STUDY OF PYRENE DEGRADATION BY HALOPHILIC BACTERIA UNDER HYPERSALINE ENVIRONMENT

BY

FITRI BUDIYANTO

A Thesis Presented to the

DEANSHIP OF GRADUATE STUDIES

KING FAHD UNIVERSITY OF PETROLEUM & MINERALS

DHAHRAN, SAUDI ARABIA

In Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

In

ENVIRONMENTAL SCIENCE

JANUARY, 2017

KING FAHD UNIVERSITY OF PETROLEUM & MINERALS

DHAHRAN- 31261, SAUDI ARABIA

DEANSHIP OF GRADUATE STUDIES

This thesis, written by **FITRI BUDIYANTO** under the direction of his thesis advisor and approved by his thesis committee, has been presented and accepted by the Dean of Graduate Studies, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN ENVIRONMENTAL SCIENCE.**

Dr. Assad Al-Thukair
(Advisor)

Dr. Abdulaziz Al-Shaibani
Department Chairman

Dr. Alexis Nzila
(Member)

Dr. Salam A. Zummo
Dean of Graduate Studies

Dr. Musa Mohammed Musa
(Member)

27/2/17
Date

Dedication

To my beloved family

ACKNOWLEDGMENTS

In the name of Allah, the most gracious, most compassionate, most merciful and all the praises and thanks be to Allah that has given me the opportunity and capability to finish my study in KFUPM.

I am deeply indebted and most sincere appreciation goes to my advisor, Dr. Assad Al-Thukair, for knowledge, patience, encouragements, and guidance he has shown me over the past few years that I have been with this department, be it scientifically, personally, or academically.

I would like to thank my thesis committee members for their support and sharing their knowledge over the years: Dr. Alexis Nzila and Dr. Musa Mohammed Musa.

My thanks also are addressed to my wonderful family: my parents, for their endless pray, love and support; my brother and sisters, for keeping my spirit and encouragement during my master journey; and lastly — to my wife for your endless love, support and encouragement has been most influential and inspiring.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

PAHs	:	Polycyclic Aromatic Hydrocarbons
USEPA	:	United State Environmental Protection Agency
LMW	:	Low Molecular Weight
HMW	:	High Molecular Weight
SEM	:	Scanning Electron Microscope
AG	:	Arabian Gulf
PSU	:	Practical Salinity Unit
GC-MS	:	Gas Chromatography-Mass Spectrometry
BH	:	Bushnell Hash
OD	:	Optical Density
Dt	:	Doubling time
CFU	:	Colony Forming Unit
PYR	:	Pyrene
Phen	:	Phenanthrene
Anth	:	Anthracene
Naph	:	Naphthalene
SA	:	Salicylic Acid

ABSTRACT

Full Name : Fitri Budiyanto

Thesis Title : Study of pyrene degradation by halophilic bacteria under hypersaline environment

Major Field : Environmental Science

Date of Degree : January 2016

Contamination of aromatic compounds such as pyrene is becoming a growing worldwide concern since these compounds are recalcitrant to bio-degrade, therefore persist longer in the environment. This situation is even worse when the- contamination occurs in a hypersaline condition. Several technologies have been developed to remove pyrene contamination. These include physical and chemical treatments. Generally, physical treatment is costly, and the chemical approaches lead to the generation of toxic by-product waste. However, biological treatment offers an alternative option since this approach is relatively cheap and environmentally friendly. Overall, the goal of this study was to isolate and characterize halophilic bacteria that can be used in bioremediation strategies to remove pyrene contaminant in the saline environment. This study consisted of isolating halophile bacteria from contaminated areas and evaluating their ability to grow in various conditions of temperature (10, 25, 37 and 50)ºC, pH (3, 5, 7 and 9), salinity (0, 5, 10, 15, 20)% w/v and initial pyrene concentration (1, 5, 50, 100, 1000) mg/l. The ability of these bacteria to grow in presence of other aromatic pollutants (Salicylic acid (one ring)), Naphthalene (2 rings), Phenanthrene and Anthracene (3 rings) was investigated also.

We isolated two bacteria stains in 10% NaCl, *Idiomarina* sp. 10PY1A, and *Halomonas shengliensis* 10PY2B, and one strain in 20% NaCl, *Halomonas smyrnensis* 20PY1A. These three strains could efficiently grow at alkaline conditions but at acidic conditions (pH 3 and pH 5),. The doubling time of *Idiomarina* sp. 10PY1A, *Halomonas shengliensis* 10PY2B and *Halomonas smyrnensis* 20PY1A at pH 7 was 26 hours, 30 hours and 24 hours, respectively, while the doubling time at pH 9 was 25 hours, 41 hours and 20 hours, respectively. Furthermore, no growth for all isolated strains was observed at 50 ºC, and the optimum growth for all isolated strains was observed at 25-37 ºC. Moreover, all isolates grew in all tested salinity except salinity 0%. The optimum salinity for *Idiomarina* sp. 10PY1A, *Halomonas shengliensis* 10PY2B and *Halomonas smyrnensis* 20PY1A was 5%, 10%, and 15%, respectively. The results also show that increase in pyrene concentration was associated with inhibition of growth of 3 strains. On the other hand, during the test of several aromatic compounds as a sole carbon source, the growth rate of all strains correlated negatively with the number of rings of the tested aromatic compounds.

Finally, the fastest degradation rate was observed in by *Halomonas smyrnensis* 20PY1A at 37 ºC and pH 7. The next step of this research work is to test the ability of theses strains to biodegrade pyrene in laboratory pilot scale.

Keywords: hypersaline, halophilic bacteria, pyrene, temperature, pH, salinity.

ملخص الرسالة

الاسم الكامل : فيطري بوديانتو

عنوان الرسالة **:** دراسة تدهور بيرين من قبل البكتيريا المحبة للملوحة تحت البيئات ذات العالية الملوحة

التخصص **:** علوم البيئية

تاريخ الدرجة العلمية : الأول كانون2016/ ديسمبر

لتلوث من المركبات العطرية مثل البيرين أصبح مصدر قلق متزايد في جميع أنحاء العالم لأن هذه المركبات صعبة التتحلل، وبالتالي تستمر لفترة أطول في البيئة .هذا الوضع يكون اسوء عندما يحدث التلوث بهذة المركبات في بيئات وظروف عالية الملوحة .لقد تم تطوير العديد من التقنيات لإزالة التلوث بمركب الببيرين .وتشمل هذه االمعالجات الفيزيائية والكيميائية .بصورة عامة المعالجة الفيزيائية تعتبر مكلفة، اما المعالجة الكيميائية فانها تؤدي إلى توليد النفايات السامة من قبل المركبات الكيميائية المستخدمة. لذا فان المعالجة البيولوجية تقدم خيار بديل لأن هذا النهج هو رخيصة نسبيا وصديقة للبيئة . ان الهدف من هذه الدراسة هو عزل وتوصيف البكتريا المحبة للملوحة والتي يمكن استخدامها في استراتيجيات المعالجة البيولوجية لإزالة التلوث بمركب البيرين في البيئة المالحة .تتألف هذه الدراسة من عزل أصناف من البكتيريا التي لها القدرة على تحمل نسب عالية من الملح ، والتي تم عزلها من المناطق الملوثة وتقييم قدرتها على النمو تحت ظروف مختلفة (10، 25، 37 و 50) Cº، ودرجة الحموضة (3 و 5 و 7و 9)، والملوحة (0، 5، 10، 15 (20٪ ث / ت وتركيز بيرين الأولي (1، 5، 50، 100، 1000) ملغم / لتر .كما تم التحقيق أيضا في قدرة هذه البكتيريا على النمو في وجود ملوثات عطرية اخرى مثل حمض الساليسيليك (1حلقه)، النفثالين (2 حلقات)، فينانثرين وأنثراسين (الحلقة 3) أيضا.

لقد قمنا بعزل نوعين من البكتيريا في 10٪ كلوريد الصوديوم، *Idiomarina sp.* 10PY1A و *Halomonas shengliensi* 10PY2B، وسلالة واحدة في 20٪ كلوريد الصوديوم، *Halomonas. Smyrnensis* 20PY1A وهذه السلالات الثلاث يمكن ان تنمو في ظروف قلوية بصورة افضل ، كما يمكن أن تنمو في الظروف الحمضية،كدرجة الحموضة 3 و 5. أن الوقت المضاعفة *Idiomarina sp* 10PY1A ، *Halomonas shengliensis* 10PY2B *Halomonas. Smyrnensis* 20PY1A في درجة الحموضة 7 هي 26 ساعة، 30 ساعة و 24 ساعة، على التوالي، في حين كان االوقت المضاعفة في درجة الحموضة 9 هي 25 ، 41 و 20 ساعة، على التوالي .وعلاوة على ذلك، لم يلاحظ أي نمو لجميع سلالات معزولة في 50 درجة مئويه . º كما تم اكتشاف ان النمو الأمثل لجميع سلالات المعزولة في 25 º-27 درجة مئوية وان الوقت المضاعف.*Idiomarina sp.* 10PY1A في درجة حرارة 10 و 25 و 37 هو28º ، 24 ساعة، 27 ساعة، على التوالي .اما *Halomonas shengliens* 10PY2B في درجة حرارة 10و 25 و 37 ، فهي º 25 C ساعة، 23 ساعة و 30 ساعة، على التوالي .اما الوقت المضاعفة*Halomonas. Smyrnensis*0 20PY1A في درجة حرارة 10، 25، 37 وكان º 31 ، 29 و 24 ساعة، على التوالي .وعلاوة على ذلك، فان جميع الأنواع المعزولة لم تستطع النمو في الملوحة 0٪ . ان الملوحة المثلى للنمو للانواع *Idiomarina sp.* 10PY1A ، *Halomonas shengliensis* 10PY2B و *Halomonas. Smyrnensi* 20PY1A هي 5٪ و 10٪ و 15٪ على التوالي .كما ان زيادة تركيز البيرين لها تأثير مثبط لجميع السلالات .من ناحية أخرى، وخلال اختبار العديد من المركبات العطرية كمصدر وحيد للكربون،

فإن معدل نمو جميع السلالات المعزولة لديها علاقة سلبية مع عدد الحلقات في المركبات العطرية التي تم اختبارها.
لقد كان أسرع معدل تحلل بواسطة و *Halomonas. Smyrnensi* 20PY1A في 37 ºC ودرجة الحموضة 7.

أن الخطوة التالية والمقترحة لتكمله هذا العمل البحثي هو اختبار قدرة هذه السلالات التي تحلل البيرين من خلال نموذج تجريبي لمفاعل مصغر يتم عملة في المختبر.

كلمات البحث: الملوحة والبكتيريا المحبة للملوحة، بيرين، ودرجة الحرارة، ودرجة الحموضة والملوحة.

CHAPTER 1

INTRODUCTION

1.1 Background overview

Polycyclic Aromatic Hydrocarbons (PAHs) are organic compounds containing two or more fused aromatic rings, and their recalcitrant presence in an environment leads for toxic, mutagenic and carcinogenic cases for living organism [1]. This pollutant performs photo-mutagenicity, a process allowing these PAHs to be more reactive and more hazardous under sunlight radiation [2]. PAHs can be classified as persistent pollutants staying longer in an environment due to their hydrophobic character and less relativity (and reduced reactivity) [3]. The source of PAHs can be natural sources such as oil seepage, volcano eruption and fire forest or from anthropogenic sources. An anthropogenic source like incomplete combustion of hydrocarbons (oil, wood), waste and biomass become a major source of PAH in an environment [4]. United State Environmental Protection Agency (USEPA) classifies 16 PAHs in the list of hazardous pollutants: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene and indeno [2, 5, 6].

PAHs can be classified into two groups according to their molecular weight: Low Molecular Weight (LMW) with 2 or 3 fused aromatic rings and High Molecular Weight (HMW) with 4-6 fused aromatic rings [7]. PAHs with LMW such as anthracene, phenanthrene, and naphthalene are more soluble in water than non-aromatic fractions of organic compounds like alkanes and can be easily degraded. However, PAHs with HMW such as benzo(a)pyrene and benzo(a)anthracene take more effort to be degraded by microorganisms [8]. Furthermore, PAHs genotoxicity escalates as the molecular size of PAH increases [9]. Moreover, the presence of PAHs has been influenced by both biotic and abiotic factor like chemical processes, bioaccumulation, and transformation by microbes (called biodegradation) [10]; this biodegradation process has been used to remedy the waste containing PAHs. Bioremediation is a process in which organic pollutant like PAHs is consumed by organisms as a carbon source however the mechanism of microbes to be able to degrade toxic compound remains unclear [11].

The archaea [11] and fungi [12] are common among microbes utilized for biodegradation. The gram-negative bacteria from genus: *Flavobacterium*, *Pseudomonas*, *Bukholderia*, and *Sphingomonas* or gram positive bacteria of genus: *Bacillus*, *Mycobacterium*, and *Rhodococcus* are commonly used for PAHs biodegradation [13, 14]. Those strains of bacteria were isolated from a non-saline soil sample. Unfortunately, the challenge of these PAHs' decontamination processes increases by the presence of salt. Non-halophilic bacteria are not able to live in the hypersaline condition [15]. Hypersalinity leads to hyperosmotic conditions promoting plasmolysis and cell dehydration. Also, these conditions inhibit enzyme activity decreasing respiratory, growth, and reproduction rate of bacteria [16].

Hypersaline environments featured by high salt concentrations of > 35 g/L [17] is characterized by its distinctive biodiversity and physical-chemical properties [18–23]. This environment is constructed by two factors: natural forces inducing high salt conditions like the emersion in the Dead Sea [24–26] and human-made manipulation [27]. The examples of human activities releasing hypersaline water are desalination plant and oil production [21, 22]. Desalination plant produces brine in which the salt concentration reaches 2.5 times higher than the sea water salinity [30]. Hypersaline water also naturally co-exists in petroleum and natural gas reservoir and mostly becomes a by-product of these exploration activities [31].

Produced water in the oil production process varied for each well. In the oil production, produced water is considerably large compared with the oil itself and the aged well is recorded to have remarkable water: oil ratio. The water to oil ratio is estimated at 3:1 (average) or at around 250 million barrel of produced water each day compared to 80 million barrel of oil produced per day. Regrettably, 5% of this hypersaline water is generally discarded into surface water and the others are re-injected to the production process [32]. Oil production by Kingdom of Saudi Arabia in 2015 reached 10.19 million barrel per day [33]. That means over 30 thousand barrels of produced water are released in Saudi Arabia in each day from this activity only. This produced water is prosperous of hydrophobic organic compounds such as PAHs.

1.2 Problem statement

Since the kingdom has run the oil industry, the pollution of organic compounds is unavoidable. The demand of the kingdom for a green method that scientifically proven in

reducing organic pollutants is substantial [34]. Massive technologies to encounter the pollution are installed throughout the industrial areas in the Kingdom [35]. Such pollutants in hypersaline conditions, mostly from produced water during oil exploration, grants distinguished challenge in the treatment. Chemical compounds inside this produced water like PAHs render a hazard to the environment [36]. Physical and chemical treatment offer successful outcome in reducing PAHs, however, an expensive capital for operation and technology become inevitable drawback [37, 38]. The biological agent provides a rewarding alternative by declining cost and minimizing waste [15, 39].

A hypersaline condition delivers an obstacle for a microorganism to live. Only specific bacteria exist in such condition. The unique biodiversity of microbes living in the hypersaline environment, called halophile, give distinctive approach on biodegradation process. Yet, the study of halophilic bacteria in the kingdom is limited especially in its application in dealing with PAHs pollution. This research aims at studying biodegradation of pyrene, an HMW PAH. In many types of research, pyrene becomes a model for studying HMW PAHs due to its close character with other carcinogenic HMW PAHs [40]. In addition, the selection of pyrene as an object of study falls to the presence of pyrene in water produced from the oil industry.

1.3 Objectives

The main objective of this research is to find halophilic bacteria that have the ability to degrade pyrene under hypersaline environment. Some specific objectives have been proposed to fulfill the main objectives which are to:

1. Isolate a strain of bacteria from petroleum contaminated soil samples that have the ability to degrade pyrene at high salinity medium
2. Identify isolated halophile bacteria species using 16S RNA sequences and construct the bacteria Phylogenic tree.
3. Characterize the morphology of bacteria species using light microscopy and Scanning Electron Microscope (SEM).
4. Study the effect of various conditions: temperature (10, 25, 37 and 50 ^{o}C), pH (3, 5, 7, and 9), salinity (0, 5, 10, 15, and 20 % NaCl), initial pyrene concentration (1, 5, 50, 100, and 1000 ppm) on the ability of isolated strains to degrade pyrene also to quantify pyrene concentration during biodegradation process using GC. Also, to test the ability of strains to grow in different aromatic compounds (salicylic acid, naphthalene, phenanthrene, anthracene).

CHAPTER 2

LITERATURE REVIEW

2.1 Arabian Gulf: A potential habitat to discover halophilic bacteria degrading organic compound

Arabian Gulf (AG) is a semi-closed water, an extension of Indian Ocean in an arid region [41]. The location is challenging AG with high evaporation rate, low precipitation, and low freshwater intake, leading AG to have high salinity [42]. The evaporation rate is exceeding 2 m/year with a precipitation rate of 0.15 m/year and river discharge of 0.15-0.19 m/year [43]. The highest evaporation rate develops during summer especially near coastal zone and the northern part of AG [44]. The real measurement of salinity is exceeding 40 psu, higher than average global sea water salinity which is about 35 psu [45] and the salinity of the southern part of AG was reported to be up to 70 psu [46]. Another unique character of AG is the salinity, which differs between summer and winter (Figure 1).

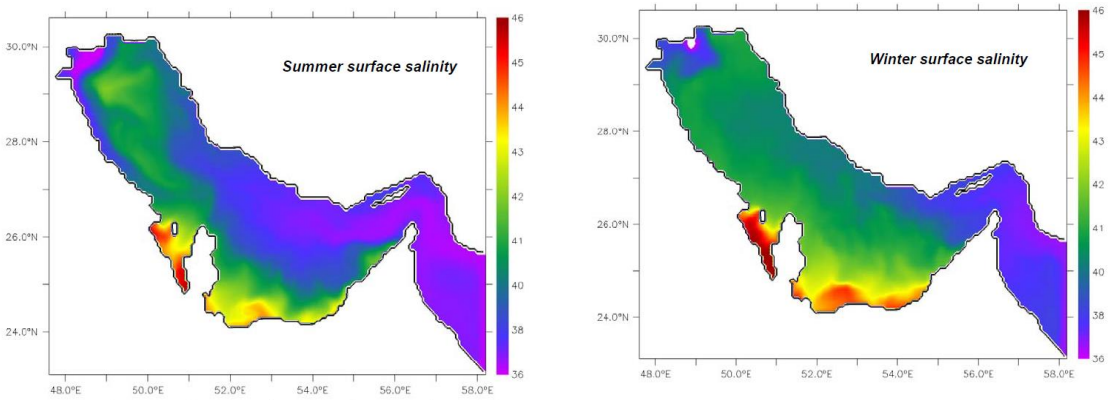


Figure 1. The salinity of seawater in AG during summer and winter [47]

Many research efforts have been conducted in AG as a naturally stressful area [48–51]. Many anthropogenic activities potentially release pollutant into AG. Industrial waste, domestic waste, mining, smelting, wastewater discharge, shipping activities become major sources of anthropogenic pollution [52], yet the oil spill in 1991 deeply spoiled the gulf [53]. The oil spill in 1991 is considered as the largest oil spill in the history with 11 million barrel of oil were intentionally released into AG from its source in Kuwait [54]. Half of this spill was drifted into an open sea and the rest was retarded, moreover, it took more than half a year for this oil to disappear. The retarded part interfered to 600 km southward to Saudi coastal line with difference consequences [55]. Only, 1,163,000 barrels of oil could be recovered from water surface behind Abu Ali Island, North of Jubail, Saudi Arabia and required about $210-450 million just for cleaning up [54]. Mortality and devastation of biodiversity by 50% to 100% was reported [56]. This oil took a long period of recovery, even for years the oil still remain in sediment and the adverse effects occur [57–59].

The natural or anthropogenic pressure in AG inflicts selection to organisms. Microbes may sustain in this harsh environment. The pressure of organic pollutant and high salinity converge some bacteria which sustain in a high saline environment. Thukair *et al.* [60] reported that *cyanobacteria* can live in a mat at extreme of different temperatures, salinity and desiccation periods. Furthermore, a previous study successfully isolated the oil-degraded halophilic, *Halomonas aquamarina* and *Alteromonas macleodii* from Kuwait coast [61].

2.2 Produced water: One of hypersaline source containing organic pollutants

Produced water as a side product of oil and gas industry contains a mixture of organic and inorganic compounds. These compounds are classified in five categories: dissolved and dispersed oil compounds (contains PAHs), dissolved minerals, production chemical compounds (antifoam, emulsion beaker), producing solid (bacteria, waxes), and dissolved gas (CO_2, H_2S). BTEX (benzene, toluene, ethyl benzene, xylenes), MTBE (methyl *tert*-butyl ether). The aromatic part of dissolved oil which is toxic to an environment cannot be removed completely by oil/water separation techniques. Produced water also contains inorganic compounds like anion, cation and heavy metal, which are hazardous to organisms. In addition, some chemicals which are injected into the well to support oil extraction like linear alkyl benzene sulfonate (LAS) and 2-alkyl-1-[N-ethylalkylamide]-2-imdazolines are released to an environment by produced water's stream. Since the oil production extracted materials from the ground reservoir, solid compartments such as bacteria, waxes, and asphaltenes are lifted to the surface and thus released to the environment via produced water's stream [62].

Several techniques have been established to treat produced water prior to its discharge to the environment. Physical, chemical and biological processes have been adopted as basic principles of produced water treatment [62]. Adsorption [63] and filtration have been well adapted to physical treatment for produced water. Coagulant [64], resin, zeolite, modified zeolite, organo-clay, activated sludge, carbonaceous substances, and carbon active, are adsorbents that widely use to remove organic

compounds in produced water [65–72]. Electrochemical technology [73], electrodialysis, carbon aerogel-based capacitive deionization technology, macro porous polymer extraction, nanofiltration and reverse osmosis membrane have been developed [74–78]. However, these technologies faced several problems such as they are able to remove certain types of compounds only, like ions or metals. In addition, the performance of instrument has been influenced by high salinity, temperature, pH and the concentration of solid material. Beside, membrane and nano-filter technologies are expensive. Another difficulty in these techniques is the generation of chemical waste after use [62]. Biological treatment offers solutions to minimize the waste of the process [79–81]. The produced water is purposing to be conveniently treated by natural agents like bacteria. Biological activities are capable of treating produced water, however, for normal bacteria, the efficiency reduced when salinity increased [82].

2.3 Pyrene: A persistent Polycyclic Aromatic Hydrocarbon

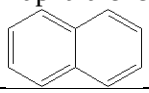
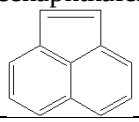
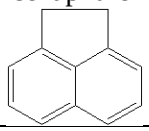
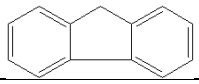
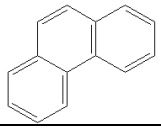
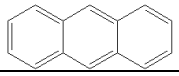
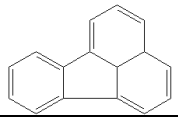
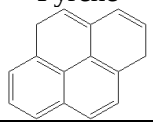
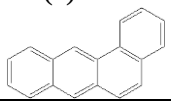
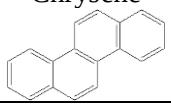
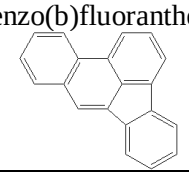
Polycyclic aromatic hydrocarbons (PAHs) are organic compound generated from incomplete oxidation or high-pressure processes of complex organic substances. Mostly, PAHs are insoluble in water nevertheless sparingly soluble in organic solvent. In addition, PAHs show low volatility promoting these substances to be a solid at room temperature. Due to their low solubility in water, PAHs concentrations in water are considered low and tend to be accumulated in sediment. PAHs become environment and health concern regarding their toxicity effect on organisms including human. The major

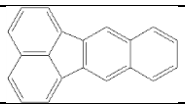
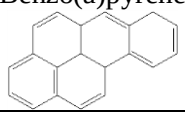
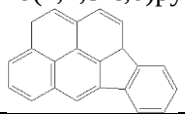
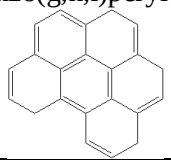
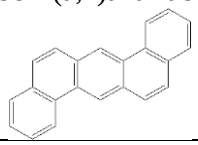
factor controlling PAHs toxicity is the formation of reactive metabolites, thus different type of PAHs exhibit different toxicity [83].

The PAHs' physicochemical properties and toxicity rank are summarized in Table 1. The potency of PAHs compound to be degraded by natural agents like microorganisms has been shown to be affected by the molecular weight of the compound itself. There is an evidence of a significantly positive correlation between molecular weight and the half-life of PAHs compounds in an environment [84]. Pyrene, a PAH-containing four fused benzene rings, has a molecular weight of 202.2506 g/mol and solubility of about 0.135 mg/L in water making this compound less soluble than LMW PAHs [85]. USEPA reported that there were no enough evidence to classify pyrene as carcinogen substance [86]. However, several regulations were established regarding pyrene concentration in drinking water: 210 µg/L (Florida); 200 µg/L (Minnesota); 250 µg/L (Wisconsin) [85].

Pyrene is released into an environment as a waste from an incomplete combustion of organic complexes, cigarette smoke, coal, oil and wood-burning stove. In the atmosphere, pyrene can be found as vapor and particulate phase. Vapor-phase pyrene can be degraded by the photochemical reaction and show half-life for 8 hours. The particulate-phase of pyrene is removed from the atmosphere by wet and dry deposition. Pyrene is well absorbed in soil and the biodegradation rate is considered slow with half-life reaching up to several years. Pyrene can be found in particulate material and sediment in the water column [85].

