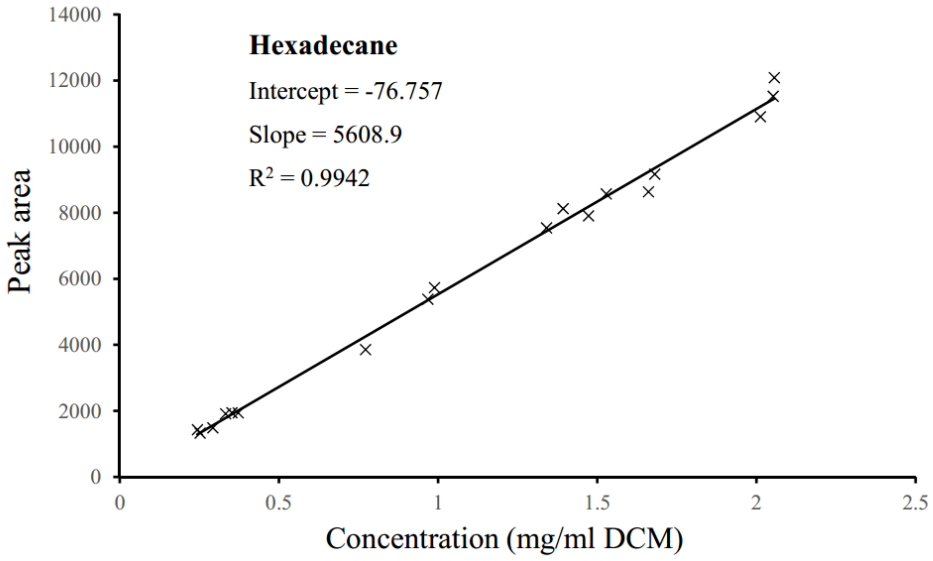
Sample standard curve for pristane.

A.2 Standard curves for hydrocarbons with Agilent 6890 series GC

In the experiments of " Effect of KCl and Na_2SO_4 on biodegradation of hexadecane", the standard curves were obtained by using an Agilent 6890 system with a flame ionization detector (FID) and a 30-m HP-1 capillary column (Agilent Technologies Co. Ltd. USA).



Sample standard curve for squalane.



Sample standard curve for hexadecane.

A.3 Calculations for extracted hydrocarbons with standard curves

1. Calculation of hydrocarbon concentration in cell culture and sterile controls

For a hydrocarbon, *i*, with peak area A_i:

$$C_{i,extract} = \frac{A_i - b}{k}$$

Where: $C_{i,extract}$ is the concentration of hydrocarbon in the extract, mg/ml DCM.

A_i is the peak area of hydrocarbon from GC-FID profile.

b is the y-intercept of the standard curve for the hydrocarbon *i*

k is the slope of the standard curve for the hydrocarbon *i*

The internal standard was added into the culture to calculate the concentration of hydrocarbon *i* in the culture, $C_{i,culture}$:

$$C_{i,culture} = \frac{C_{istd,culture}}{C_{istd,extract}} \cdot C_{i,extract}$$

Where: $C_{istd,culture}$ is the known concentration of internal standard in the culture, mg/ml DCM

$C_{istd,extract}$ is the calculated concentration of internal standard in the extract, mg/ml DCM

$C_{i,extract}$ is the calculated concentration of the hydrocarbon in the extract, mg/ml DCM

The average concentration of hydrocarbon i, $C_{avg,culture}$, is:

$$C_{avg,culture} = \frac{\sum_{j=1}^{n} C_{i,culture,j}}{n}$$

Where: n is the number of replicates

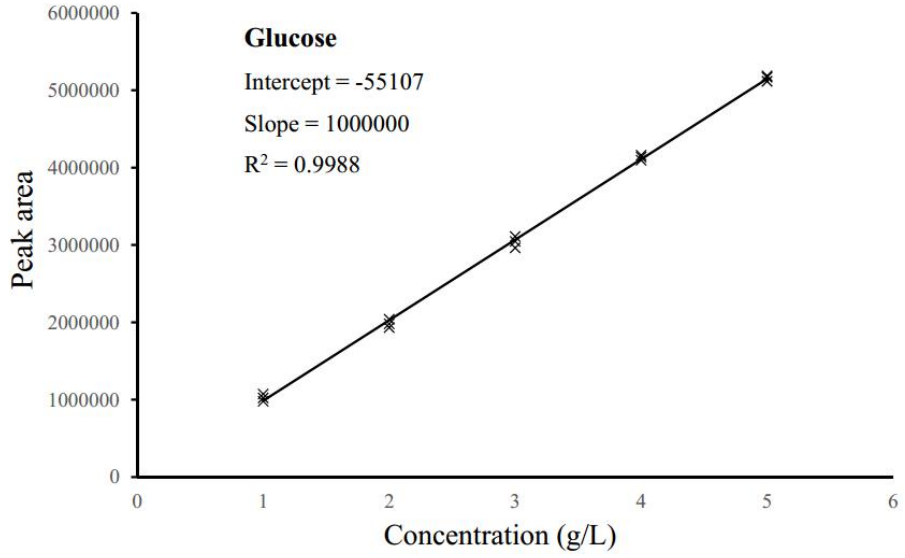
Note: Standard deviation (S.D.) is used for descriptive error bars.

Appendix B Standard curve and calculations for glucose analyses

B.1 Standard curve for glucose

This appendix presents the standard curve of glucose from HPLC quantitation, showing the linear relationship between the samples' mass concentration and the corresponding peak area obtained from HPLC profile.

The standard curves were obtained by using an Agilent 1200 series HPLC (Agilent Technologies Co. Ltd., USA) with Refractive Index Detector (RID) and Aminex HPX-87H Column (BIO-RAD Laboratories, Inc., Hercules, CA).



Sample standard curve for for glucose.

B.2 Calculation of glucose concentration in cell culture and sterile controls

For glucose, g, with peak area A_g:

$$C_{g,culture} = \frac{A_g - b}{k}$$

Where: $C_{g,culture}$ is the concentration of glucose in the culture, g/L.

A_g is the peak area of glucose from HPLC profile.

b is the y-intercept of the standard curve for the glucose

k is the slope of the standard curve for the glucose

The average concentration of glucose g, $C_{avg,culture}$, is:

$$C_{avg,culture} = \frac{\sum_{j=1}^{n} C_{g,culture,j}}{n}$$

Where: n is the number of replicates

Note: Standard deviation (S.D.) is used for descriptive error bars.