Table 1. The physical character, carcinogenic potency and toxicity rank of 16 PAHs as USEPA's list for organic hazardous pollutant [5]

types of PAHs	MW [(b)]	Solubility [(c)]	Classification	Toxicity rank
Naphthalene 	128	31.7	USEPA : D IARC :3	80
Acenaphthalene 	152	16.1	USEPA : D IARC :N/A	N/A
Acenaphthene 	154	3.9	USEPA: N/A IARC : N/A	168
Fluorene 	166	1.9	USEPA: N/A IARC: 3	N/A
Phenanthrene 	178	1.15	USEPA: D IARC : 3	246
Anthracene 	178	0.043 to 0.075	USEPA: D IARC : 3	N/A
Fluoranthene 	202	2.6×10^{-1}	USEPA: D IARC : 3	138
Pyrene 	202	0.135	USEPA: D IARC : 3	254
Benzo(a)anthracene 	228	9.4×10^{-3}	USEPA: B2 IARC : 2A	37
Chrysene 	228	2.0×10^{-3} to 6.3×10^{-3}	USEPA: B2 IARC : 3	141
Benzo(b)fluoranthene 	252	1.5×10^{-3}	USEPA: B2 IARC : 2B	10
Benzo(k)fluoranthene	252	8.0×10^{-4}	USEPA: B2	61

			IARC : 2B	
Benzo(a)pyrene 	252	1.6×10^{-3}	USEPA: B2 IARC : 2A	8
Indeno(1,2,3-c,d)pyrene 	276	2.2×10^{-5}	USEPA: B2 IARC : 2B	174
Benzo(g,h,i)perylene 	276	2.6×10^{-5}	USEPA: D IARC : 3	N/A
Dibenz(a,h)anthracene 	278	2.49×10^{-3}	USEPA: B2 IARC : 2A	15

[(b)] Molecular weight

[(c)] Aqueous solubility at room temperature (mg/L)

USEPA, U.S. Environmental Protection Agency:

B2 : probable human carcinogen;

D : not classifiable.

IARC, International Agency for Research on Cancer:

2A : Probable human carcinogen;

2B : Possible Human Carcinogen;

3 : Not classifiable

N/A : data not available

[a] reference [31] and [32]

2.4 Halophilic Bacteria

2.4.1 Phylogenic tree of halophilic bacteria

Microorganisms can be grouped by the requirement of salt concentration as: non-halophiles (less than 0.2 M NaCl); halotolerant or slight halophiles (0.2-0.5 M NaCl); moderate halophile (0.5-2.5 M NaCl); and extreme halophile (2.5-5.5 M NaCl) [88]. The term halophilic belongs to all microorganisms that sustain and live under saline

environment [89] including Bacteria, Archaea, and Eukaryote (Figure 2). This term is also becoming new taxa and a new name in the microbial taxonomy [90]. The order of *Halobacteriales,* in the domain of Archaea, holds one family, *Halobacteriaceae* consisting all gram-negative halophilic organism with 36 genera and 129 species [91–93]. The discovery of this first halophilic organism under *halobacteriaceae* was over 100 years ago by the study of the red, purple and pink color of the aquatic environment [94].

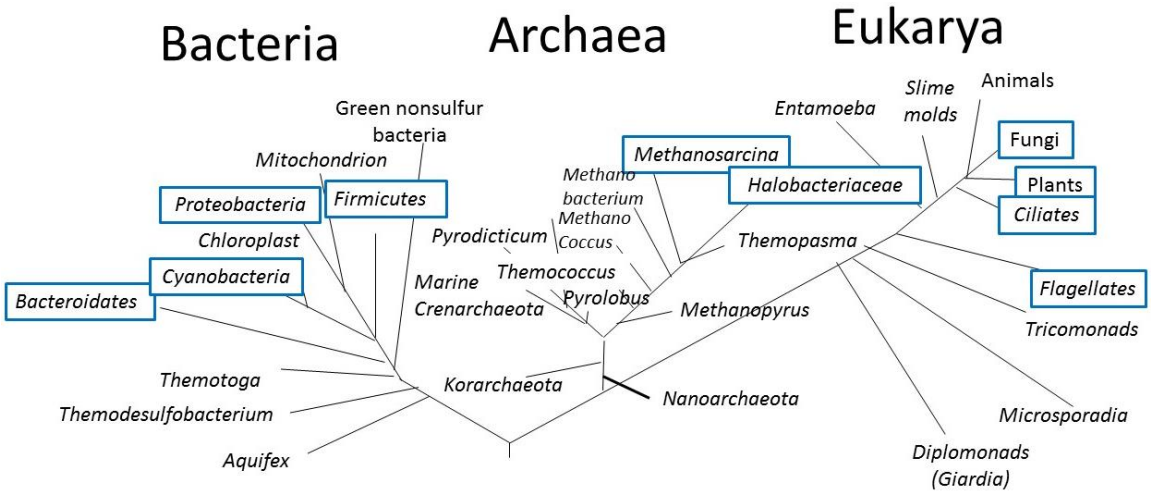


Figure 2. Phylogeny tree based on rRNA analysis for the distribution of the halophilic organism. The box indicates the groups having at least one representative of the halophilic organism [95]**.**

The class of *Gammaproteobacteria* is the richest class among phylum Proteobacteria, consist of more than 250 genera. This class is very important especially in medical and health issues due to some pathogens belong to this class such as *Escherichia*, *Vibrio*, *Salmonella*, *Yersinia* and *Pseudomonas* [96-97]. *Gammaproteobacteria* was divided into 10 orders: *Enterobacteriales*, *Pasteurellales*, *Vibrionales*, *Alteromonadales*, *Pseudomonadales*, *Oceanospirillales*, *Thiotrichales*, *Legionellales*, *Chomatiales*, *Xanthomonadales* [96].

Most of the halophilic bacteria are aerobic or methanogenic, however, fermentative halophiles are very rare, consist of only 25 species [98]. The fermentative halophiles have been found only in Phylum of Firmicutes, a class of Clostridia, the order of Halanaerobiales. The fermentative halophiles are used in catalysis of lactose in bioprocess industry [98].

The biodegradation of PAHs by aerobic bacteria involves monooxygenase or dioxygenase by adding atomic oxygen into the aromatic ring and transform the ring to central intermediates like catechol and protocatechuate. The intermediates then are cleaved by ortho- or meta- dioxygenases such as Catechol 2,3-dioxygenase to make intermediary metabolites like acetyl Co-A, succinyl Co-A, and pyruvate to be fed to Krebs cycle [99]. The degradation of pyrene by *Mycobacterium. Vanbalenii* PYR-1 involves 27 enzymes to transform pyrene to acetyl Co-A and succinyl Co-A: 14 enzymes are responsible for cleaving pyrene to a phthalate, 6 responsible for degrading phthalate to protocatechuate and 7 enzymes to transform protocatechuate to acetyl Co-A and succinyl Co-A [100].

2.4.2 Morphology and Physiology of Halophiles

The extreme halophiles are very diverse in morphology: flat, discs (*Halococccus*), triangles (*Haloarcula*), rectangles (*Halobacterium*), rods or even cocci; also some of them are immotile either gram positive or gram negative [101]. The halophiles which belong to halobacteriaceae are unique based on their dependence of salt, all of the species in this family cannot survive in fresh water [91]. Halophilic bacteria possess unique characters enabling them to survive in the high concentrations of salt. First, halophilic bacteria exhibit highly negative surface charge of halophile proteins. This condition

allows them to be more flexible at high concentrations of salt in which non-halophile bacteria become an aggregate and stiff. Second, the activity and stability of most halophile's enzyme positively correlate with salt concentration [88].

Moreover, halophiles adapted two osmoregulation strategies to be able to live in saline condition: first is ion regulation in which it accumulates ion (K^+, Na^+, Cl^-) from surrounding environment, the second is accumulation or synthesizing specific organic osmolytes (amino acid and sugar) within its cytoplasm. Halophilic bacteria regulate their inner Na^+ concentration thru Na^+/H^+ antiporters and they preserve the balance between inside and outside salt concentration by maintaining K^+ influx [88]. Also, halophilic bacteria are able to accumulate solute from their environment or produce those solute such as sugars, amino acids, glycine betaine, trehalose, and ectoine [102]. These two strategies are to maintain the osmotic pressure between inside and outside of the cell [103]. The second type of strategy by accumulating organic osmolytes is mainly used by most of the halophiles due to the less adaptation of organelles of the cell to non-organic ion and high salt concentration [103].

2.4.3 The Novel Industrial Application of Halophiles

Halophiles become prevalent agents in industries as its survival in the saline environment. The utilization of these halophiles spread from energy issue, bacteria by-product industry until environmental issue (Table 2). The choice mostly falls to the cost efficiency in bioprocess. Within an industry utilizing bioprocess as the main core, the contamination becomes a great threat and non-economical procedure often applies to encounter the contamination. However, the contamination is scant in the very selective environment like hypersaline condition. Halophiles which sustain in hypersaline

condition become the only choice to make bioprocess industry compete with chemical industry [98, 104].

The adaptability of halophiles in hypersaline environment frequently stimulates them to excrete biochemical such as enzyme, bio-surfactant or bio-polymer [105–110]. For example, the strain of *Marinobacter* sp. GA CAS9 was used to synthesize alkaline protease [111]. Moreover, the biomass from halophile can be treated as a raw material in the industry of energy alternative [112]. The coming prospect of halophile is the utilization in environmental issues. Many studies proofed the ability of halophiles in degrading pollutants [16, 113, 114], especially organic compounds such as PAHs. Piubeli *et al.* [115] reported the ability of *Halomonas* sp. to reduce phenol, benzoic acid, and para-hydroxybenzoic acid and the capability of the strain to degrade those pollutants enhanced with the addition of nitrogen, phosphorus, and other carbon sources.

Opposing the limitation study of pyrene degradation by halophilic bacteria, numerous studies have been done to understand another type of bacteria to degrade pyrene in non-saline condition [116]–[123]. *Mycobacterium* sp. strain A1-PYR, *Bacilus subtilis, Mycobacterium vanbaaleni,* and soil bacteria *Acinetobacter* strain USTB-X were able to grow in pyrene [124–127]. The family *Halomonadaceae* of order *Oceanospirillales* [128] and family *Idiomarinaceae* of order *Alteromodales* [129] are also utilized in environmental issue.

Table 2. The application of halophiles in many industrial sectors

Application	Product	Strain	NaCl	Reference
Bio-surfactant production		*Marispirillum indicum, Brevibacterium* sp., *Modicisalibacter*	6-20%	[130]

		tunisiensis, Halomonas shengliensis, Idiomarina sp., *Marinobacter flavimaris, Brevibacterium* sp., *Dietzia* sp., *Idiomarina zobelii*		
	Rhamnolipidic bio-surfactant	*Pseudomonas stutzeri*	5%	[105]
Production of bio-polymer	Polyhyxyalkanoate (PHA)	*Pseudomonas, Hydrogenovora, Hydrogenophaga*	-	[106]
	Poly (3-hydroxyalkanoic acid)	*Halomonas salina, Halomonas maura, Haomonas marina*	5%	[107]
Food fermentation	fermented fish, meat, fruit, vegetable	*Halobacterium, Halococcus, Bacillus*	-	[98]
	Fermented shrimp	*Halalkalicoccus jeotgali*	At least 10%	[131]
Enzyme production	Protease	*Marinobacter* sp. GA CAS9 *Halobacterium* sp. LBU 50301	25%	[108], [111]
	Amylase	*Amphibacillus* sp.	25%	[109]
	Proteinase	*Tetragenococcus halophilus*	0-25%	[110]
Environmental	Denitrification	*Vibrio diabolicus*	3%	[113]
	Nutrient removal	*Zunongwangia profunda*	4-10%	[114]
	Dye degradation	*Halomonas* sp.		[16]
	Phenol degradation	*Acidobacterium* sp. *Chloroflexus* sp.	10-17%	[132]
	Many organic compunds	*Halomonas organivorans*	7.5-10%	[133]
	Phenol degradation	*Haloarcula*	20%	[134]
	COD and phenol reduction	*Halomonas* sp.	10%	[115]
	Benzene degradation	*Arhodomonas* sp. *Strain Seminole, Arhodomonas* sp. *Strain Rozel*	3-18%	[135]

2.5 Biodegradation of pyrene

Studies of the biodegradation of pyrene by halophilic bacteria are demanding additional efforts because of the limitation of information, especially in the Kingdom of Saudi Arabia (Table 3). Most of the study of halophilic bacteria in biodegradation of pyrene is limited to aerobic group belonging to gram-negative halophilic bacteria of a family of *Halobacteriaceae* [1, 32]. Furthermore, the study of pyrene degradation in the Kingdom of Saudi Arabia by halophilic bacteria is very limited. Two publications by Life Science Department, King Fahd University of Petroleum and Minerals on pyrene degradation were found, however, these studies were on non-halophilic bacteria [121, 136].

Erdogmus et al. [1] studied the isolates of *Halorubacterium piscisalsi*, *Halorubrum ezzemoulense*, *Halobacterium salinarum*, *Haloarcula hispanica*, *Haloferax* sp., *Halorubrum* sp., and *Haloarcula* sp., and found that they were capable of degrading pyrene at 160 ppm. However, none of these isolates could survive at 200 ppm of pyrene. In addition, these isolates showed promising outcome in degrading other aromatic compounds, such as *p*-hydroxy-benzoic acid, naphthalene, and phenanthrene evident by the growth in these compounds at concentration 200 ppm of concentration [1]. The difficulty in degrading pyrene when used as the only carbon source was also illustrated (pyrene above certain concentration also inhibits growth) [1].

The growth of bacteria can be enhanced by adding supplementary compounds like yeast extract and compost. Bonfá *et al.* [32] observed the enhanced growth of *Haloferax alexandrius* strain KCTC 12962 and *Haloferax* sp. CS1-9 in the mixture of pyrene, naphthalene, anthracene, phenanthrene, benzo(a)anthracene with an addition of yeast

extract. Arulazhagan and Basudevan [9] reported the difference of degradability of pyrene under consortium and single strain treatment. The consortium of moderately halophilic bacteria like *Ochobactrum* sp., *Enterobacter cloacae*, *Stenotrophomonas maltophilia* gave good results for pyrene, benzo(a)pyrene and LMW PAHs degradation. This study also described the limitation of pyrene degradation: the initial concentration of pyrene. The degradability of pyrene is stranded by the increase of initial pyrene concentration. Moreover, *in situ* experiment by Sharma *et al.* [14] in soil contaminated sites explained the half-life of fluorine, phenanthrene, anthracene, and pyrene decreased by the addition of compost to soil site.

Table 3. The application of microbes either halophiles or non-halophiles in degrading PAHs

Origin of sample	**NaCl (%)**	**PAHs**	**Bacteria**	**Ref**
Halophilic bacteria				
brine	20	**pyrene**, phenanthrene, naphthalene, p-hydroxybenzoic acid	*Haloferax* sp. *Halobacterium piscicalsi* *Halorubrum ezzemoulense* *Halorubrum* sp. *Halobacterium salinarum* *Haloarcula* sp.	[1]
water, sediment	20	**pyrene**, naphthalene, anthracene, phenanthrene, benzo(a)anthracene	*Haloferax Alexandrinus* *Haloferax Prahovense* *Haloferax sulfurifontis*	[32]
soil	0.5-15	**pyrene**, phenanthrene, naphthalene, anthracene, benzo(a)pyrene	*Martelella*	[137]
crude oil contaminated soil	2-12	**pyrene**, anthracene, naphthalene, phenanthrene	*Pseudomonas* sp.	[138]
water	3	phenanthrene, fluorene,	*Ochrobactrum;* *Enterobacter cloacae,*	[9]

		benzo(a)pyrene	*Stenotraphomonas maltophilia*	
sea water	3-9	phenanthrene, benzo(a)pyrene	*Achromobacter;* *Marinobacter,* *Rhodanobacter*	[3]
soil	0.1-15	anthracene	*Martelella*	[31]
sediment	20	p-hydroxy benzoic acid	*Haloferax* sp.	[139]
water	22.5	phenanthrene, dibenzothiophene, anthracene	*Haloarcula argentinensi* *Haloferax volcanii* *Haloferax alexandrinus*	[140]
sediment	0-20	phenanthrene naphthalene anthracene	*Marinobacter nanhaiticus* sp. Nov.	[141]
sediment	6- 23	crude oil, phenanthrene, octadecane	*Open circles Haloferax* sp. *Closed circles Haloferax* sp. *open triangles Halobacterium* sp. *closed triangles Halococcus*	[11]
None (no information about) halophilic bacteria				
petroleum refinery waste	-	fluorine, phenanthrene, **pyrene**, chrysene	*Arthrobacter* sp. UG50	[116]
brackish water	-	**pyrene** phenanthrene	*Stenotrophomonas acidaminiphila*	[117]
sediment, water	-	Phenanthrene naphthalene	*Vibrio parahaemolyticus*	[118]
-	-	naphthalene	*Marinobacter* strain NCE312	[119]
soil	-	mixed PAHs	*Pseudomonas* strain MTS-1 *Stenotrophonas* strain MTS-2 *Agrobacterium* strain MTS-4	[120]

			Trabulsiella strain MTS-6 *Cupriavidus* strain MTS-7 *Pseudumonas* strain KC3 *Bacillus* strain KC5	
-	-	**pyrene**	*Mycobacterium vanbaalenii*	[125]
oil contaminated site	-	**pyrene**	*Burkholderia fungorum* *Caulobacter* sp. T2A12002	[121]
soil	-	**pyrene** naphthalene fluorine phenanthrene,	*Acinobacter* strain USTB-X	[127]
soil	-	**pyrene** benzo(a)pyrene	*Bacillus subtilis*	[126]
sediment	-	**pyrene**	*Sphingomona* *Pseudomonas* *Sphingobium*	[122]
sediment		**pyrene** phenanthrene fluoranthrene	*Sphingomonas* *Mycobacterium*	[123]

2.6 The limitation of pyrene degradation by Halophilic bacteria

The challenge of degrading pyrene is increasing by the presence of salt in the pyrene-containing environment. At least two major factors deeply influence the success of degrading pyrene in an environment by halophilic. The first factor is the availability of oxygen [13, 137]. In aerobic biodegradation process, less oxygen concentration in sediment bed reduces the biodegradation rate of bacteria [13]. However, in microcosm study, pyrene degradation could be done by *Pseudomonas floresens* under anaerobic condition even the rate was slower than aerobic condition [143]. The second factor that influences degradation of pyrene is the less bioavailability of HMW PAHs in the presence of salt. Chemically, the HMW PAHs are sparingly soluble in water. Hence, the presence of salt in hypersaline condition increases the insolubility, called “salting out” phenomena, making the HMW PAHs even less soluble in water and thus tend to

precipitate in solid form [144]. This very low solubility of this compound is addressing impediment of a biological agent for getting access to carbon source and degrading the compound [145].

Several preferences have been addressed to overcome this obstacle, thus restricting the growth of bacteria, for example, the use of consortium rather than a single strain bacterium. Many studies reported the superiority of consortium by synergistic effect among strains over a single strain bacterium in degrading pyrene. Sharma et al. described that 7-28% of pyrene could be degraded within 7 days by *S. marcescens* L-11, *S. rochei* PAH-13, *P. chrysosporium* VV-18, acted as a single strain, however, these three strains acted as a concert were capable of eliminating 39.2% of pyrene [14]. Hence, the consortium of *Pseudomonas* strain MTS-1, *Stenotrophomonas* strain MTS-2, *Agrobacterium* strain MTS-4, *Trabulsiella* strain MTS-6, *Cupriavidus* strain MTS-7, *Pseudomonas* strain KC3 and *Bacillus* strain KC5 was able to degrade pyrene 18-40% greater and faster than those strains acted as a single bacterium [120]. Another approach to overcoming the slow growth of bacteria in an elevated concentration of salt is the addition of surfactant to reduce the hydrophobicity. Some halophilic bacteria produce biosurfactant during its utilization of PAHs or other hydrocarbons. *Shewanella alga, Thalassospira* sp., *Idiomarina* sp., [146] and *Bacillus licheniformis* [147] produced biosurfactant during its growth under the presence of hydrocarbon. The extracellular surfactant produced by bacteria increases the formation of droplet affecting the stability of the water-hydrocarbon emulsion, therefore, improving the mass transfer between hydrocarbon and water. The ultimate result is the intensification of bacteria uptake over

hydrocarbon [148]. The process could be manipulated by adding the synthetic surfactant, however, some synthetic surfactant may be toxic to bacteria [149].

CHAPTER 3

MATERIAL AND METHODS

In order to meet the objectives of the proposed work, the following methodology was performed. The first step was to enrich halophilic bacteria by culturing petroleum-contaminated soil samples in the presence of pyrene as a sole source of carbon. This isolation was carried out in high salinity (10, 20 and 25 % w/v of NaCl) in a minimum mineral medium. The isolated strain was identified by either morphology or molecular analysis. Thereafter, the ability of each strain in biodegrading pyrene would be investigated by observing the growth and residual concentration of pyrene. The detailed methodology is summarized in Figure 3.

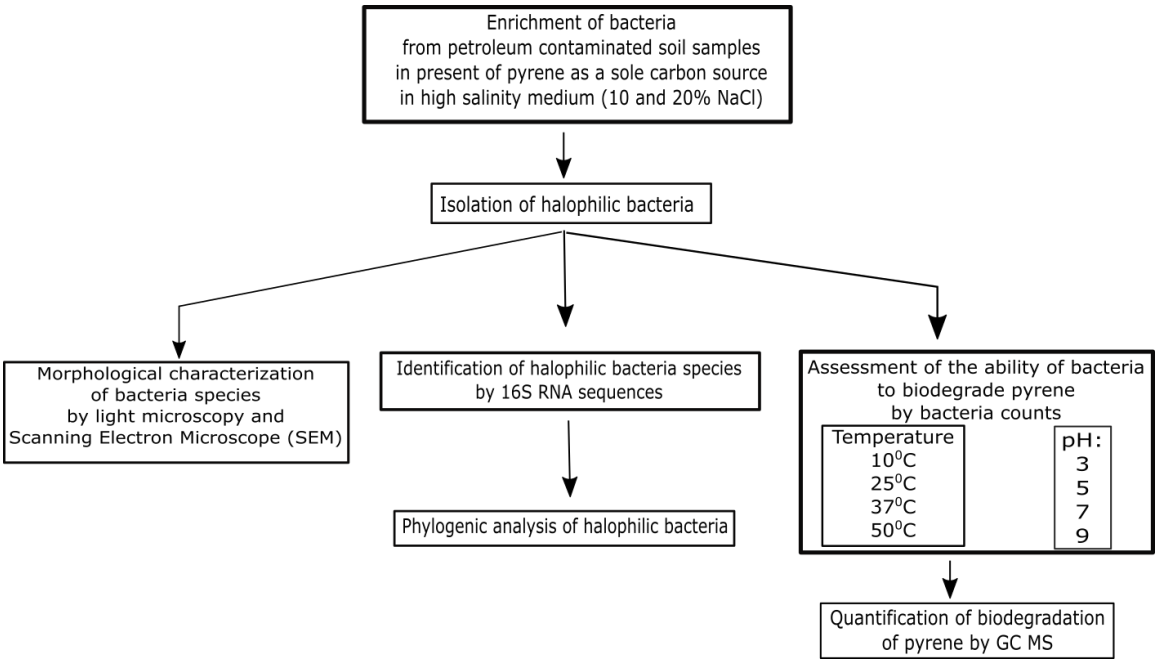


Figure 3. The performed methodology in this experiment

3.1 The Origin of sample

The petroleum contaminated soil sample was collected from Jubail area within the geographical position of 27°06′46.53″ N 49°22′24.53″ E (Figure 4).

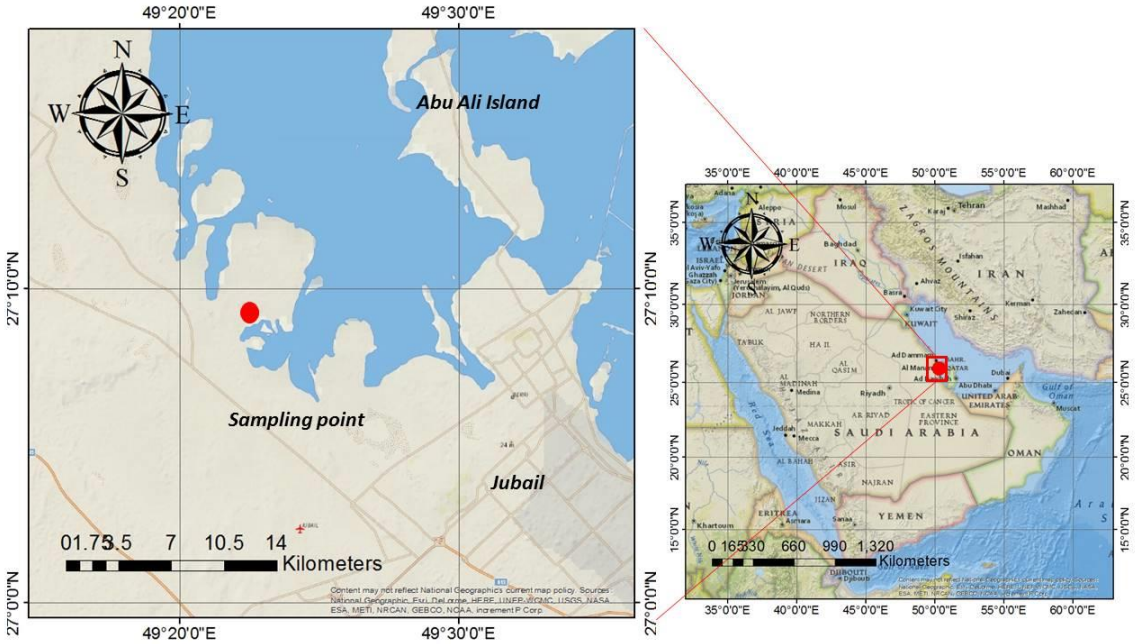


Figure 4. The sampling point of petroleum contaminated soil sample.

3.2 Preparation of the media

Bushnell-Haas medium was prepared for enrichment and isolation of halophilic bacteria capable of degrading pyrene [150]. Bushnell-Haas (BH) is a common medium, minimum mineral medium, used for microbiological degradation of hydrocarbon. The composition of BH was as follows: 0.2 g/L magnesium sulphate ($MgSO_4$), 0.02 g/L calcium chloride ($CaCl_2$), 1 g/L monopotassium phosphate (KH_2PO_4), 1 g/L dipotassium phosphate (K_2HPO_4), 1 g/L ammonium nitrate (NH_4NO_3), 0.05 g/L ferric chloride ($FeCl_3$). During the enrichment, the salt concentration would be varied at 10%, and 20% of NaCl. The media was autoclaved for 4 hours at 121ºC for sterilization prior to being used.

3.3 Enrichment and isolation of bacteria

The enrichment and isolation of halophiles used in this study were developed from Gomes *et al.* [130]. An aerobic enrichment culture was prepared in volumetric flask contained with 0.1% (w/v) pyrene as a carbon source in 100 mL BH media. 1 gram samples were added into media in the proportion of 1:100 (w/v) and the salinity was varied by 10, 20 and 25% of NaCl. Cultures were incubated in aerobic condition at 37 ºC on a rotary shaker at 100 rpm for 21 days. The sample underwent 3 times of transfers prior the isolation of bacteria onto the solid medium. During the transfer, the growth of bacteria was measured by bacteria Optical Density methods (OD_{600}=1). The isolation was conducted by streaking the culture onto solid media in the similar composition. The plates then were kept at 37ºC until the growth of bacteria was observed. The certain colony of bacteria was transferred to another pyrene + BH solid medium then the solid medium was incubated until the growth. This process in transferring single colony was repeated until the single colony observed, at least 5 times of transfer during this study. The last step of isolation was cryopreserved those single colony on solid media.

3.4 Morphological characterization of isolates

3.4.1 **Morphological characterization of isolates by light microscopy**

Colony shape was analyzed using light-microscope. This step involved culture preparation followed by microscopy procedure [127]. First, the isolated strains were streaked onto nutrient agar plates. Then the agars were incubated within 48 hours at 37ºC. Second, after the growth was observed, colonies' form, elevation, and margin were inspected under dissecting microscope. Third, the cellular identification was conducted

after heat fixing of isolates on glass slides using Zeiss Imager D2 compound light microscope equipped with AxioCam MRc camera and Axio Vision software.

3.4.2 **Morphological characterization of isolates by Electron Microscopy**

The morphological characterization of isolates was performed using Scanning Electron Microscope JEOL at accelerating voltage of 10 keV. The preparation of samples for electron microscopy utilized fixation and dehydration principal. The isolated strains used for identification were wet-mount cultures after 48 hours incubation at 37 ºC. The isolates were prepared by fixation onto glass slides utilizing 2.5% of formaldehyde [151, 152]. The sample was harvested directly from an agar plate, flooding the surface of agar plate by 2.5% formaldehyde in distilled water. The mixture of the sample then underwent dilution of 100 fold. Thereafter, a drop of the sample with formaldehyde-fixed onto slide's surface, the isolates were dehydrated by series of ethanol concentration (10, 25, 50, 70, 96 and 100%). Then the gold-coated sample was placed onto aluminum foil disk and was examined under SEM under dried air condition[152–154].

3.5 Molecular identification of isolates

The molecular identification procedure was adopted from Nzila *et al.* [155]. The 16S rRNA analysis was performed by the company Royal Life Science, Pvt. Ltd., Secunderabab, India. The main process of characterization of bacteria involves two main steps, first is DNA extraction and second is RNA amplification. The sample in agar media was prepared wet-mount to be sent to a company.

The amplification of 16S rRNA gene utilized the obtained-DNA supernatant. 2 primers were used in polymerase chain reaction for amplification of 16S rRNA gene:

Primer 27F (AGAGTTTGATCMTGGCTCAG), and 1492R (TACGGYTACCTTGTTACGACTT). The following were the procedure of PCR program: 35 amplification cycles at 94ºC for 45 sec, 55ºC for 60 sec and 72ºC for 60 sec. The generated 16S rRNA gene then was sequenced using Big Dey terminator circle sequencing kit (Applied BioSystems, USA) utilizing two primers: 518F(CCAGCAGCCGCGGTAATACG) and 800R (TACCAGGGTATCTAATCC) then the sequence was encoded by an automated DNA sequencing system (Applied BioSystems model 3730XL, USA). Afterward, The BLASTIN facility of the National Center for Biotechnology Information was used to identify the sequence of 16S rRNA (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [153, 96].

3.6 Phylogenic analysis of bacteria isolates

The construction of phylogenic tree was performed using MEGA7.0.14 software. The sequence of 16S rRNA was aligned using ClustalW [156], then the strength of the relationship was evaluated by boot-strap analysis using 1000 replicates of the neighbour-joining data for tree topography. The evolutionary distance was computed using the Kitamura 2-parameter method [148], [150]–[152].

3.7 The observation of strains growth under the presence of pyrene

The ability of isolated strains to grow in the presence of pyrene was assessed using bacteria count methods every 3 days. In brief, the isolated strain of halophilic bacteria from the BH agar was transferred into the mix solution of 2.5 mg of pyrene in 50 mL BH media. Then, the growth of bacteria was observed [150]. The salt concentration of media was varied by 10% and 20%. The temperature was varied by 10, 25, 37, and 50ºC. The

addition of pyrene was varied by 0.05, 0.25, 2.5, 5, 50 mg per 50 mL of BH media. The standardization of initial bacteria was performed by initial OD (1 mL of culture) of 0.8-1 (around 10^7 CFU/mL). The growth of bacteria was observed over 18 days in the presence of 2.5 mg pyrene in 50 mL of BH medium as initial concentration and the growth will be observed by bacteria count (modified from [32]). Pyrene as carbon source was dissolved with dimethyl sulfoxide prior the addition into medium and the dimethyl sulfoxide was evaporated prior the addition of culture. Non-inoculated controls were prepared and treated under the same conditions. In extended experiment, salinity was varied by 0, 5, 10, 15, 20% w/v NaCl. The other aromatic compounds were tested also: Salicylic acid (1 ring); Naphthalene (2 rings); Phenanthrene (3 rings), Anthracene (3 rings). During these 2 experiments, the temperature and pH were adjusted to 37 ºC and 7, respectively.

3.8 Quantification of pyrene biodegradation by halophilic bacteria

The quantification of residual pyrene concentration during degradation process by halophilic bacteria was executed using liquid-liquid extraction followed by the GC-MS procedure. The experiments were carried out within the respective salinity: The strains isolated in 10% salinity were tested in 10% of salinity and the strain isolated in 20% salinity was incubated in medium with 20% of salinity. The experiments were conducted in a standard condition in which the pH of media was adjusted at 7±0.1 and temperature was set at 37±0.1ºC. The duplication of the experiment was undertaken to ensure precision of the experiment. The control (non-inoculated sample) was passing through the similar protocol.

A set of experiment was prepared for 18 days period of incubation and every 3 days the sample was extracted. Consequently, 7 flasks were prepared for samples with

inoculum and 7 flasks were prepared for abiotic control. For each flask, 0.5% (w/v) of pyrene (in dimethyl sulfoxide) was added into 49 mL of BH medium to make a final solution containing 2.5 mg of pyrene per 50 mL of BH medium. Dimethyl sulfoxide is capable to dissolved pyrene [160] and decreases the surface tension of dissolved pyrene enhancing the bioavailability [161]. For sample flasks, 1 ml of isolates with OD of 0.8-1 was added into prepared BH media. Within the scheduled period of incubation, the respective sample was extracted thru 30 minutes of sonication followed by 5 minutes of liquid-liquid extraction using acetyl acetate (GC grade). 1.5 mL of extract was experienced room-temperature evaporation. The remaining chemical was re-dissolved using chloroform (GC grade) prior injection into GC-MS.

The last step is the injection of samples into GC-MS. Gas Chromatography-Mass Spectrometry (GC-MS) was used to measure the pyrene concentration with the following GC Temperature program:

1. Initial temperature: 55 ºC for 15 minutes
2. Ramp 1: temperature increase of 6 ºC/min until 250 ºC within 10 minutes
3. Ramp 2: holding temperature at 290 ºC for 15 minutes

During the analysis, the mass spectrum of compounds would be detected by mass spectrometer sensor and the chromatogram was build. The mass spectrum of pyrene was detected after the retention time of 47.59 minutes. The peak area from the chromatogram was analyzed to calculate the pyrene concentration. The control would be analyzing in the same protocol.

3.9 Data analysis

All growth profiles were drawn using origin® 2016 with $\log_{10}$ scale for the Y-axis. The statistical analysis (ANOVA) was carried out using MINITAB 17®. Furthermore, to analyze the growth rate, the doubling time of isolated strains was computed for each experimental data. This doubling time was computed using growth rate constant. The growth rate constant was obtained by fitting the data to the best prediction of exponential growth (generated by excel software). Adopted from Nzila *et al.*[162], the growth rate constant and doubling time were formulated as follow:

$$\boldsymbol{Q_t = Q_0 e^{kt}} \quad \textbf{(1)}$$

$$\boldsymbol{db = \frac{Ln\,(2)}{k}} \quad \textbf{(2)}$$

Qt and Q_0 are bacterial countings at time t and initial bacteria count (in CFU/ml), k is the growth rate constant and t is time. db is doubling time which is negatively correlated with bacteria growth. Values for dt were plotted using origin® 2016.

On the other hand, the degradation rate of pyrene was formulated as follow [163]:

$$\boldsymbol{C_t = C_0 e^{-kt}} \quad \textbf{(3)}$$

Which: Ct and C_0 are pyrene concentration at time t and initial pyrene concentration (in ppm or ppm) while k is the degradation rate constant (in day^{-1}).

CHAPTER 4

RESULTS

4.1 Isolation of consortia by enrichment

The Consortia of halophilic bacteria capable of degrading pyrene was selected by enrichment at salinity 10%, 20%, and 25% NaCl w/v, using petroleum contaminated soil samples. Incubation of liquid media containing soil sample at the aforementioned salinity in presence of pyrene (0.1 %, w/v) for 21 days resulted in OD of approximately 1, indicating the presence and growth of bacteria. Two consortia were isolated, one corresponding to the culture at 10%, 10ConsA, and another at 20% NaCl, and the consortium was named 20 ConsB. On the other hand, the culture medium at 25% NaCl did not yield any bacteria. The further step of the investigation was to isolate individual colonies present in these consortia.

4.2 The isolation of individual strains or colonies

The colonies isolation was carried out by streaking the consortia on agar solid media followed by incubation. The solid media consisted of 0.1% (w/v) pyrene in BH agar containing the respective NaCl concentrations. The streaking of consortia on agar plate led to the growth of several colonies. The morphological analysis of each solid plate led to the isolation of the 2 individual strains from 10ConsA and one single strain from 20 ConsB. To ascertain the purity of these strains, each of them was transferred to agar plates and cultured, and this process was repeated 5 times. 10PY1A and 10PY2B; were the strains from 10ConsA, and the strain 20PY1A was identified from 20 ConsB.

4.3 Colony Characterization

These three strains were then characterized by light microscopy and electron microscopy. As indicated in Table 4, 10PY1A has a circular form, a flat elevation, and an entire margin, with the size of 3.5-4.5 µm in length and 0.4-0.72 µm in width; while 10PY2B was punctiform (form), convex (elevation) and entire margin. Its size was 0.79-1.29 µm in length and 0.57-0.71 µm in width. The morphological characteristics of 20PY1A are identical to 10PY1A (circular, flat, entire) and its size was 1.17-1.75 µm in length and 0.67-0.83 µm in width. All the 3 strains were gram negative, and 10PY1A and 20PY1A are rods, while 10PY2B is a short rod bacterium. The detailed morphological structures based on light and electron microscopes are shown in micrographs in Figure 5, Figure 6 and Figure 7.

Table 4. The morphological observation of isolated strains

Salinity (wt/V)	**Isolate**	**Gram staining**	**Light microscopy**			**SEM**
			Form	Elevation	Margin	(L × W) µm
10%	10 PY 1A	Negative	Circular	Flat	Entire	4.07 × 0.52
	10 PY 2B	Negative	Punctiform	Convex	Entire	0.96 × 0.64
20%	20 PY 1A	Negative	Circular	Flat	Entire	1.53 × 0.75

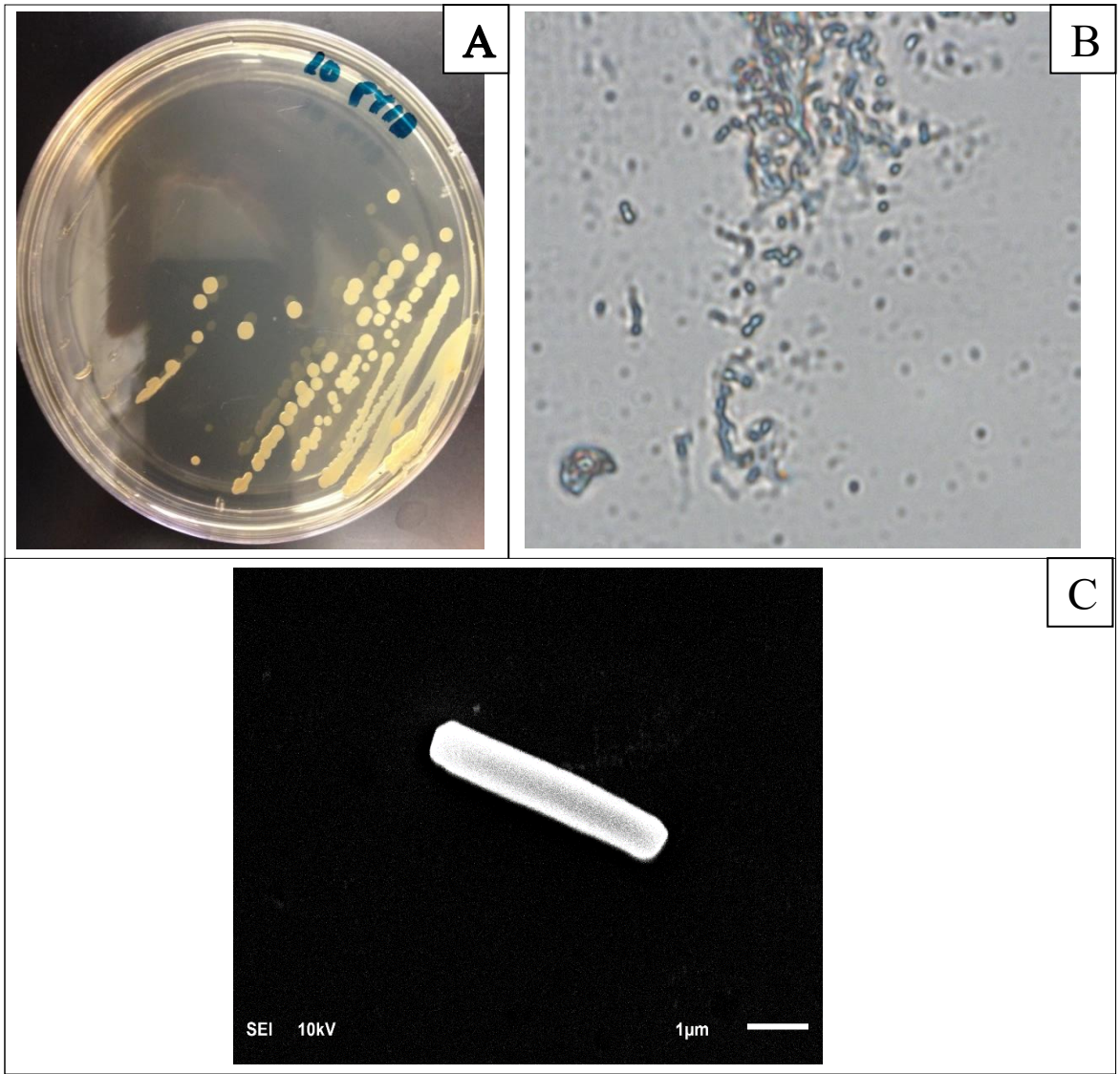


Figure 5. The Morphological observation of isolated strain 10PY1A: A) Colony form of isolate; B) micrograph of light microscopy based on ×100 magnification, C) micrograph of Scanning Electron Microscope

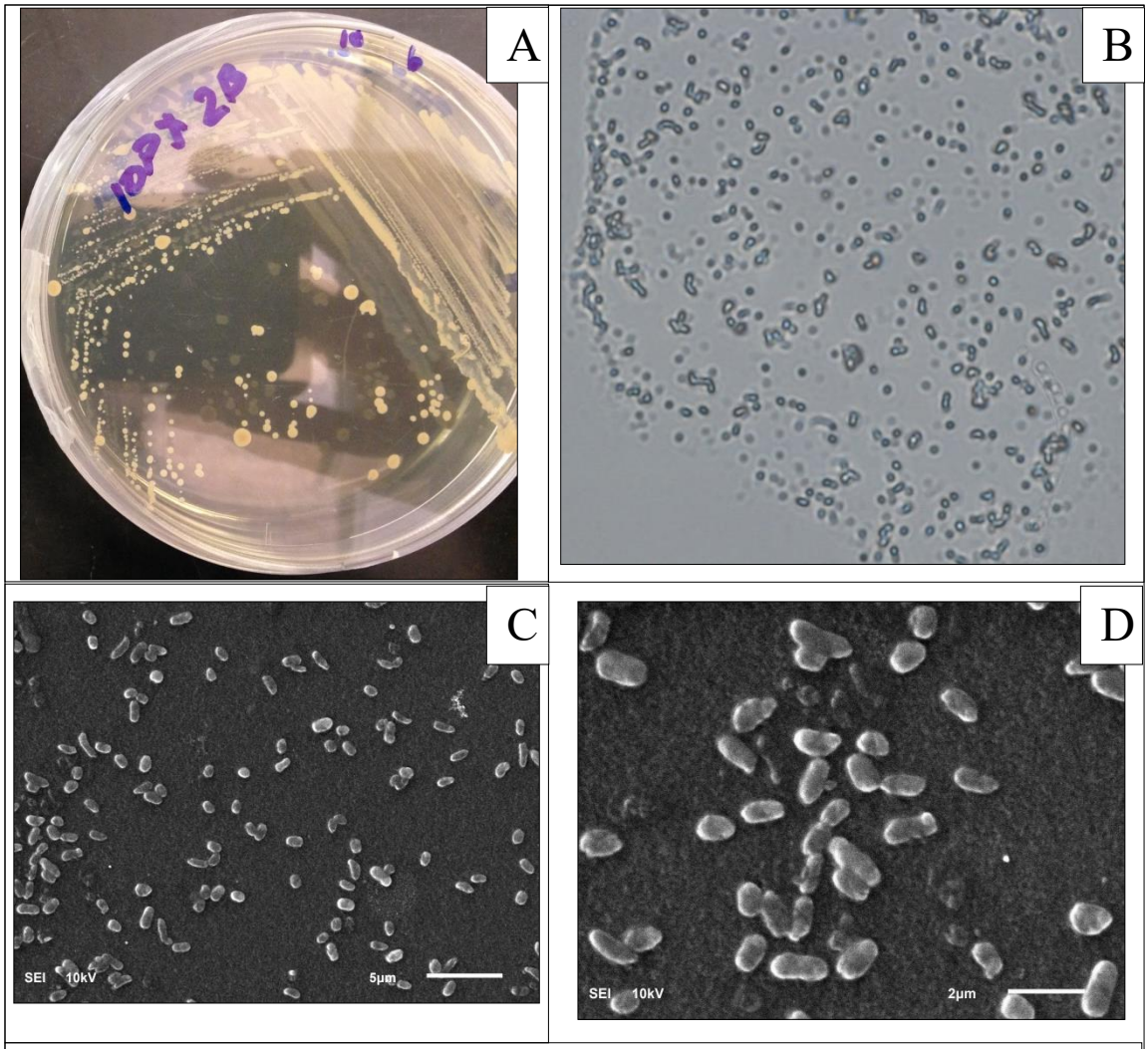


Figure 6. The morphological observation of isolated strain 10 PY2B: a) Colony form of the isolate, B) micrograph of light microscope based on × 100 magnification, C) and D) micrographs of Scanning Electron Microscope.

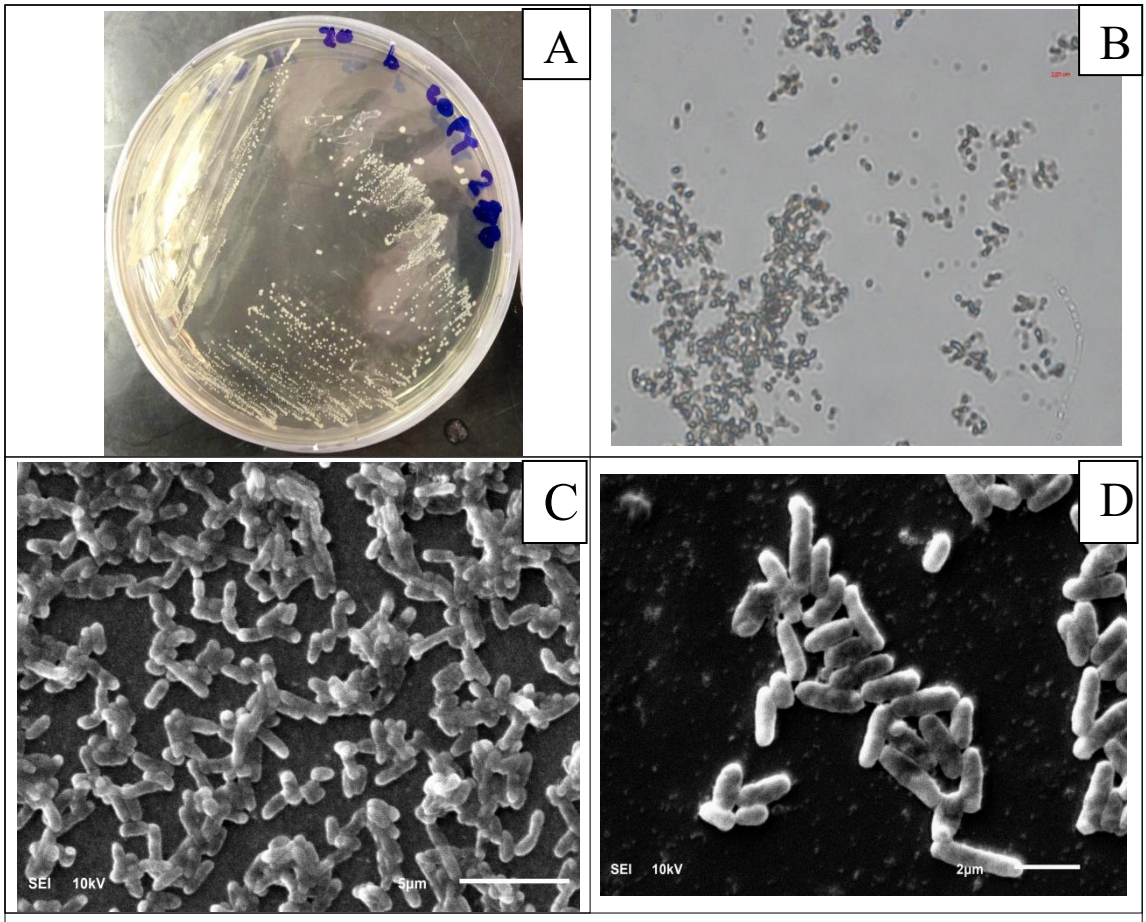


Figure 7. The morphological characterization of isolated strain 20PY1A: A) Colony form of isolate, B) micrograph of light microscope based on ×100 magnification; C) and D) micrographs of Scanning Electron Microscope

4.4 Species identification by molecular techniques

4.4.1 **Bacteria preparation**

Each strain of bacteria, 10PY1A, 10PY2B, 20PY1A, was prepared in nutrient media for rRNA sequence analysis. Prior, each strain was cultured in huge amount (100 mL) of nutrient media in respected salinity then the cell was harvested. These harvested cells were sent for analysis of 16s rRNA analysis.

4.4.2 **16s rRNA sequence and species identification**

The molecular identification was performed by 16s ribosomal RNA, partial sequence, and this sequence was compared to the available sequences in the database of the nucleotide sequence (National Center for Biotechnology Information, NCBI). The name of species was proposed based on 99% homology, and if this homology was less than 99%, thus the species could not be defined.

The analysis of 16S rRNA of 10PY1A showed the higher homology of 98% was reported as *Idiomarina* strains. This strain could not be ascribed a species, thus it was named *Idiomarina* sp. On the other hands, strain 10PY2B was identified as *Halomonas shengliensis* and 20PY1A as *Halomonas smyrnensis* since they share 99% of homology with these species. These three strains belong to the class of Gammaproteobacteria (Table 5). The access numbers of the 16S rRNA sequences of these 3 strains in the NCBI database are provided in Table 5.

-Table 5. **The BLAST analysis of 16s rRNA sequence for each isolate**

Strain	similarity	Description	Accession
10PY1A	99%	*Idiomarina* sp.	KU308250.1
10PY2B	99%	*Halomonas shengliensis*	KU308251.1
20PY1A	99%	*Halomonas smyrnensis*	KU308252.1

4.4.3 **Phylogeny tree**

Using the rRNA sequence of isolated strains and rRNA sequence from NCBI database, the phylogeny tree had been drawn using MEGA® 7 software. Prior, the rRNA sequence of isolated strains was aligned using Clustal W from the same software. Then, the phylogeny tree was developed to understand the evolutionary history of strains. In brief overview, the isolated strain 10PY1A was not genetically related to strains 10PY2B and 20PY1A (Figure 8). From this phylogeny tree, it was confirmed that strain 10PY1A was related to *Idiomarina* sp. as well as the *Idiomarina piscisalsi*. Furthermore, this phylogeny tree analysis also confirmed that the strains 10PY2B and 20PY1A are members of genus *Halomonas.* Isolate 10PY2B was *Halomonas shengliensis* and isolate 20PY1A was *Halomonas smyrnensis* (Figure 9).

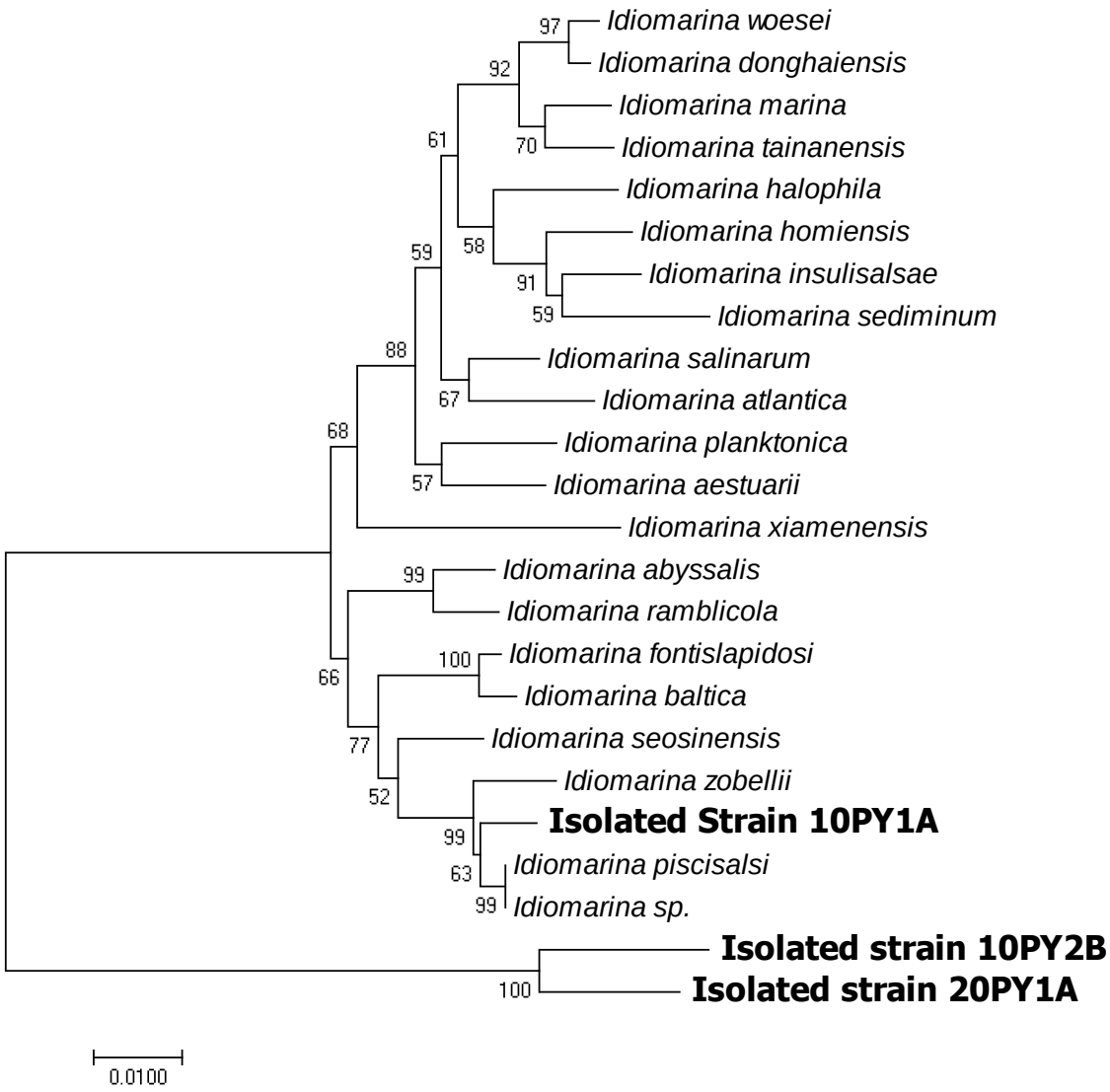


Figure 8. Phylogeny tree of isolate 10PY1A: MEGA 7 software analysis

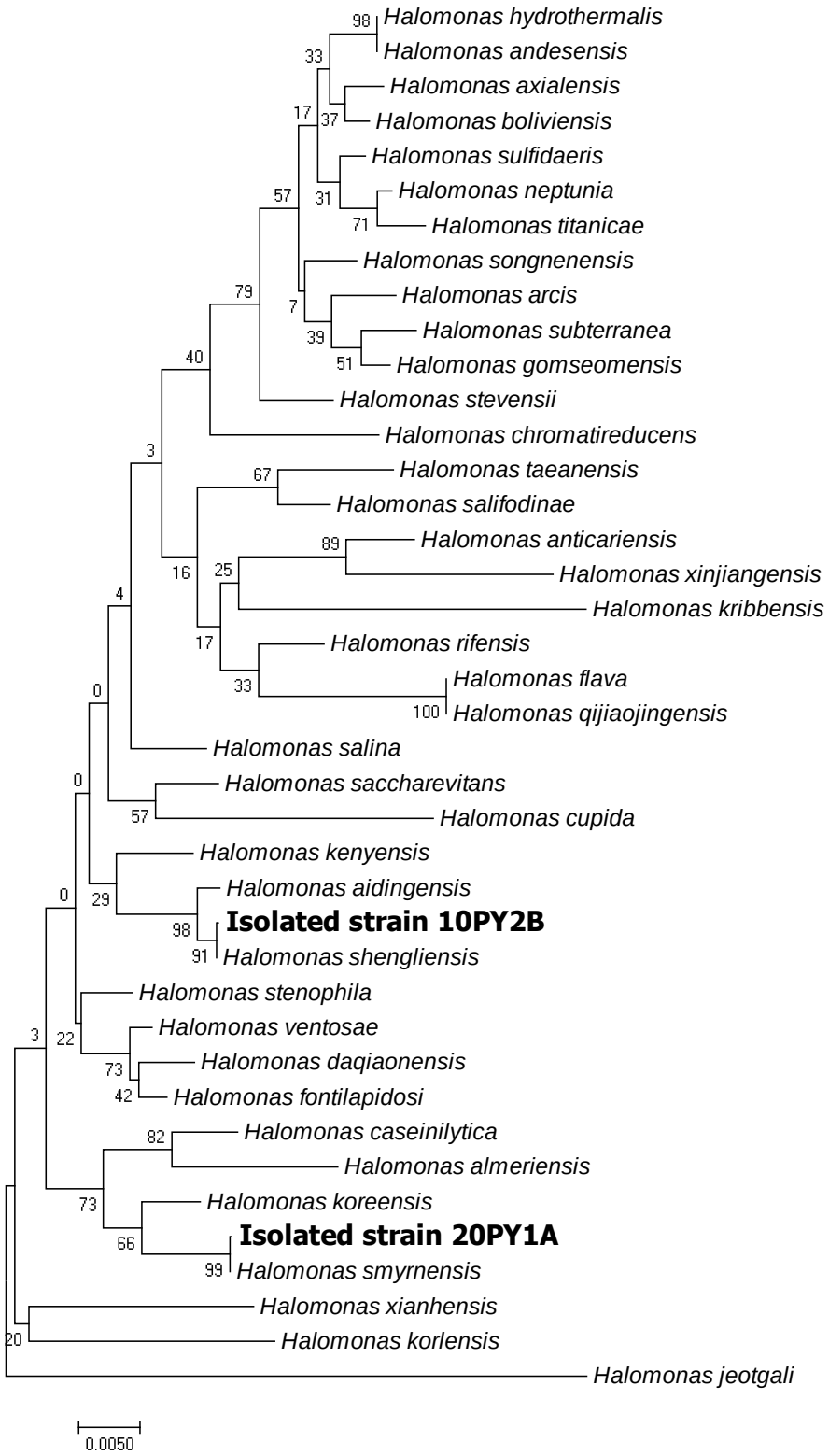


Figure 9. Phylogeny tree of isolate 10PY2B and 20PY1A: MEGA 7 software analysis

4.5 The growth of isolates under different environmental conditions

4.5.1 Standard conditions of growth

In view of further characterizing these strains, their growth profile was first investigated in the normal conditions (37 ºC, and pH 7, and salinity 10% NaCl for *Idiomarina piscisalci* 10PY1A and *Halomonas shengliensis* 10PY2B, and 20% NaCl for *Halomonas smyrnensis* 20PY1A). All cultures were carried out in presence of 2.5 mg of pyrene in 50 mL BH media.

The cultures were initiated with 2×10^6 CFU/mL bacterial counts, and the growth was monitored every 3 days by bacterial count. The results are summarized in Figure 10. Overall, these strains reached the maximum growth within 6-9 days, with maximum count falling between $2\text{-}8\times10^8$ CFU/mL. The growth profile indicated no significant difference among strains ($p>0.05$).

The population of strain *Idiomarina* sp. 10PY1A increased to 566×10^6 CFU/mL after 9 days then decreased to 39×10^6 CFU/mL at day 18. Starting with 2.6×10^6 CFU/mL, the population of *Halomonas shengliensis* 10PY2B grew to 257×10^6 CFU/mL then reduced to about 50×10^6 CFU/mL after 18 days. On the other hand, the population of *Halomonas smyrnensis* 20PY1A reached to 740×10^6 CFU/mL at day 9. However, the population drastically decreased to 4×10^6 CFU/mL after 18 days (Figure 10).

The computation of doubling time (dt) revealed the difference of growth among strains ($p<0.05$), *Halomonas smyrnensis* 20PY1A was the fastest growing strain with a dt value at 24 hours. *Idiomarina* sp. 10PY1A was the second fastest growing isolate (dt= 26 hours), and the slowest was *Halomonas shengliensis* 10PY2B (dt = 30 hours) (Figure 11).

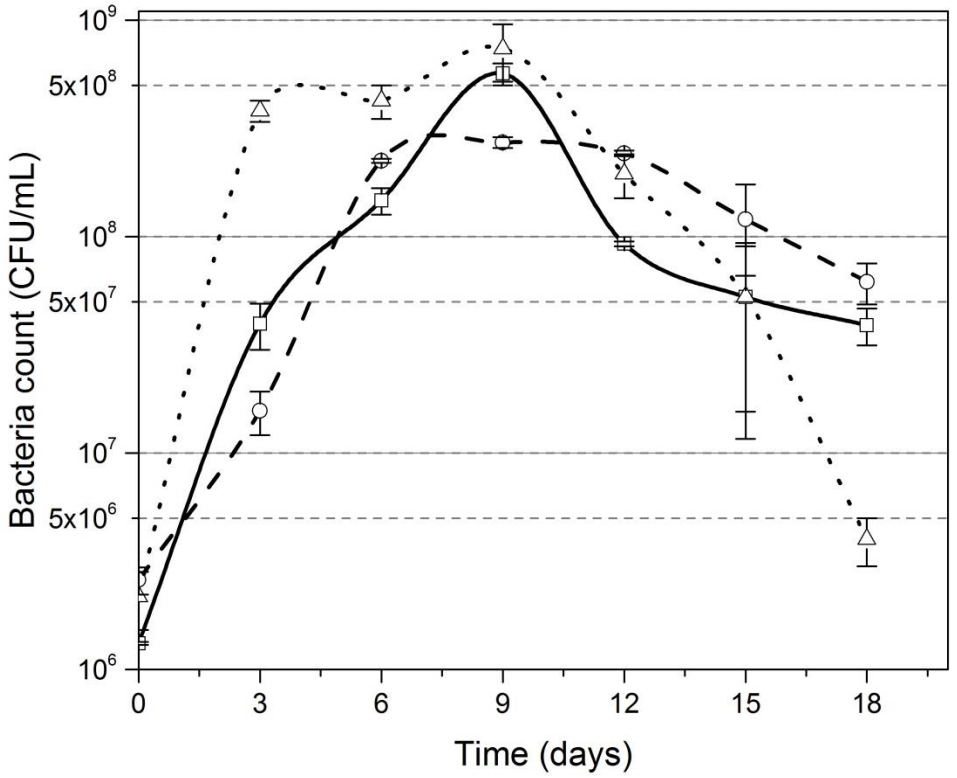


Figure 10. The growth profile of isolates: *Idiomarina* sp. 10PY1A (—□—); *Halomonas shengliensis* 10PY2B (- -○- -); *Halomonas smyrnensis* 20PY1A (· ·△· ·). The Y axis is presented in anti-Log base 10.

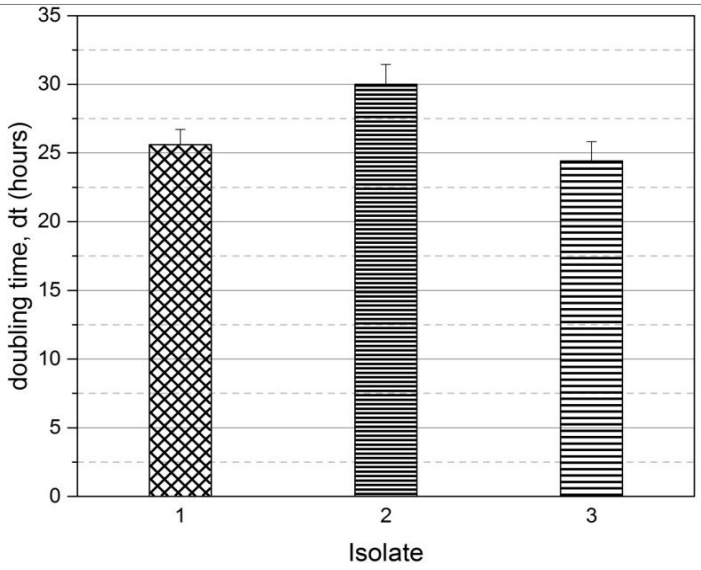


Figure 11. The doubling time of isolates at 37 ºC, pH 7: (1) *Idiomarina* sp. 10PY1A; (2) *Halomonas shengliensis* 10PY2B; (3) *Halomonas smyrnensis* 20PY1A

4.5.2 **Investigation of the optimum conditions of growth**

To identify the optimum conditions of growth of *Idiomarina* sp. 10PY1A, *Halomonas shengliensis* 10PY2B and *Halomonas smyrnensis* 20PY1A, several conditions were tested: pH (3, 5, 7 and 9); Temperature (10, 25, 37 and 50 ºC), Salinity (0%, 5%, 10%, 15% and 20%) and amount of pyrene added (0.05, 0.25, 2.5, 5 and 50 mg).

4.5.2.1 The effect of pH

***Idiomarina* sp. 10PY1A**

Starting with 1.3-2×10^6 CFU/mL, the growth of the *Idiomarina* sp. 10PY1A increased to a maximum count within 9 days at pH 7 and pH 9, and the growth profiles between these pHs were not significantly different ($p>0.05$). Within 9 days, the population reached 5.66×10^8 CFU/mL at pH 7 and rose to 5.6×10^8 CFU/mL bacterial counting at pH 9. Opposite, no growth was observed at pH 3 and pH 5. Using computation of doubling time (dt), the growth at pH 9 was faster than that at pH 7 since dt was 25 hours for pH 9 and 26 hours for pH 7. Hence, computation of dt was not carried out at pH 3 and pH 5 because no growth observed at these pH (figure 15).

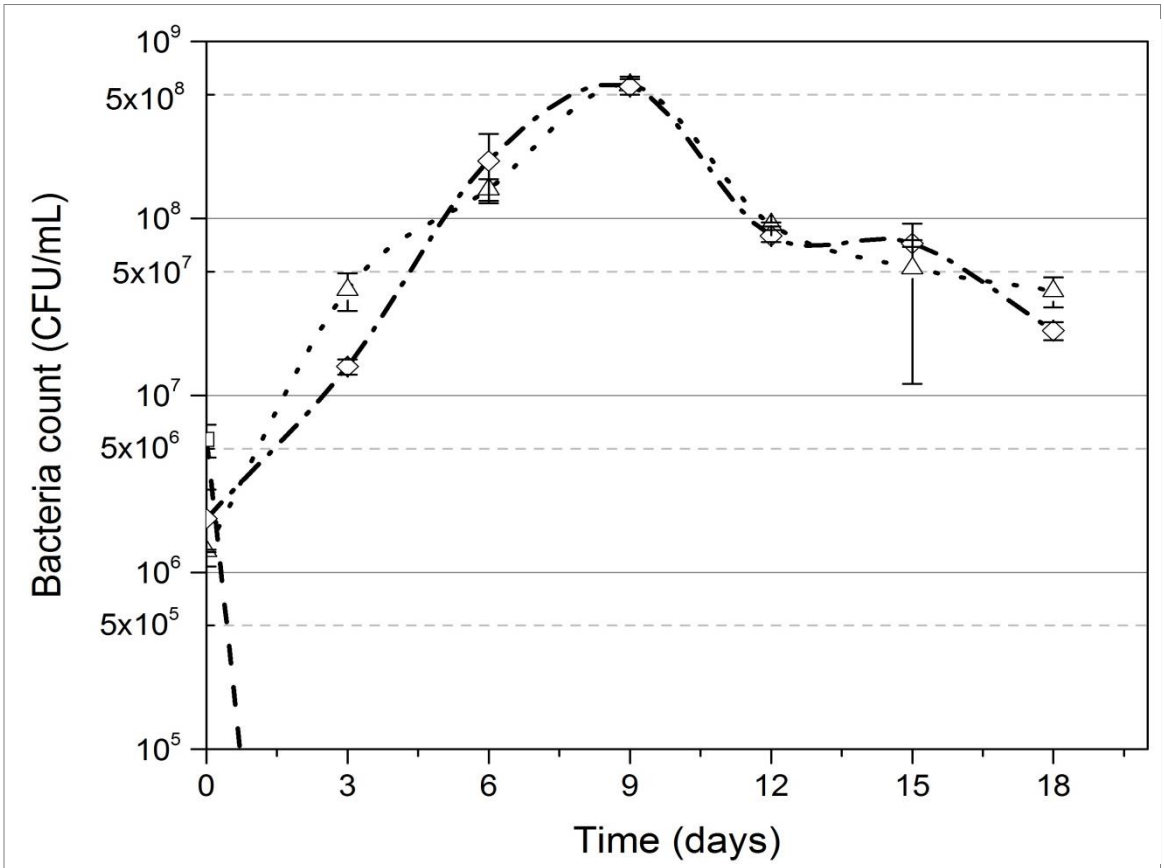


Figure 12. The growth profiles of *Idiomarina* sp. 10PY1A at various pHs: pH 5 (- - □ - -); pH 7 (··· △ ···); pH 9 (-·- ◇ -·-). No growth of strain at pH 3 since day 0. Y axis is presented in anti-Log base 10.

***Halomonas shengliensis* 10 PY 2B**

The culture of *Halomonas shengliensis* 10PY2B was initiated with 2.6-3×10^6 CFU/mL. The population rose to 2.72×10^8 CFU/mL at pH 7. On the other hand, the population grew to maximum bacterial count at 8.9×10^7 CFU/mL at pH 9. As in the previous experiment, no growth was observed at pH 3 and pH 5. The detailed growth profile was presented in Figure 13. The computation of dt revealed that at pH 7, the population growth was the fastest growth rate since dt was 30 hours and the dt at pH 7 was 41 hours. In addition, the dt at pH 3 and pH 5 was not computed since no growth was observed (Figure 15).

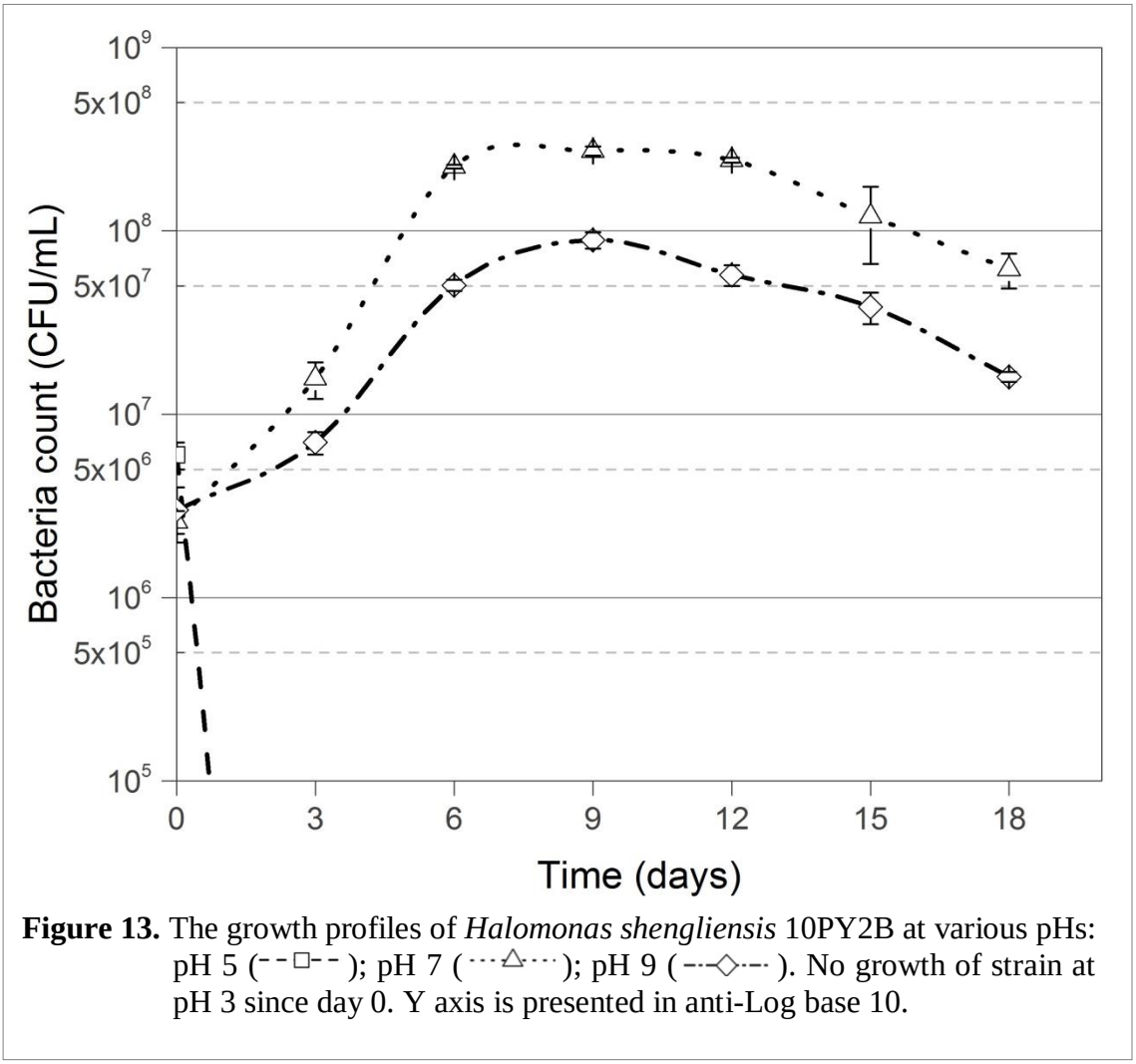


Figure 13. The growth profiles of *Halomonas shengliensis* 10PY2B at various pHs: pH 5 (- - □ - -); pH 7 (··· △ ···); pH 9 (-·-◇-·-). No growth of strain at pH 3 since day 0. Y axis is presented in anti-Log base 10.

***Halomonas smyrnensis* 20PY1A**

The growth profile of *Halomonas smyrnensis* 20PY1A was initiated with 1.5-2.1×10^6 CFU/mL. The population grew to 7.4×10^8 CFU/mL bacterial counting at pH 7 and rose to 7.1×10^8 CFU/mL bacterial counting at pH 9 after 9 days. However, no growth was monitored at pH 3 and pH 5. The growth profile of the strains was presented in Figure 14. The computation of dt expressed that the growth at pH 9 (dt= 19 hours) was faster than at pH 7 (dt= 24 hours) (Figure 15).

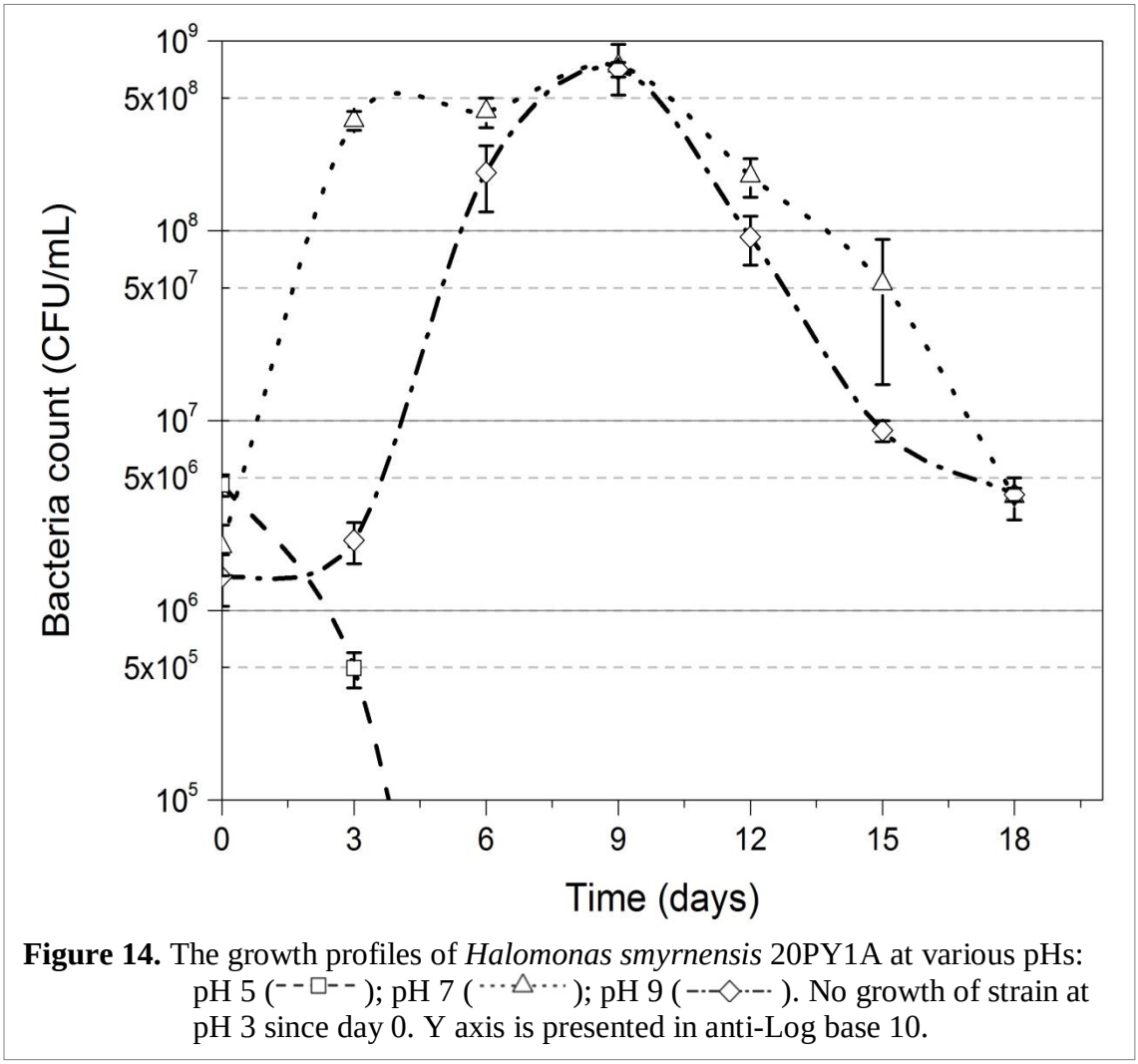


Figure 14. The growth profiles of *Halomonas smyrnensis* 20PY1A at various pHs: pH 5 (- - □ - -); pH 7 (··· △ ···); pH 9 (-·-◇-·-). No growth of strain at pH 3 since day 0. Y axis is presented in anti-Log base 10.

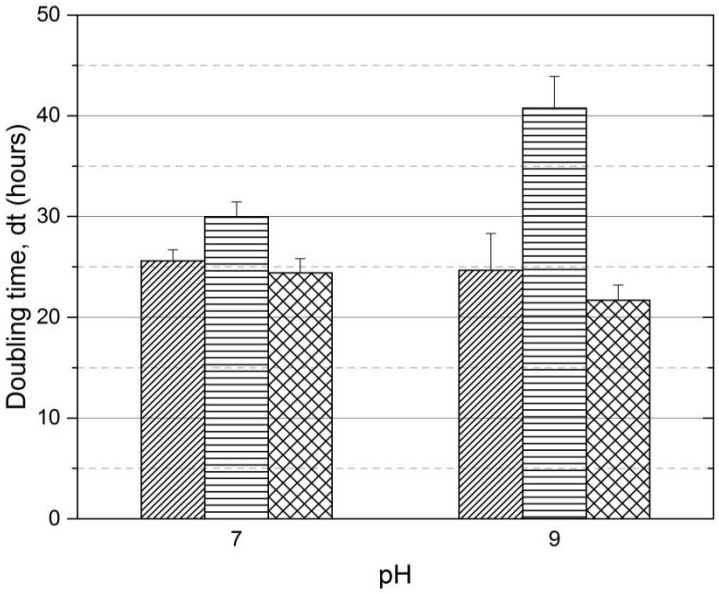


Figure 15. The doubling time at various pHs: *Idiomarina* sp. 10PY1A (▨); *Halomonas shengliensis* 10PY2B (▤); *Halomonas smyrnensis* 20PY1A (▩)

4.5.2.2 Effect of Temperature

***Idiomarina* sp. 10PY1A**

The growth of *Idiomarina* sp. 10PY1A was started with 1.25×10^{6} CFU/mL. The population increased to $2.73\text{-}5.66\times10^{8}$ CFU/mL at 10 ºC, 25 ºC, and 37 ºC. However, no growth was observed at 50 ºC. The detailed growth profile of this strain was presented in Figure 16. From the computation of dt, the growth at 25 ºC was the fastest rate with dt=23 hours followed by that at 37 ºC (dt= 25 hours) and that at 10 ºC (dt= 27 hours) (Figure 19).

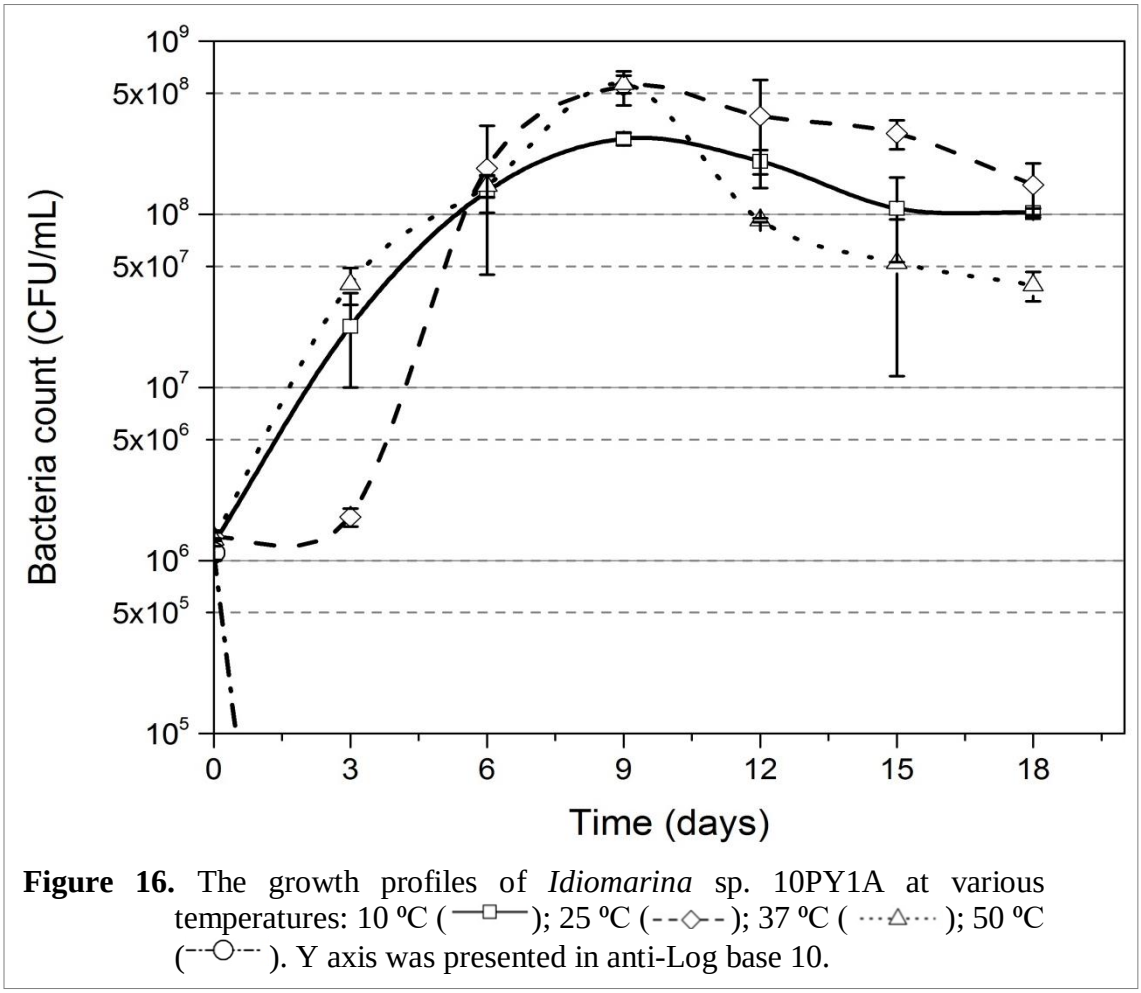


Figure 16. The growth profiles of *Idiomarina* sp. 10PY1A at various temperatures: 10 ºC (—□—); 25 ºC (- -◇- -); 37 ºC (···△···); 50 ºC (-·-○-·-). Y axis was presented in anti-Log base 10.

***Halomonas shengliensis* 10PY2B**

The growth of *Halomonas shengliensis* 10PY2B was started with 0.9-2.5×10^6 CFU/mL bacterial count. The maximum bacterial count was achieved within 6-12 days, and all temperature gave similar behavior. The population rose to 4.08×10^8 CFU/mL within 9 days at 10 ºC, increased to 3.22×10^8 CFU/mL within 6 days at 25 ºC and grew to 2.72×10^6 CFU/mL within 9 days at 37 ºC. Conversely, no growth was observed at 50 ºC. The growth profile was presented in Figure 17. The computation of dt indicated the fastest growth occur at 25 ºC (dt= 23 hours) followed by 10 ºC (dt= 25 hours) and 37 ºC

(dt= 30 hours). Since no growth was observed at 50 ºC, no computation of dt was carried out (Figure 19).

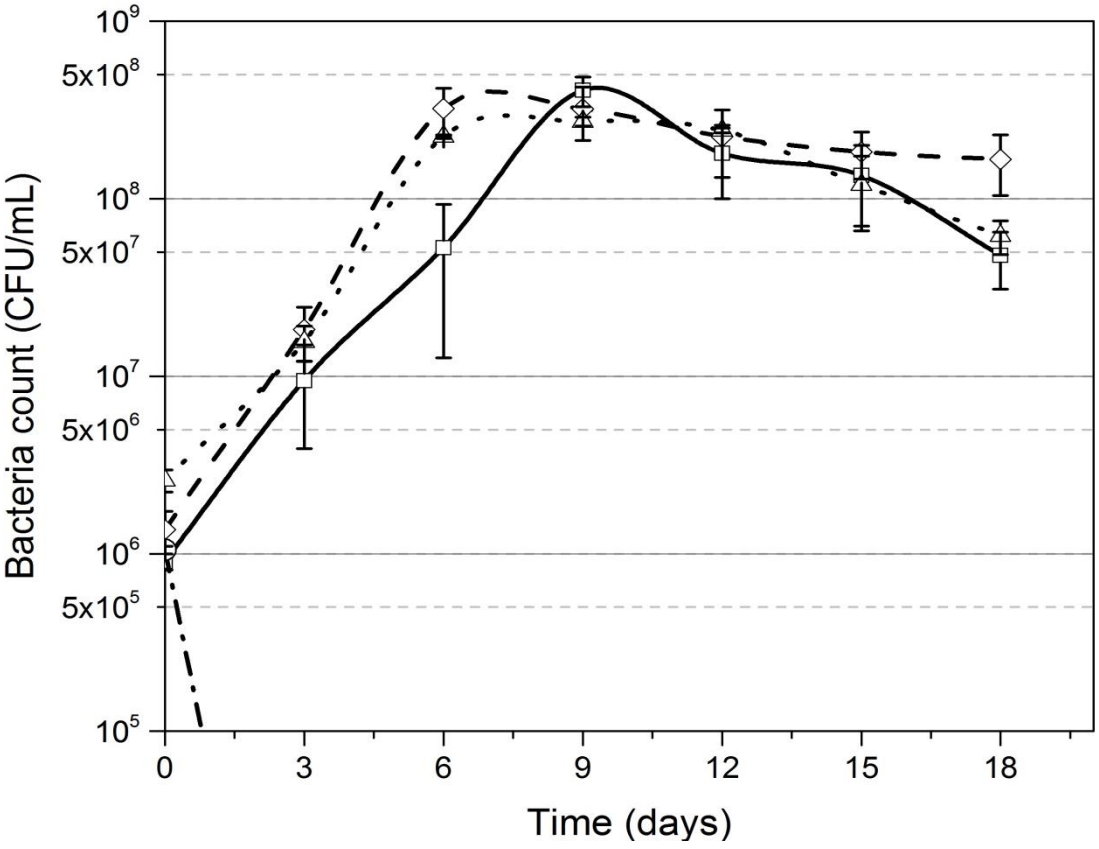


Figure 17. The growth profiles of *Halomonas shengliensis* 10PY2B at various temperatures: 10 ºC (—□—); 25 ºC (- -◇- -); 37 ºC (···△···); 50 ºC (-·○·-). Y axis is presented in anti-Log base 10.

***Halomonas smyrnensis* 20PY1A**

Starting with initial culture 1.5-2×10^6 CFU/mL, the strain was able to grow at maximum bacterial count within 9 days. The culture grew to 2.15-5.01×10^8 CFU/mL at 10 ºC, 25 ºC, and 37 ºC. However, no growth was observed at 50 ºC. The growth profile was presented in Figure 18. The computation of dt revealed the fastest growth was at 37 ºC (dt= 24 hours), the second was at 25 ºC (dt= 29 hours) and the slowest was at 10 ºC (dt=

31 hours). However, no computation on dt was carried out at 50 ºC since no growth was observed (Figure 19).

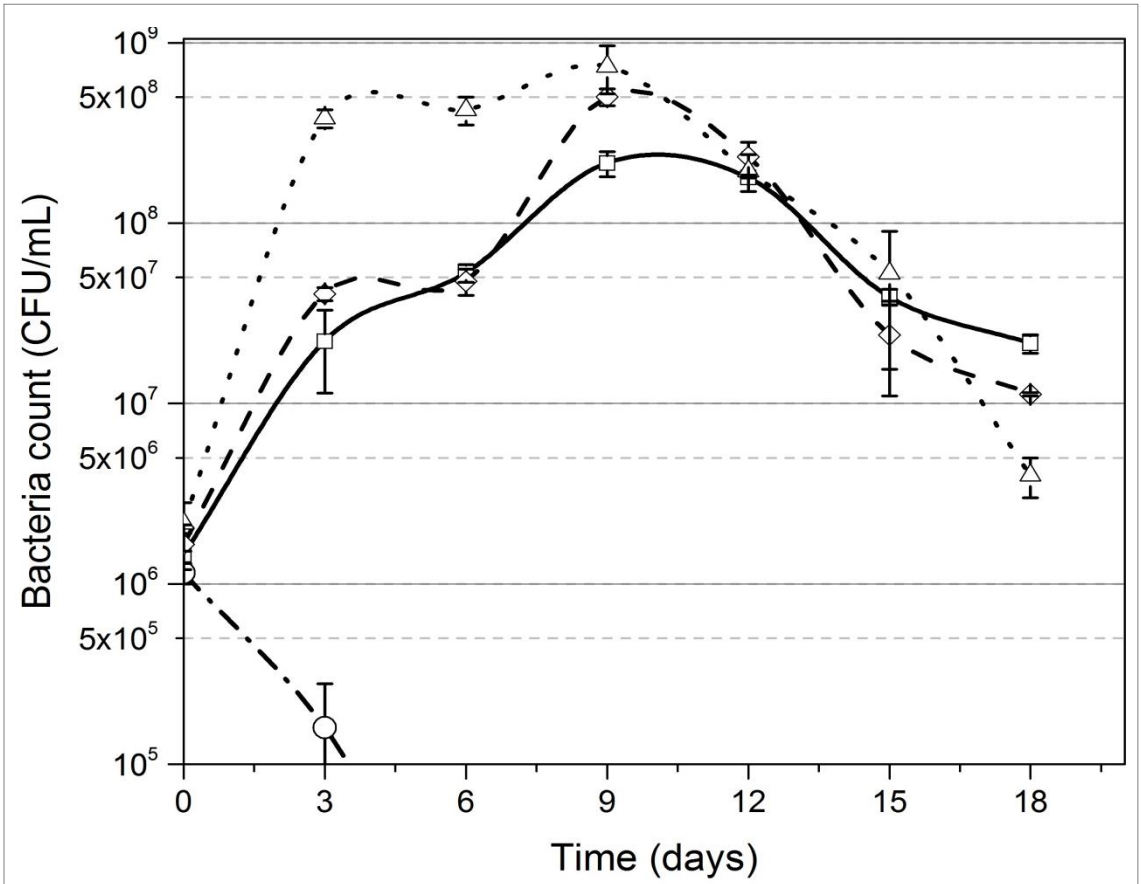


Figure 18. The growth profiles of *Halomonas smyrnensis* 20PY1A at various temperatures: 10 ºC (—□—); 25 ºC (- - ◇ - -); 37 ºC (···△···); 50 ºC (-··○··-). Y axis is represented in anti-Log base 10.

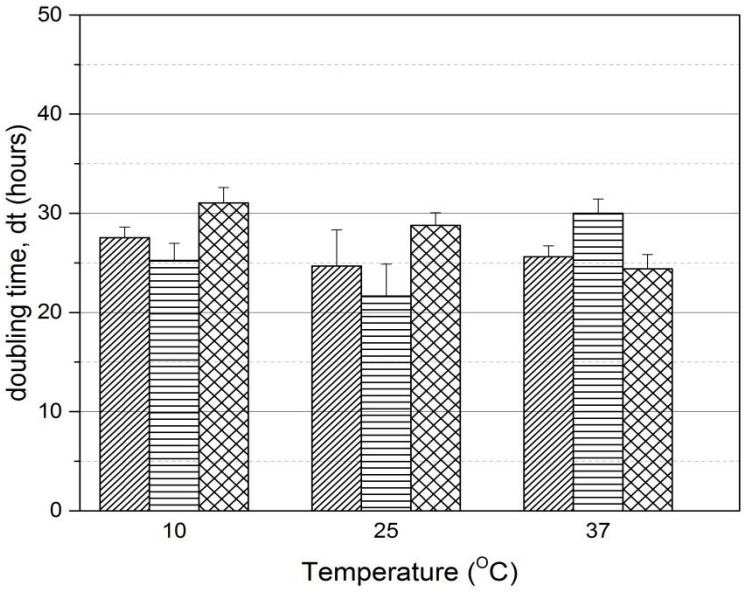


Figure 19. The doubling time at various temperatures: *Idiomarina* sp. 10PY1A ( ); *Halomonas shengliensis* 10PY2B ( ); *Halomonas smyrnensis* 20PY1A ( )

4.5.2.3 The effect of salinity

***Idiomarina* sp. 10PY1A**

The growth of *Idiomarina* sp. 10PY1A was initiated with 1.2-3×10^6 CFU/mL. The culture reached its maximum bacterial count at 6-9 days. The culture grew to 4.35×10^8 CFU/mL within 6 days at 5% salinity. Hence, the culture increased to 5.66×10^8 CFU/mL within 9 days at salinity 10%, rose to 3.2×10^7 CFU/mL within 6 days at salinity 15% and reached to 5×10^6 CFU/mL within 6 days at salinity 20%. However, no growth was observed at salinity 0%. The growth profile was presented in Figure 20. The computation of dt expressed the best salinity for this strain to grow was at salinity 5% (dt= 18 hours), followed by salinity 10% (dt= 26 hours), salinity 15% (dt= 31 hours and the slowest at

salinity 20% (dt= 136 hours). Since no growth observed at salinity 0%, the computation of dt was not carried (Figure 23).

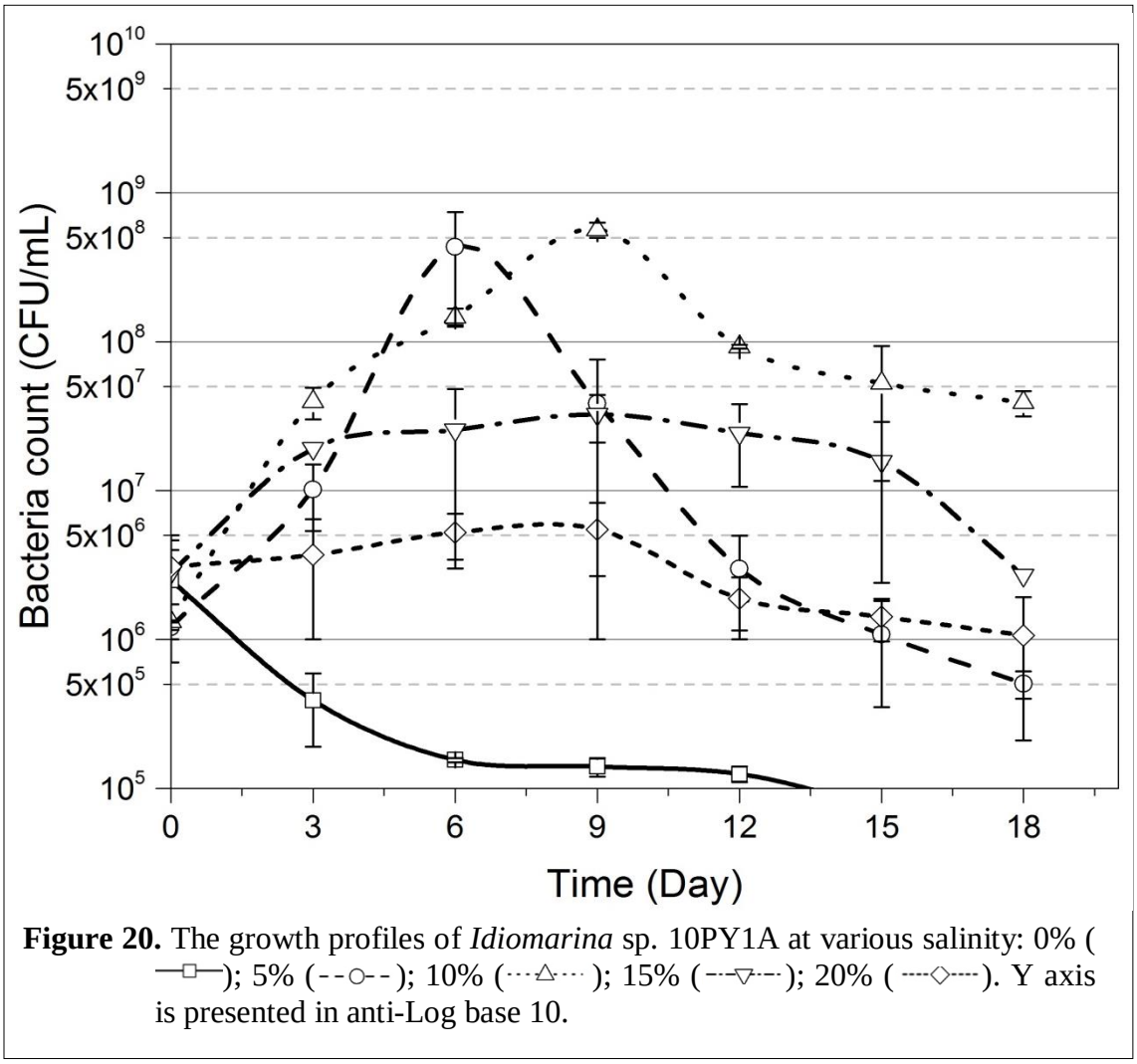


Figure 20. The growth profiles of *Idiomarina* sp. 10PY1A at various salinity: 0% (—□—); 5% (- - ○ - -); 10% (⋯△⋯); 15% (-·-▽-·-); 20% (····◇····). Y axis is presented in anti-Log base 10.

***Halomonas shengliensis* 10PY2B**

Starting with 0.3-2×10^6 CFU/mL bacterial count, the growth of *Halomonas shengliensis* 10PY2B was monitored. The culture grew to the maximum bacterial count within 6-9 days. The culture grew to 3.5×10^7 CFU/mL within 9 days ay salinity 5%, increased to 2.78×10^8 CFU/mL within 9 days at salinity 10% and rose to 5×10^6 CFU/mL within 6

days at salinity 15%. Yet, no growth was observed at salinity 0% and 20%. The growth profile was presented in Figure 21. The computation of dt informed that the fastest growth was at salinity 10% (dt= 30 hours) followed by salinity 15% (dt= 31 hours) and salinity 5% (dt= 38 hours) (Figure 23).

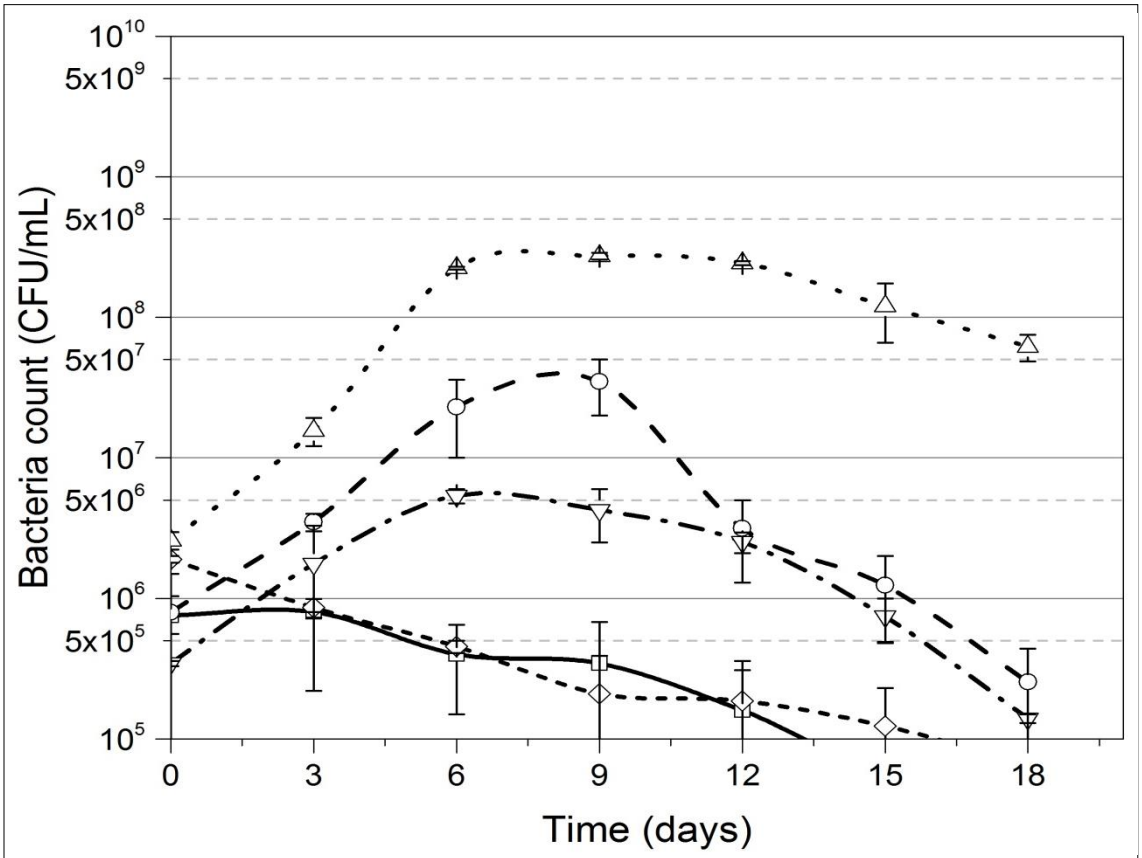


Figure 21. The growth profile of *Halomonas shengliensis* 10PY2B at various salinity: 0% (—□—); 5% (- -○- -); 10% (···△···); 15% (-·-▽-·-); 20% (----◇----). Y axis is presented in anti-Log base 10.

***Halomonas smyrnensis* 20PY1A**

Using 2.1-3.7×10^6 CFU/mL as initial culture's concentration, the monitoring of the growth of *Halomonas smyrnensis* 20PY1A was carried out every 3 days. The maximum growth was observed within 6-9 days. The culture increased to 4.1×10^8 CFU/mL within 9

days at salinity 5%, rose to 9.3×10^8 CFU/mL within 9 days at salinity 10%, grew to 9.5×10^8 CFU/mL within 6 days at salinity 15% and reached to 7.4×10^8 CFU/mL within 9 days at salinity 20%. However, no growth was monitored at salinity 0%. The growth profile was presented in Figure 22. Hence, using computation of dt, the fastest growth of strain was occur at salinity 10% (dt= 15 hours) followed by salinity 15% (dt= 21 hours), salinity 20% (dt= 24 hours) and salinity 5% (dt= 28 hours). Yet, no dt was computed at salinity 0% since no growth was monitored (Figure 23).

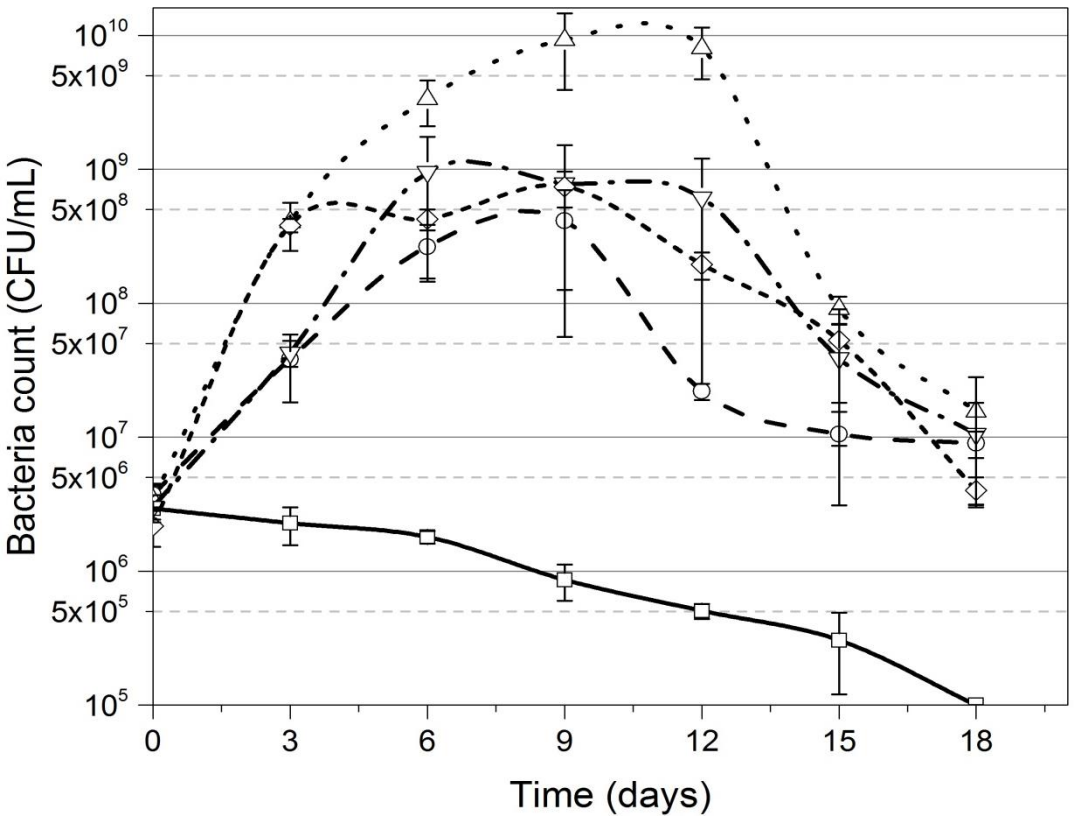


Figure 22. The growth profiles of *Halomonas smyrnensis* 20PY1A at various salinity: 0% (—□—); 5% (- -○- -); 10% (···△···); 15% (-··▽··-); 20% (····◇····). Y axis is presented in anti-Log base 10.

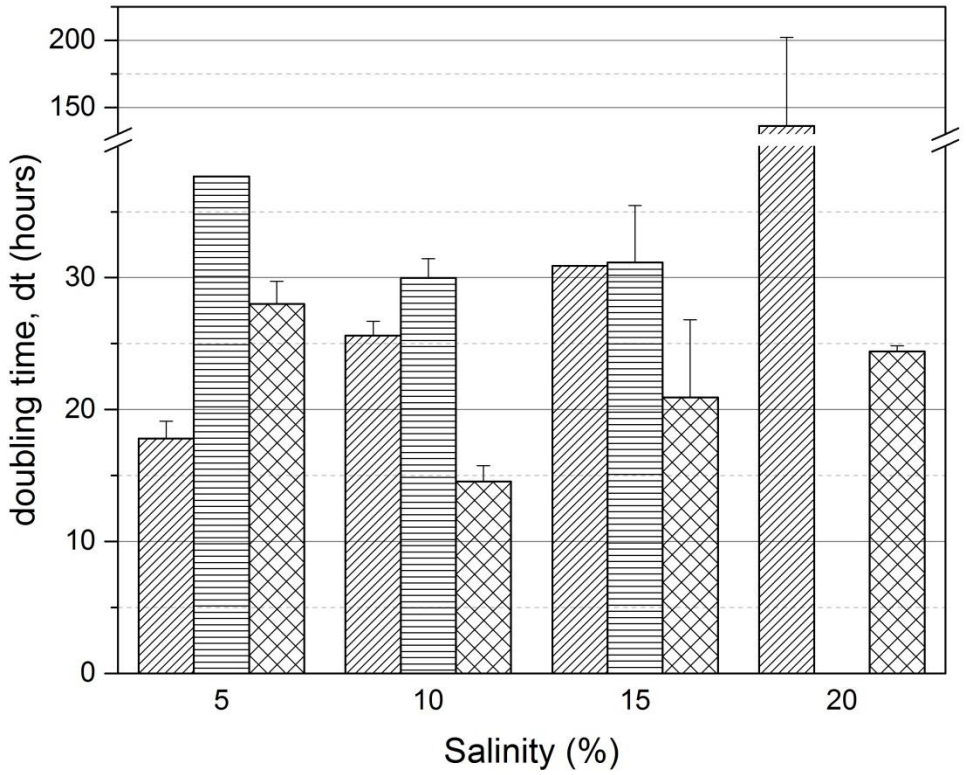


Figure 23. The doubling time at various salinity: *Idiomarina* sp. 10PY1A (▨); *Halomonas shengliensis* 10PY2B (▤); *Halomonas smyrnensis* 20PY1A (▩)

4.5.3 **Effect of initial pyrene concentration**

***Idiomarina* sp. 10PY1A**

The experiment was initiated with bacteria count range from 1.9 to 2.5×10^6 CFU/mL. The growth increased to maximum count within 3 days in presence of 0.05 and 0.25 mg of pyrene, range from 0.25 to 1.7×10^8 CFU/mL. On the other hand, the culture rose to 0.52×10^8 CFU/mL within 6 days in presence of 5 mg pyrene and no growth was monitored in presence of 50 mg of pyrene. The growth profile is presented in Figure 24. The lower the pyrene concentration, the faster the growth: 0.05 mg (dt= 10 hours), 0.25

mg (dt= 17 hours), 2.5 mg (dt= 25 hours), 5 mg (dt= 32 hours) and no computation of dt for 50 mg of pyrene as no growth was observed (figure 27).

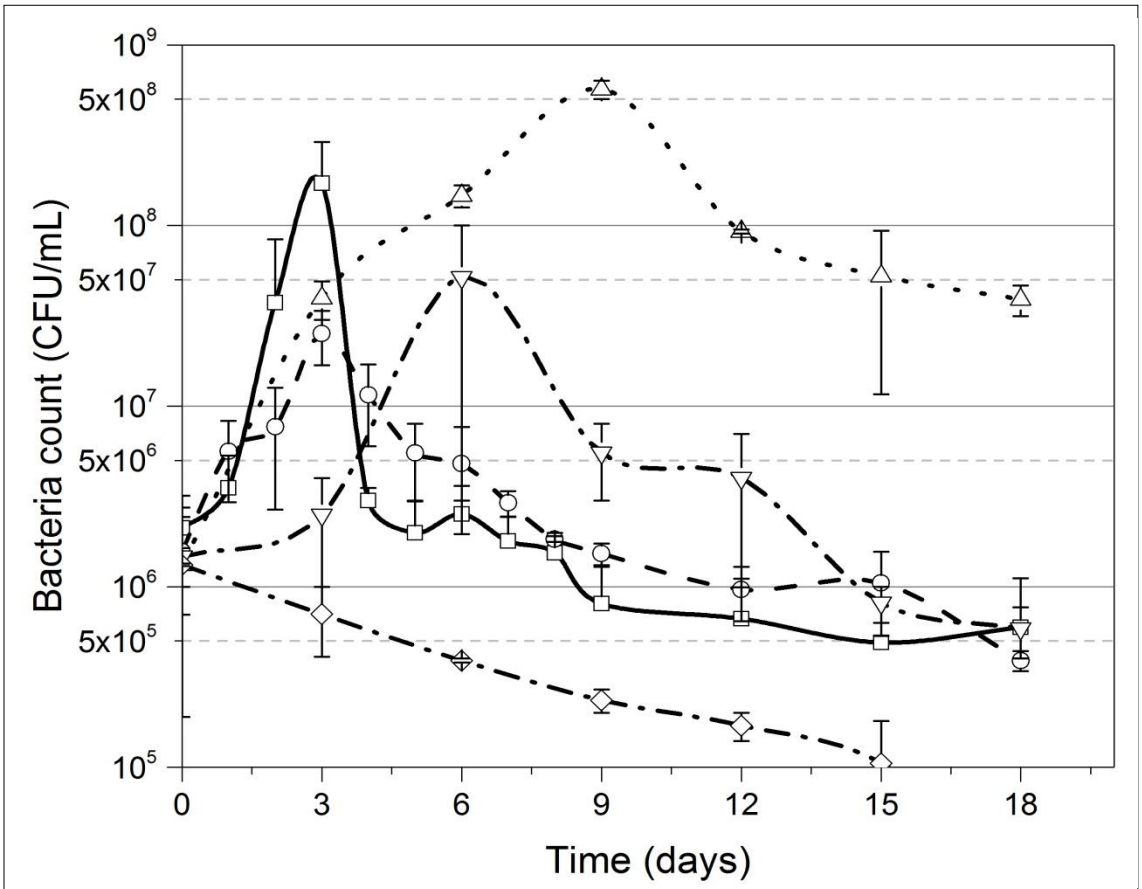


Figure 24. The growth profiles of *Idiomarina* sp. 10PY1A at various amount of pyrene added (mg): 0.05 (—□—); 0.25 ppm (- -○- -); 2.5 (···△···); 5 (-··▽··-); 50 (-··◇··-). Y axis is presented in anti-Log base 10.

***Halomonas shengliensis* 10PY2B**

Using initial culture range from 0.5-2×10^6 CFU/mL, the growth of strain was observed. Within 2 days, the population grew to the maximum count, about 2.6-5.1×10^7 CFU/mL in presence of 0.05 and 0.25 mg of pyrene. On the other hand, the growth of strain rose to 2.8×10^7 CFU/mL within 1 day in presence of 5 mg of pyrene and no growth was monitored in presence of 50 mg of pyrene. Figure 25 presented the detailed growth

profile. The dt become higher at high amount of pyrene (mg): 0.05 (dt= 6 hours), 0.25 (dt= 15 hours), 2.5 (dt= 30 hours), 5 (dt= 38 hours) and no computed dt for 50 mg of pyrene as no population growth was monitored (Figure 27).

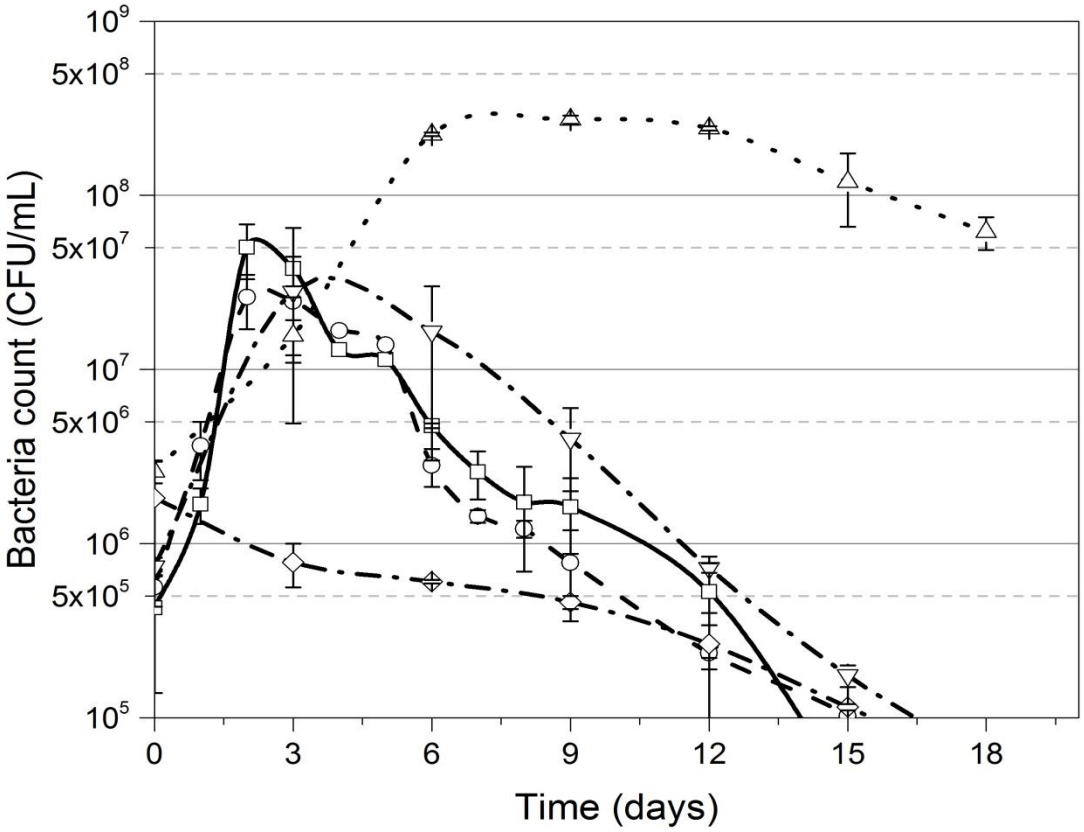


Figure 25. The growth profiles of *Halomonas shengliensis* 10PY2B at various amount of pyrene added (mg): 0.05 (—□—); 0.25 (- - ○ - -); 2.5 (··· △ ···); 5 (-·-▽-·-); 50 (-·-◇-·-). Y axis is presented in anti-Log base 10.

***Halomonas smyrnensis* 20PY1A**

Using the initial culture range 2-4.5×10^6 CFU/mL, the growth of *Halomonas smyrnensis* 20PY1A was monitored. The culture reached maximum counting after 3 days, range from 0.27-7.4×10^8 CFU/mL. However, no growth was observed in presence of 50 mg of pyrene. Figure 26 represented the detailed growth profile of *Halomonas smyrnensis* 20PY1A in presence of various amount of pyrene added. The dt become slower with the

increase of amount of pyrene added (mg): 0.05 (dt=9 hours), 0.25 (dt=12 hours), 2.5 (dt=24 hours), 5 (dt=35 hours) (Figure 27).

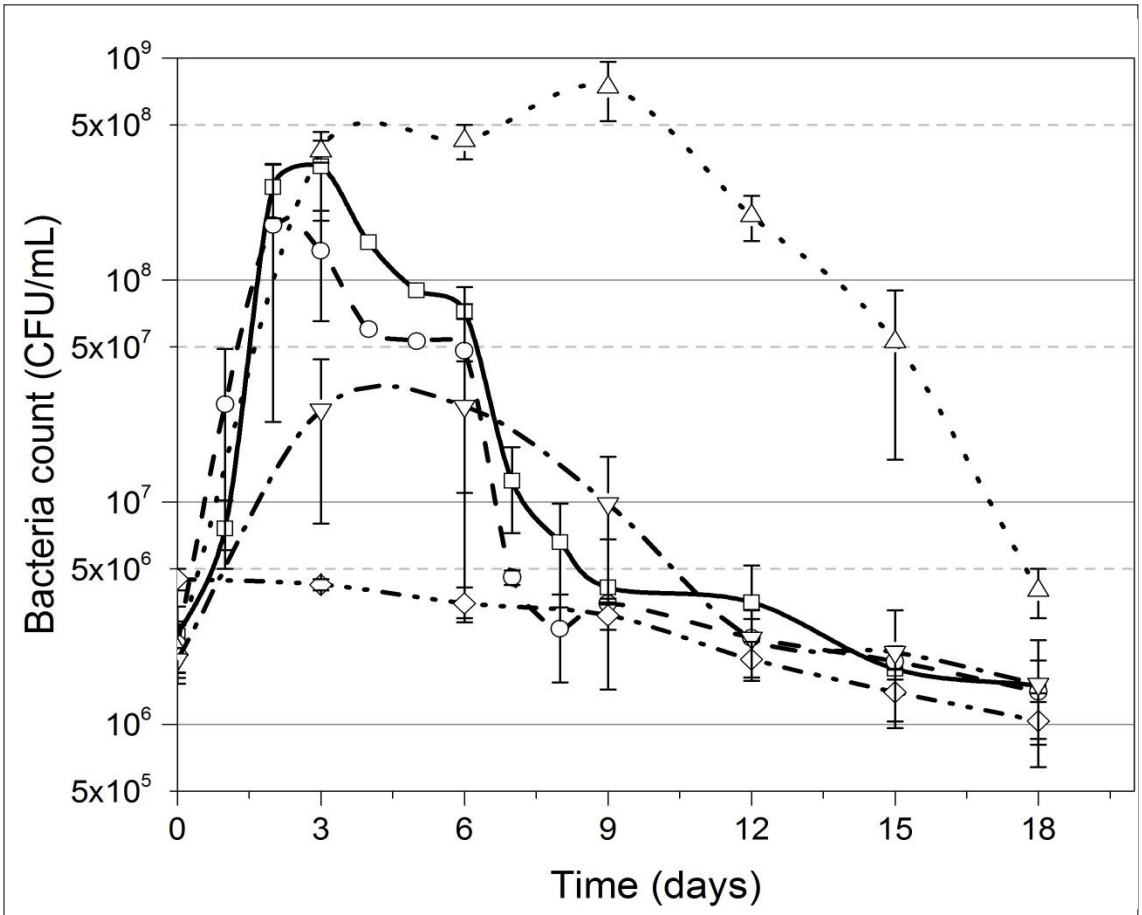


Figure 26. The growth profiles of *Idiomarina* sp. 10PY1A at various amount of pyrene added (mg): 0.05 (—□—); 0.25 (- -○- -); 2.5 (···△···); 5 (-·-▽-·-); 50 (-·-◇-·-). Y axis is presented in anti-Log base 10.

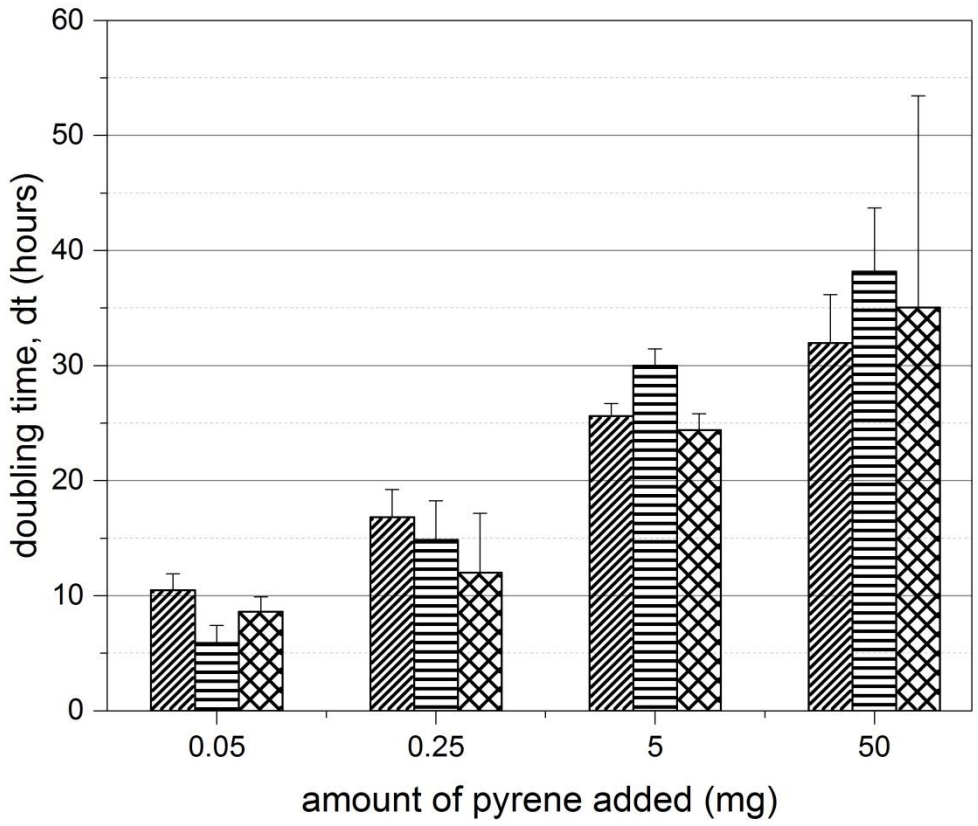


Figure 27. The doubling time at various amount of pyrene added: *Idiomarina* sp. 10PY1A (▨); *Halomonas shengliensis* 10PY2B (▤); *Halomonas smyrnensis* 20PY1A (▩).

4.6 The quantification of pyrene during incubation periods

4.6.1 **Standard curve and the mass spectrum of pyrene**

A series of known samples as an external standard was prepared to compute the pyrene concentration. Five different known samples: continuity 0; 0.5; 1.25; 2.5 and 3.75 mg of pyrene in 50 mL BH media (0; 10; 25; 50 and 75 mg/L of pyrene), were prepared as than extracted using ethyl acetate (liquid-liquid extraction protocol) followed by GC-MS analysis. The extracts injected to GC-MS and the chromatographs were obtained. The area under the curve showed on chromatography was used to predict the concentration as a function of the area under the curve. For each known samples, the area under the curve

was plotted against the concentration of known sample. The linear function of the graph was used later on for computation of unknown samples' concentration. The result on plotting, with R^2 was 0.996, the concentration as a function of the area under the curve was formulated as follow (figure 28):

$$\boldsymbol{X = \frac{(8.96x10^6 - Y)}{7.93\ x\ 10^6}} \qquad \textbf{4}$$

In which, X represented the concentration of pyrene and Y is peak area. The GC peak of pyrene came after 47.583 minutes after the injection of the sample into injector of GC-MS (Figure 29A), and the mass spectrum obtained for pyrene is shown in Figure 29B.

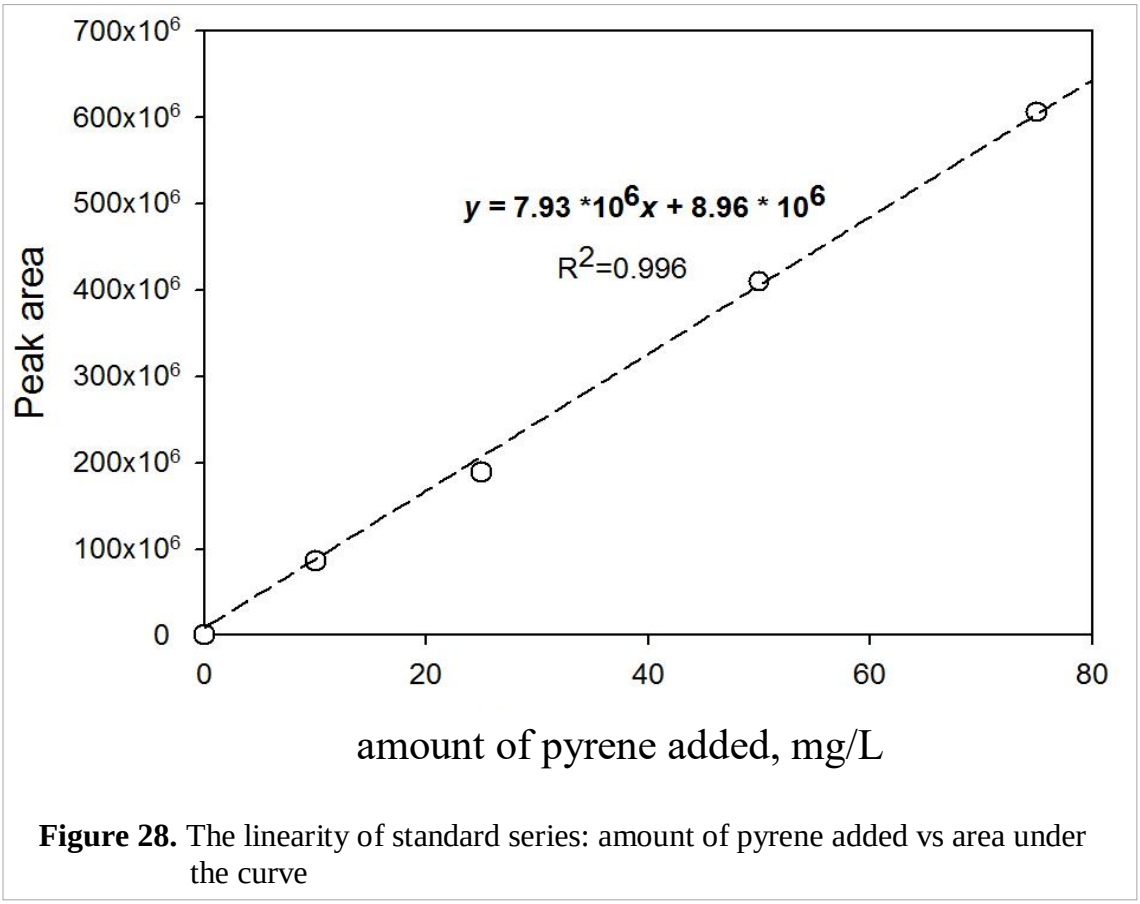


Figure 28. The linearity of standard series: amount of pyrene added vs area under the curve

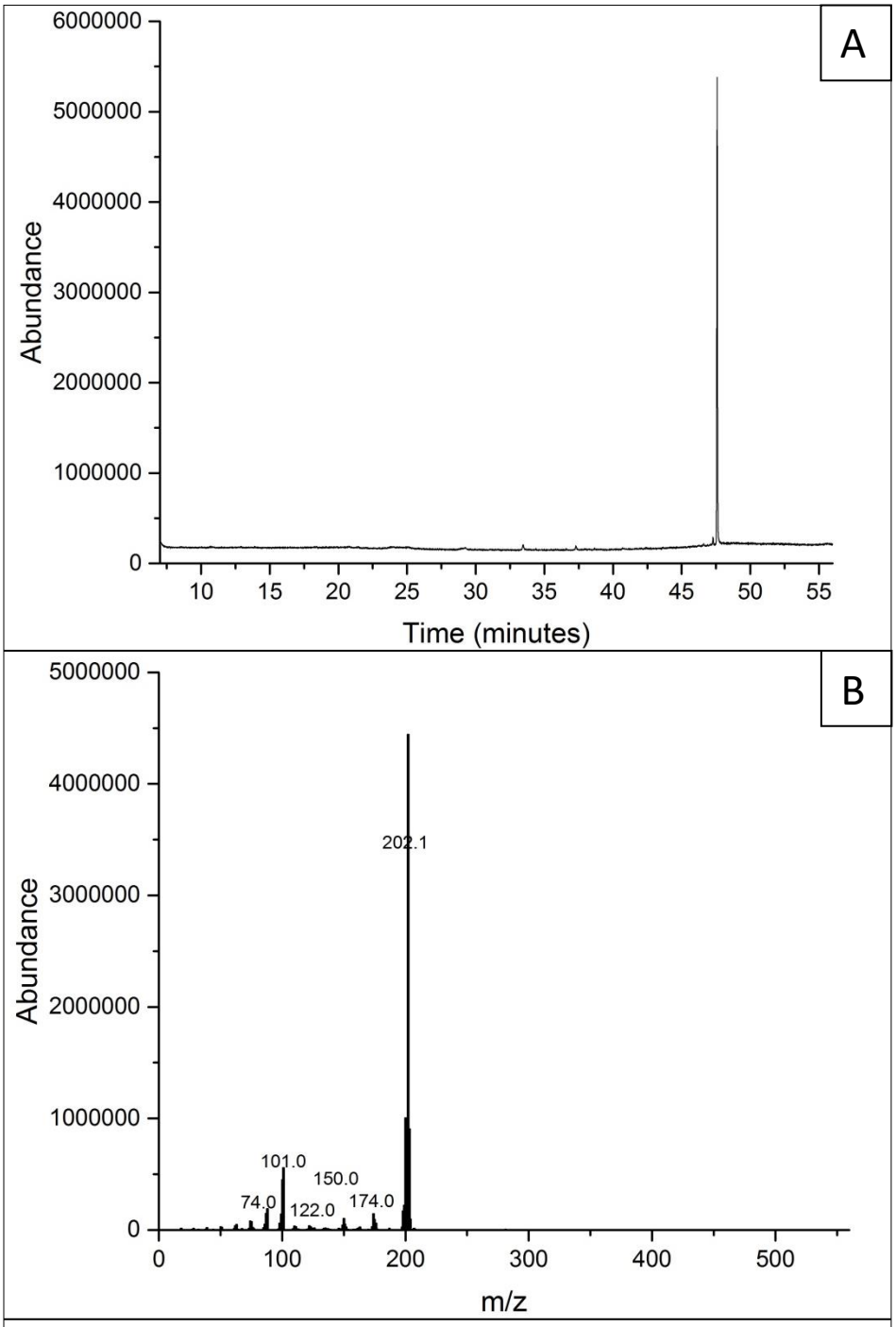


Figure 29. The chromatograph of peak area of GS-MS (A) and the mass spectrum of pyrene (B)

4.6.2 **The degradation of pyrene by *Idiomarina* sp. 10PY1A**

The experiment on quantification of pyrene was carried out using liquid-liquid extraction procedure followed by GC-MS measurement. This protocol was performed every 3 days along with counting the bacteria population. Using 2.5 mg of pyrene (50 mg/L) as initial concentration, the culture was incubated at 37 ºC and pH 7. Using 1.32×10^6 CFU/mL as initial bacteria count, the population increased to 5.66×10^8 CFU/mL within 9 days then the population decreased to 3.9×10^6 CFU/mL after 18 days. As the population rose, the pyrene concentration decreased to 51% at the end of the experiment. However, the abiotic factor reduced of pyrene concentration by 10% after 18 days. Figure 30 represented the growth profile of bacteria and residual pyrene concentration. Hence, the biodegradation rate was computed at 0.046 day^{-1} (Figure 33).

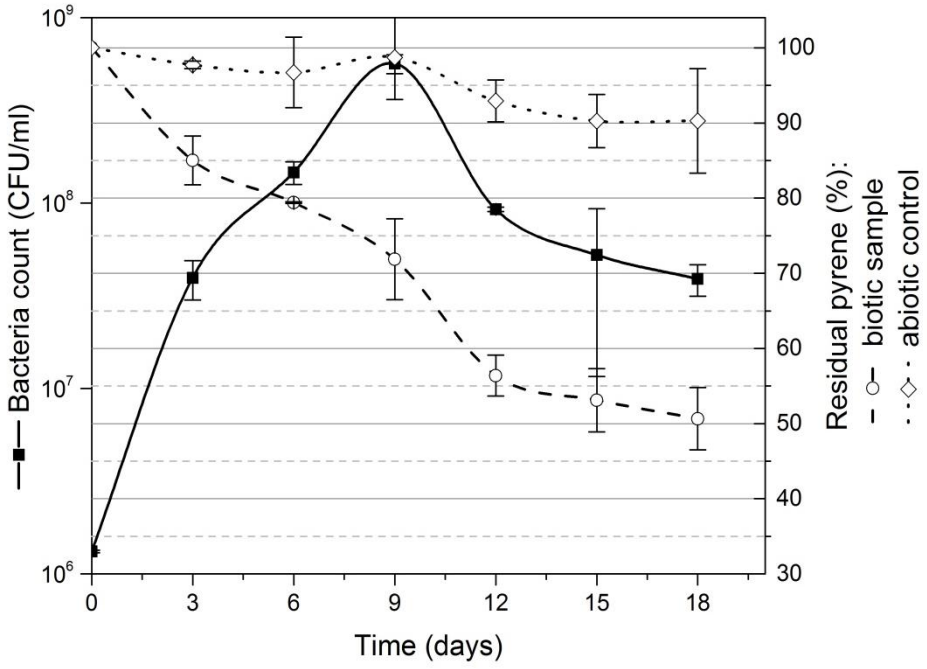


Figure 30. The growth profile of *Idiomarina* sp. 10PY1A (—■—) and the residual pyrene concentration during incubation: with inoculum (- - ○ - -); abiotic control (···◇···)

4.6.3 **The degradation of pyrene by *Halomonas shengliensis* 10PY2B**

The experiment was performed exactly as for the previous strain. Starting with 2.5×10^6 CFU/mL, the population grew to 2.74×10^8 CFU/mL within 9 days then reduced to 6.2×10^6 CFU/mL after 18 days. Within 18 days, there was only 43% of pyrene remaining. However, the abiotic reduction was observed up to 15%. The growth profile and residual pyrene concentration were presented in Figure 32. The reduction rate was 0.0455 day^{-1} (Figure 33).

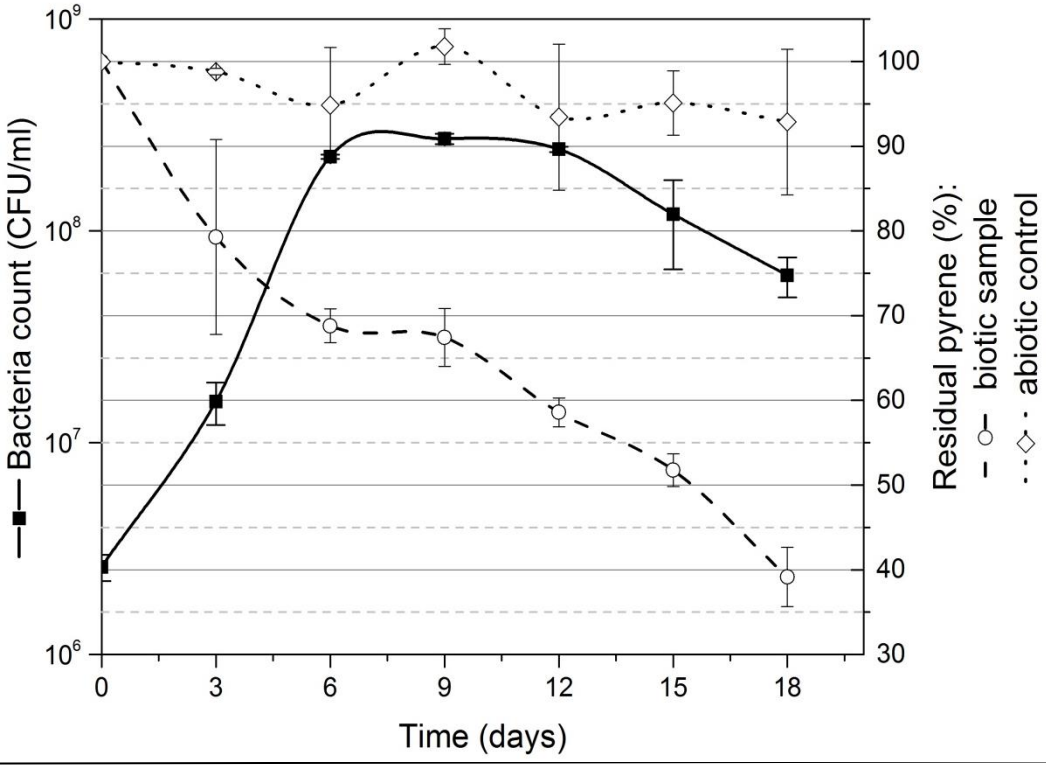


Figure 31. The growth profile of *Halomonas shengliensis* 10PY2B (—■—) and the residual pyrene concentration during incubation: sample (- - ○ - -); control (··· ◇ ···)

4.6.4 **The degradation of pyrene by *Halomonas Smyrnensis* 20PY1A**

The experiment was performed as two previous experiments on pyrene quantification. The culture was initiated at 2.17×10^6 CFU/mL and rose to 7.4×10^8 CFU/mL within 9 days then reduced to 4×10^6 CFU/mL at day 18. Pyrene was remaining at 52% at day 18, yet the abiotic reduction of pyrene was 7%. Figure 33 expressed the detailed growth profile and residual pyrene concentration. The biodegradation rate was computed about 0.0495 day^{-1} (Figure 34).

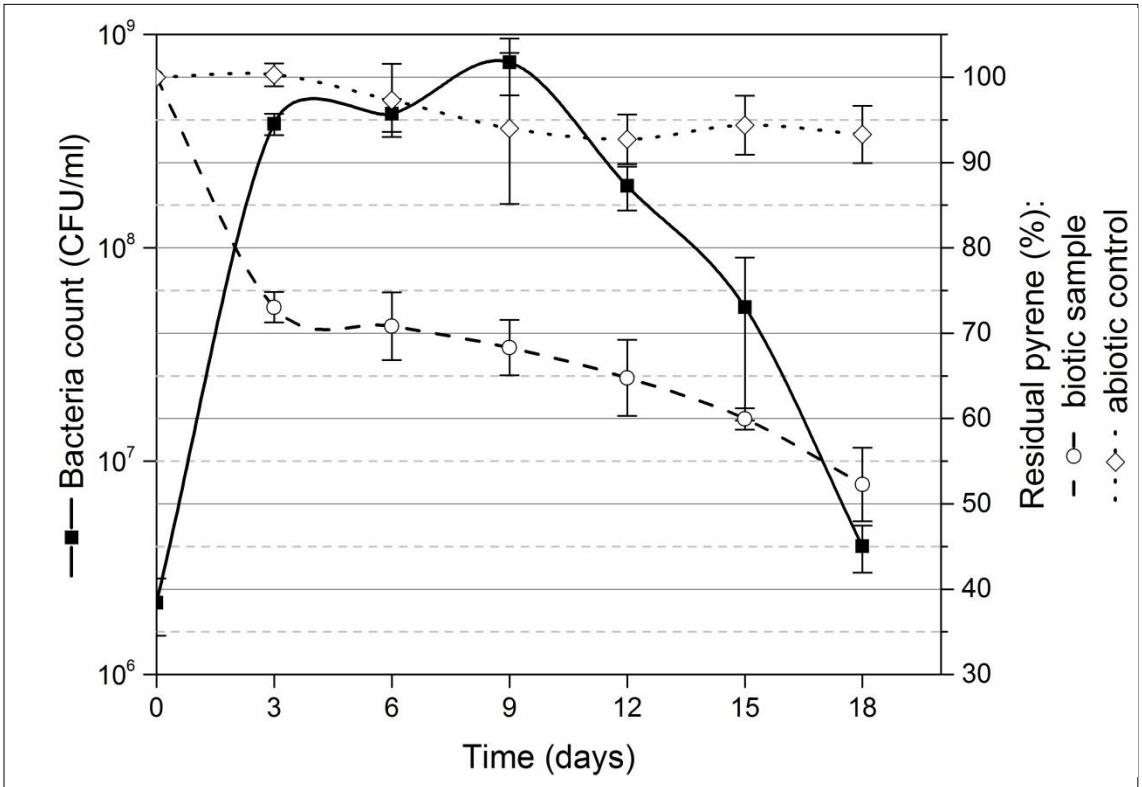


Figure 32. The growth profile of *Halomonas smyrnensis* 20PY1A (—■—) and the residual pyrene concentration during incubation: with inoculum (- -○- -); control (···◇···)

Among these three isolates, strain *Halomonas smyrnensis* 20PY1A was capable of degrading pyrene faster (not significant, $p>0.05$) than others with degradation rate at 0.0495 day^{-1}. The degradation rate performed by *Idiomarina* sp. 10PY1A and *Halomonas*

shengliensis 10PY2B was not significantly different, 0.046 day^{-1} and 0.0455 day^{-1} (figure 34).

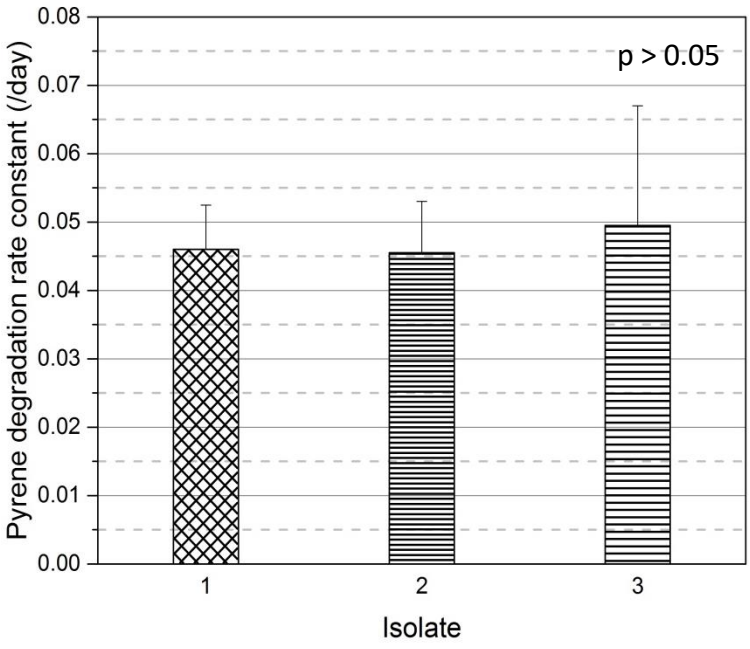


Figure 33. The degradation rate of pyrene: (1) *Idiomarina* sp. 10PY1A; (2) *Halomonas shengliensis* 10PY2B; (3) *Halomonas smyrnensis* 20PY1A

4.7 The capability of isolates to grow in the presence of different types of carbon source

4.7.1 The growth of *Idiomarina* sp. 10PY1A in the presence of different type of carbon sources

Idiomarina sp. 10PY1A was incubated in the presence of different polyaromatic compounds containing three, two or one rings such as phenanthrene (Phen), and Anthracene (Anth); also those containing two rings, naphthalene (Naph) and salicylic acid (SA). This 2.5 mg PAHs were added individually into 50 mL media and the experiments were carried out at 37 ºC and pH 7. The counting of bacteria was performed every 3 days. With the initial culture about 1×10^6 CFU/mL, the isolate was capable of

growing under these aromatic compounds. Starting with 1.2×10^6 CFU/mL, the population increased to 208×10^6 CFU/mL within 6 days in the presence of phenanthrene. However, the population climbed only to 75×10^6 CFU/mL within 6 days in the presence of anthracene. After 18 days, the population jumped below 20×10^6 CFU/mL in the presence of these 3 rings containing PAHs (Phen and Anth). Naphthalene as a sole carbon source supported the growth of population to 1400×10^6 CFU/mL within 6 days. Furthermore, the growth of population increased to 2100×10^6 CFU/mL within 3 days in the presence of Salicylic acid (Figure 34). Using the computation of dt, the growth rate was arranged in order from the fastest to the slowest as: SA (dt= 14 hours), Naph (dt= 17 hours), Phen (dt= 18 hours), Anth (dt= 21 hours) and PYR (dt= 26 hours) (Figure 37).

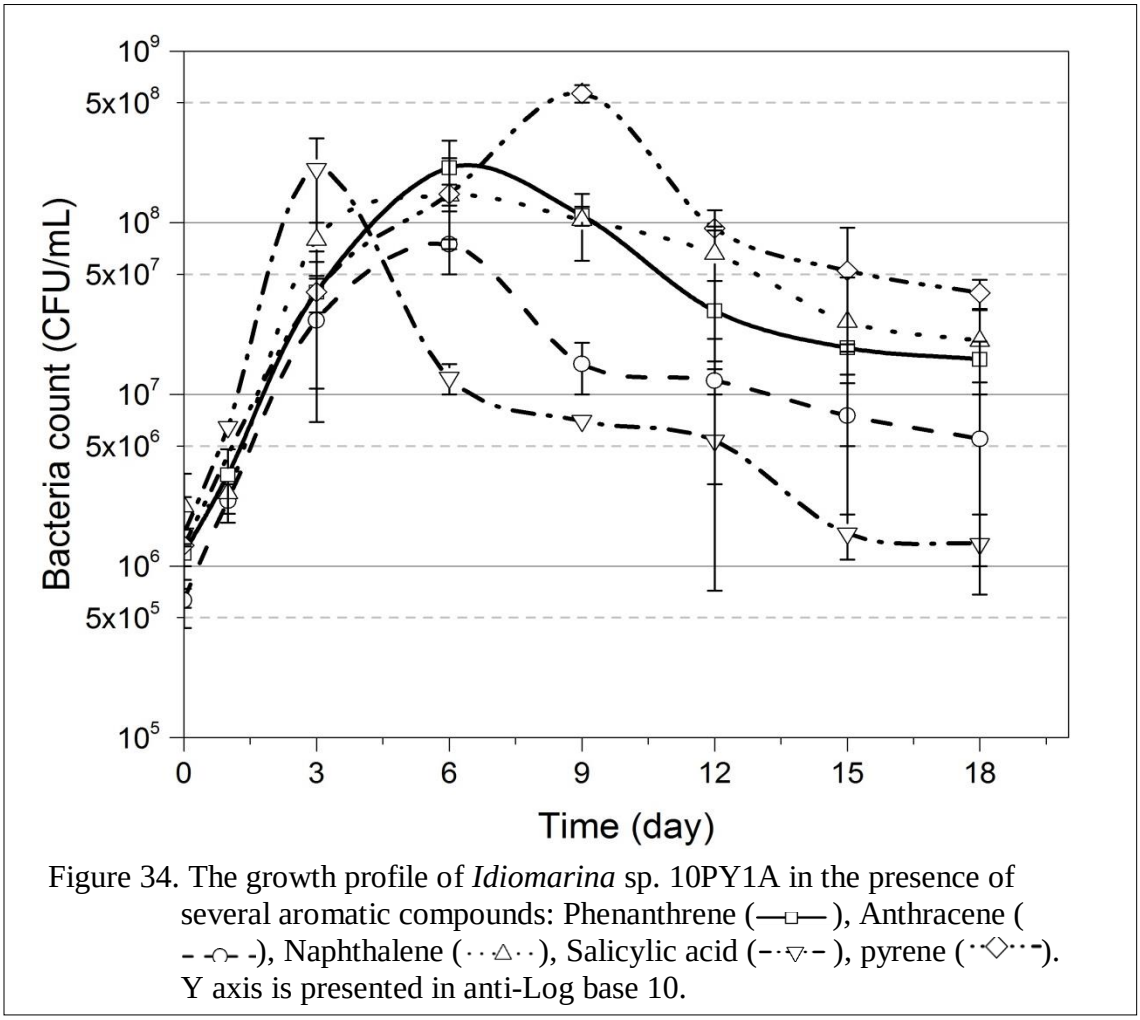


Figure 34. The growth profile of *Idiomarina* sp. 10PY1A in the presence of several aromatic compounds: Phenanthrene (—□—), Anthracene (- -○- -), Naphthalene (· · △ · ·), Salicylic acid (- · ▽ · -), pyrene (· · ◇ · · -). Y axis is presented in anti-Log base 10.

4.7.2 **The growth of *Halomonas shengliensis* 10PY2B under different types of carbon source**

The extended experiment was performed to *Halomonas shengliensis* 10PY2B, some different aromatic compounds were also added as sole carbon source. *Halomonas shengliensis* 10PY2B grew under different kind of aromatic compounds: Phen, Anth, Naph, and SA. The population grew up to 46×10^6 CFU/mL within 6 days in the presence of phenanthrene. Yet, the population increased to 7.5×10^6 CFU/mL within 6 days in the presence of anthracene. On the other hand, within 6 days, the population rose to 450×10^6 CFU/mL in the presence of naphthalene. Hence, within 3 days, the population grew to

216×10^6 CFU/mL in the presence of salicylic acid (Figure 35). The growth rate can be arranged in order from the fastest to the slowest as: SA (dt= 9 hours), Naph (dt= 14 hours), Phen (dt= 23 hours), Anth (dt= 23 hours) and PYR (dt= 30 hours) (Figure 37).

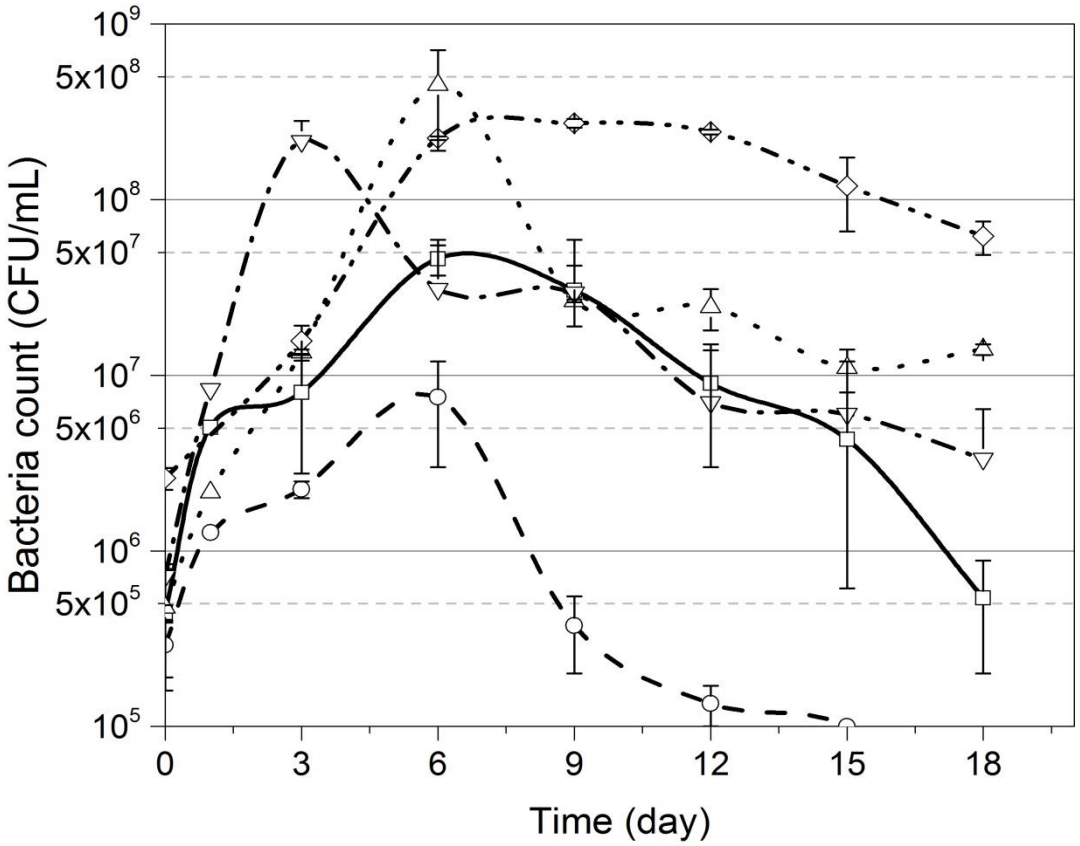


Figure 35. The growth profile of *Halomonas shengliensis* 10PY2B in presence of several aromatic compounds: Phenanthrene (—□—), Anthracene (- -○- -), Naphthalene (· · △ · ·), Salicylic acid (- · ▽ · -), pyrene (· · ◇ · · -). Y axis is presented anti Log-base 10.

4.7.3 **The growth of *Halomonas smyrnensis* 20PY1A under different types of carbon sources**

Halomonas smyrnensis 20PY1A was tested under several kinds of aromatic compounds: Phen, Anth, Naph, and SA. In the presence of Phen, the population grew until 52×10^6 CFU/mL within 6 days. The population rose to 75.5×10^6 CFU/mL within 6 days in the

presence of Anth. Hence, the population increased to 233×10^6 CFU/mL within 6 days in the presence of Naph as a sole carbon source. Furthermore, the population climbed to 936×10^6 CFU/mL in the presence of salicylic acid (Figure 36). The dt revealed growth rate as: SA (dt= 14 hours), Naph (dt= 17 hours), Phen (dt= 18 hours), Anth (dt= 21 hours) and PYR (dt= 26 hours) (Figure 37).

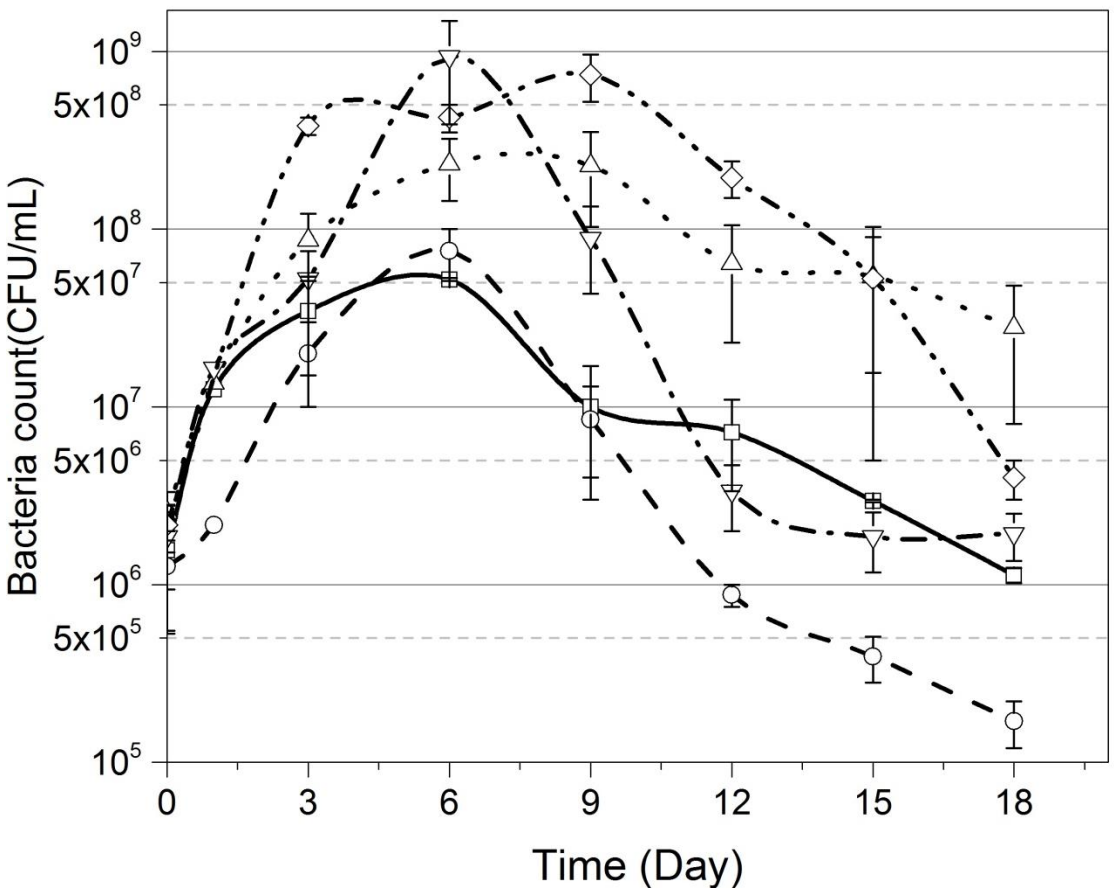


Figure 36. The growth profile of *Halomonas smyrnensis* 20PY1A in the presence of several aromatic compounds: Phenanthrene (—□—), Anthracene (- -○- -), Naphthalene (··△··), Salicylic acid (-··▽-), pyrene (··◇··). Y axis is presented in anti-Log base 10.

In general, the dt of the isolate to grow was negatively correlated with a number of rings. The lower ring (salicylic acid) is having the shortest doubling time, *Idiomarina* sp. 10PY1A (dt= 14 hours); *Halomonas shengliensis* 10PY2B (dt= 9 hours); *Halomonas*

smyrnensis 20PY1A (dt= 17 hours). Moreover, between the two 3 ring containing PAHs tested, isolates grew slower in the presence of Anth compared to the presence of Phen (Figure 37).

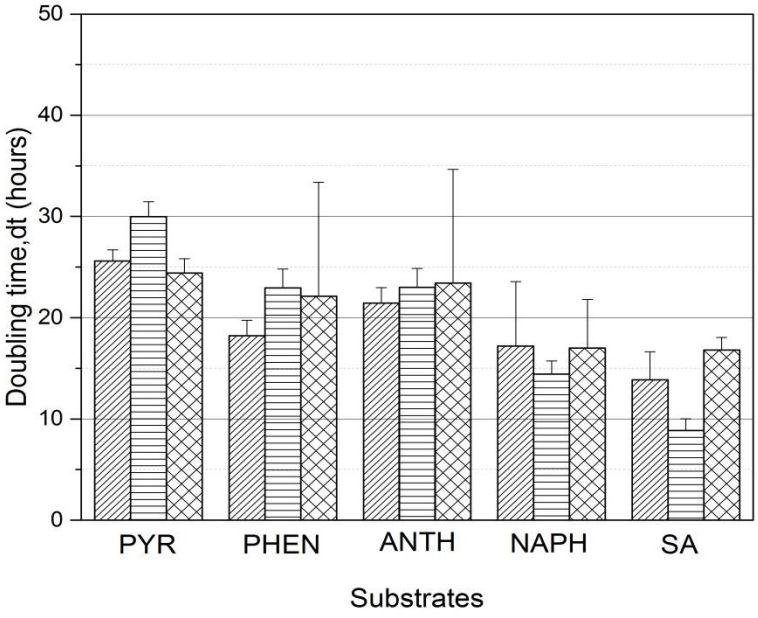


Figure 37. The doubling time of isolates in the presence of Phen, Anth, Naph, SA, PYR: *Idiomarina* sp. 10PY1A (▨); *Halomonas shengliensis* 10PY2B (▤); *Halomonas smyrnensis* 20PY1A (▩)

CHAPTER 5

DISCUSSION

5.1 Isolate characterization: Morphology and Molecular technology

Three strains were successfully isolated in this study, two were isolated under 10% NaCl concentration named 10PY1A and 10PY2B and one strain was isolated from 20% NaCl encoded with 20PY1A. Hence, the molecular analysis revealed that 10PY1A belong to family Idiomarinaceae and the others to Halomodaceae.

This study was using strain 10PY1A, genetically closed to *Idiomarina* sp., and *I. piscisalsi* sp. nov. strain TPS4-2 isolated from *pla-ra* fish in Thailand. However, the morphological characters are different. *Idiomarina piscisalsi* sp. nov.stain TPS4-2 is a slightly curved bacteria with the size of (1.2-2)×(0.3-0.4) µm [164] while *Idiomarina* sp. 10PY1A is rod-shape bacteria with size about 4.07×0.52 µm.

The family of idiomarinaceae consists of two genera, Idiomarina which was first proposed by Ivanova *et al*. [165] and Alidiomarina. Twenty-seven species were officially reported as members of genus Idiomarina: *I. abyssalis*, *I. zobellii* [165], *I. xiamenensis*, *I. aestuarii* [166], *I. aquamaris* [167], *I. baltica* [168], *I. donghaiensis* comb. nov., *I. ivanova*, *I. marina* comb. nov., *I. tainensis* comb. nov., *I. maritima* comb. nov., *I. taiwanensis* comb. nov., *I. sediminium* comb. nov., *I. homiensis* comb. nov., *I. salinarum* comb. nov., *I. insulisalsae* sp. nov. [169], *I. atlantica* sp. nov. [170], *I. seosinensis* [171] *I. fontislapidosi* sp. nov., *I. ramblicola* sp. nov. [172] *I. halophile* [173], *I. indica* [174], *I.*

loihiensis sp. nov. [175], *I. Maris* [176], *I. Planktonica* [177], *I. woesei* sp. nov. [178], and *I. piscisalsi* [164].

The genus of Idiomarina can be discovered in a wide range of environmental landscape from seashore to volcanic-deepsea. It can grow in slight salinity to hypersaline conditions (salinity at 0.5%-more than 20%) [171]. Some studies in Korea successfully isolated idiomarina from sea-shore, for example, *Idiomarina seosinensis* sp. nov. (grow at 1-20% salt) was isolated from a solar saltern consisting hypersaline water [171]. Another strain, *Idiomarina homiensis* sp. nov, was discovered in seashore sand in Korea living in 0.5-15% of salt (optimum 3-5%) and pH between 5 and 10 [179]. Some species in Idiomarinaceae also lives in an extreme environment along the bottom of the sea. *Idiomarina loihiensis* was firstly discovered in an underwater hydrothermal vent (1300 m depth). Since it inhabits extreme conditions, *I. loihiensis* survives in a wide variation of physical conditions, temperatures (4–46 °C) and salinities (from 0.5 to 20% NaCl) [180].

In addition, two isolates, 10PY2B and 20PY1A belonging to genera of Halomonas were identified in this experiment. These isolates have a rod shape with the size range from about 1 µm in length and 0.7 µm in width. This description was in line with a description from Poli *et al.* (2013) in their report of novel species *Halomonas smyrnensis*. Furthermore, their study discovered 4 subspecies of *Halomonas smyrnensis*, and these 4 subspecies, coded with AAD6^{T}, AAD4, AA17 and AAD21, were different in some aspects. Even these four subspecies are having a rod shape, they are different in the colony pigmentation. Subspesies of *Halomonas smyrnensis* AAD6^{T} was having Dark-yellow color, AAD21 was having cream-yellow color, AAD4 and AAD17 have a cream color [181]. On the other hand, the *Halomonas shengliensis* 10PY2B which was studied

in this experiment, exhibits the same morphological character with *Halomonas shengliensis* SL014B-85^T, a novel *Halomonas* species discover in 2007 by Wang *et al*. They described *Halomonas shengliensis* as rod shape bacteria with the range of size at 1.0-1.6 in length and 0.6-0.8 in width, moreover their profound study explained that this species is having several lateral flagella [182].

There are 45 species recorded as a part of the genus *Halomonas* [183] and most of the description indicated that *Halomonas* is a negative, rod bacteria. However, there are variations in this rod shape. *Halomonas salina* CECT 5288^T was reported to have a short rod shape [184] and *Halomonas maura* CECT 5298^T as a curved rod bacteria [185]. Moreover, extensive research on *Halomonas* has been conducted to figure out the potency of *Halomonas* on the production of the biopolymer. *Halomonas stenophila* HK30 [186], *Halomonas ventosae* and *Halomonas anticariensis* [187] were scientifically proven on their capacity on the production of exopolysaccharides, a biopolymer used for its bioactive property and widely utilized in industry, pharmacy, agriculture and many fields. *Halomonas* sp. also studied in levan production [188]. Levan is a microbial polysaccharide and one of its function is a raw material for stabilizer, emulsifier, and thickener [189]. In addition, some species in the genera of *Halomonas* were extensively studied for its bioremediation aspect. *Halomonas organivorans* is an efficient halophilic bacteria in biodegrading organic compounds like phenol, benzoate, and salicylate [19, 133, 183].

This study supported other research on halophilic bacteria to degrade organic compounds, especially in PolyAromatic Hydrocarbon. Various environment conditions were tested to *Idiomarina* sp. 10PY1A, *Halomonas shengliensis* 10PY2B and *Halomonas*

smyrnensis 20PY1A in degrading pyrene (4 rings Aromatic Hydrocarbon) as a sole carbon source.

5.2 Isolates growth at various conditions in the presence of pyrene as a sole carbon source

The growth of isolated strain *Idiomarina* sp. 10PY1A, *Halomonas shengliensis* 10PY2B and *Halomonas smyrnensis* 20PY1A in the presence of pyrene as a sole carbon source was observed within 18 days over various variety of conditions: temperature (10, 25, 37 and 50 ºC), pH (3, 5, 7, 9), salinity [0, 5, 10, 15, 20 % (w/v)], in presence of pyrene (0.05; 0.25; 2.5, 5, 50 mg in 50 mL BH media).

The previous research revealed many capabilities of halophilic in degrading pyrene. Pyrene concentrations at 25, 50, 75, 100 mg/L were degraded by *Ochrobactrum* sp. VA1 at 71, 64, 60.5, 32% within 4 days [190]. In addition, Erdogmus *et al*., (2013) revealed that *Haloferax* sp. C24, *Haloferax* sp. C27, *Halobacterium piscisalsi* C37, *Halobacterium ezzemoulense* C41, *Halorubrum* sp. C43, *Halorubrum ezzemoulense* C46, *Halorubrum hispanica* C50, *Halorubrum salinarum* C51, and *Haloarcula* sp. C52 grew up to 160 ppm of pyrene however at 200 ppm of pyrene, these strains were not growing [1].

5.2.1 The effect of pH on the growth of isolated strains

Alkaline conditions were optimum for growth of all isolated strains in this study. Thus, pH is playing a role in controlling the solubility of pollutant, transport process and enzyme activity [144, 190]. *Idiomarina* sp. 10PY1A, *Halomonas smyrnensis* 10PY2B

and *Halomonas shengliensis* 20PY1A was non-acidophilic bacteria due to their incapability to grow under acidic conditions, pH 3 and pH 5. The acidic conditions improve solubility of pyrene, however, such conditions reduce the activity of enzyme [192]. This study was inline with Sitdhipol *et al*., (2013) on *Idiomarina piscisalsi* sp. nov., Poli *et al.* (2013) on *Halomonas smyrnensis* sp. nov., Wang *et al.* (2007) on *Halomonas shengliensis* sp. nov [164, 181, 182].

On the JCM media (Japan collection of Microorganism) no. 377 with casamino, glutamic acid, yeast extract and many minerals, *Idiomarina piscislasi* sp. nov. was capable of growing under pH range 6-9 (optimum at 8) [164]. Since its first report on this species was in 2013, no official publication can be found about this species especially its application. On the other hand, using the medium A containing yeast extract and other minerals, Poli *et al.* (2013) discovered different pH range from 4 subspecies of *Halomonas smyrnensis* sp. nov. as follow: *Halomonas smyrnensis* AAD6^{T} (5.5-8.5); *Halomonas smyrnensis* AAD4 (6.5-7.5); *Halomonas smyrnensis* AAD 17(6.5-7); *Halomonas smyrnensis* AAD21 (6.5-8.0) [181]. This isolate was intensively studied for its production of levan [189]. This finding was analogous with the observation of Wang *et al.* [182]. Furthermore, using artificial sea water containing peptone, yeast extract, and many minerals, Wang *et al.* (2007) described that *Halomonas shengliensis* sp. nov. was capable of growing at pH range 8.0-9.0 with the optimum growth observed at pH 8.5 [182]. The recent study in this species by Gomes *et al*. [130] revealed that *Halomonas shengliensis* MCAT 10 degraded 57% of pyrene within 20 days under 9% of NaCl at 28 ^{o}C, pH of 7

Many studies were carried out on the effect of pH on PAHs degradation. optimum pyrene degradation of Halophilic *Thalassospira* sp. TSL5-1 achieved at pH 7.4 (42.5%), however at pH 6.1 the degradation fell to 6.2% within 25 days [40]. Phenanthrene, a 3-ring PAH, was efficiently degraded by *Martelella* sp AD-3 at pH 9 [137]. On the opposite, non-halophile *Micobacterium vanbaalenii* PYR-1 was able to degrade pyrene and phenanthrene faster at pH 6.4, compared with pH 7.5 [192].

5.2.2 The effect of temperature on the growth of isolated strains

In this study, the effect of various temperature on the growth of the isolated strains has been tested in presence of pyrene. These three strains sustained growth at 10, 25 and 37 ºC, yet no growth was observed at 50 ºC, and the optimum temperature of growth was between 25 and 37 ºC. This range of temperature resembles the temperature during isolation and temperature from the origin of the sample. The annual seawater temperature variation in the Arabian gulf is about 19.5-30.9 ºC, and the air temperature varied between < 10 ºC to > 40 ºC [43].

This study complemented the finding of Sitdhipol *et al.* on *Idiomarina piscisalsi* sp. nov. which shows the optimum growth of this isolate on JCM media was between 30-37º C and maximum temperature at 45 ºC [164]. More similarity is found with to *Halomonas shengliensis* 10PY2B and *Halomonas Smyrnensis* 20PY1A since the optimum growth in the presence of pyrene recorded at Temperature 25ºC. However, in another study, in which on media A and artificial sea water were used, the optimum growth of *Halomonas shengliensis* and *Halomonas smyrnensis* were 30 ºC and 37 ºC, respectively [181, 182].

Temperature affected the microbial composition and rate of organic compound metabolism by isolates [191]. Nedwell, D.B (1999) reported that the temperature affected the stability of membrane, causing ineffective and in some condition a failure, of protein's transport. This inefficiency of protein's transport will influence the capability of a microorganism to acquire the substrate accordingly, the deficiency of nutrient occur and the retarded growth rate is assured [192, 193]. Yet, the biotransformation or biodegradation of organic compound may proceed in certain temperature level [195], [196]. Mesocosm study conducted by Cuolon *et al.* (2005) in the sub-Antarctic environment illustrated the feasibility of degradation of organic compounds, TPH and PAHs, at temperature 10ºC [197]. However, the process was slow, in extent, the lag phase was longer compare with higher temperature [198]. In general, the degradation of PAHs is slower at the lower temperature [198]. The consortium bacteria studied by Sartoros *et al*. (2005) showed the reduction in biodegradation efficiency of pyrene at temperature 10 ºC, with the degradation efficiency of 2 mg/L of initial pyrene was measured at 66.1% (temperature at 25 ºC) and 61.5% (temperature at 10 ºC) within 100 days. However, other study found the opposite result [199], the temperature did not significantly affect the biodegradation of phenanthrene. The psychrotrophic bacteria from the creosote-contaminated site showed no difference in the degradation rate of phenanthrene between 10 ºC and 25 ºC (14%) [199].

5.2.3 The effect of salinity on the growth of the isolates

The isolates had been exposed to several salinity from the absence of NaCl (0%), varied to 5, 10, 15 and 20% NaCl. However, none of the isolates could grow in the absence of NaCl. This range of salinity resembles the salinity used during isolation. Disintegration or

dehydration of cell wall become a major impact on salinity alteration, also the depletion of dissolved oxygen in environment and reduction of the bioavailability of substrate may happen [145].

The finding of this study was in line with Sitdhipol *et al*. (2013) on *Idiomarina piscisalsi* sp. nov. which is living in the salinity range of 3%-25% (w/v). *Halomonas shengiensis* sp. nov reported by Wang *et al*. (2007) grew optimally at 5-15% (w/v) NaCl on Medium A [182]. In addition, *Halomonas smyrnensis* sp. nov. was reported to grow in the salinity range of, 3%-20% (w/v) [181].

In another study on PAHs, Halophilic *Thalassospira* sp. TSL5-1 (isolated at 5% NaCl) exhibited a degradation capability of pyrene at moderate salinity: the optimum degradation of pyrene by this strain was 3.5-5% NaCl. The degradation rate decreased at low salinity 0 and 1.5 %, and at higher salinity (more than 9.5%) [40]. Likewise the consortium of genera Pseudomonas completely degraded phenanthrene (initial concentration 100 ppm) within 4 days at salinity 10% and temperature 30º C. However, the degradation rate decreased when the salinity increased [15].

5.2.4 The effect of initial pyrene concentration on the growth of isolates

The amount of pyrene had been varied, and both pH and temperature were adjusted at 7 and 37 ºC, respectively. Isolate *Idiomarina* sp. 10PY1A and *Halomonas shengliensis* 10PY2B were exposed to 10% NaCl, while *Halomonas smyrnensis* 20PY1A was grown at 20% NaCl.

The initial pyrene concentration has a negative correlation with the growth rate of all isolates in which the three isolates grew faster in low initial pyrene concentration. The toxic property of pyrene at higher concentration inhibited the growth of isolates. The

study of Feyza *et al*. (2013) showed the same outcome, there was no growth observed in initial pyrene at 200 ppm and in the low pyrene concentration, the growth is limited to the availability of carbon source over time [200]. Radzi *et al.* (2015) confirmed this finding, the growth was observed until concentration pyrene at 150 mg/L [201]. However, in their research, they claimed that the origin-source of isolate determined the capability of the isolate in degrading pyrene.

5.3 The degradation rate of pyrene by isolated strains

The isolated strains were grown in the presence of 2.5 mg of pyrene (50 mg/L) at temperature 37 ºC and pH 7, and the decrease of pyrene in media was monitored. The growing isolated strains in BH media were subjected to liquid-liquid extraction followed by the GC-MS procedure to measure the residual pyrene concentration.

The fastest degradation of pyrene was observed with *Halomonas smyrnensis* 20PY1A, with the rate of 0.0495 day^{-1}, followed by *Idiomarina* sp. 10PY1A and *Halomonas shengiensis* 10PY2B. Zhou et *et al*. (2016) discovered that *Thalassospira* sp. strain TSL 5-1 capable of degrading 44% pyrene within 25 days [40]. A *Mycobacterium* sp. strain KR2 was able to degrade 60% of pyrene within 8 days at 20 ºC [202]. Another research revealed the capability of consortium in degrading pyrene. The consortium of *Orchrobacter* sp., *Enterobacter cloacae* and *Stenotrophomonas maltophilia* was able to degrade 89%, 88%, 74%, 52% pyrene at 5, 10, 50 and 100 ppm, respectively within 4 days [203].

The genera of *Idiomarina* and *Halomonas* were explored for their potency on biotechnology. *Idiomarina* sp. strain N11 was capable of growing in the presence of naphthalene, and up to 52.78% was biodegraded from 200 mg/L initial naphthalene

within 7 days [146]. Above 400 mg/L of naphthalene, no degradation was observed in this strain. *Ochrobactrum* sp. VA1 showed a decrease of degradation efficiency with the increase of pyrene concentration: 25 ppm (71%); 50 ppm (64%); 75% (60.5%); 100 ppm (32%) [190]. Other strains of halomonas exhibited various utilities. *Halomonas stenophila* HK30 was utilized to produce haloglycan, an exopolysaccharide [186] and *Halomonas stenophila* sp. nov. produced sulphate exopolysaccharides [204]. *Halomonas campisalis* reported in its biodegradability of benzoate and salicylate [205]. *Halomonas* sp. PH2-2 living at salinity 5-25% was recorded to degrade phenol up to 1100 ppm [206].

5.4 The growth of isolated strain in the presence of other aromatic compounds as a sole carbon source

The extended study proved the growth of isolates under different Aromatic Compounds: Phenanthrene, Anthracene, Naphthalene, and Salicylic Acid as a sole carbon source. In general, the number of rings has a negative correlation with the growth rate of isolates. SA has the lower number of aromatic compound studied in this experiment and was associated with the highest growth for the 3 tested strains.

A similar conclusion has also been reported by Coulun *et al*. (2005) on analysis of Pyrene, Phenanthrene, dibenzothiophenes, and Naphthalene. The decrease in the degradation rate of those compounds was a function of size/number of the ring of PAHs [197] for the impact of the substrate solubility. *Pseudomonas florescens* degraded Anth, Phen and PYR to an undetectable level (<0.001 ppm) within 12-80 hours, 12-44 hours, 24-72 hours [143]. A strain of halophilic may be isolated under certain organic/aromatic compound, yet the isolated strain was able to degrade a wide variety of substrate. For example, moderately halophilic *Halomonas organivorans* isolated using benzoic acid was

able to degrade many aromatic acids: salicylic, cinnamic, benzoic, phenyl acetic, p-cuomaric, p-aminosalicylic acid, ferulic, p-hydroxybenzoic [207].

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The petroleum contaminated soil sample from sea-shore of Jubail area was used as a source of halophilic bacteria capable of degrading pyrene, by enrichment techniques in various salinity conditions. Two strains, 10PY1A and 10PY2B, were isolated in 10% NaCl salinity, while one strain, 20PY1A, was isolated at 20% NaCl. Morphological and molecular analyses were conducted to ascertain the species: 10PY1A belonged to *Idiomarina* sp., 10PY2B was *Halomonas shengliensis* and 20PY1A was *Halomonas smyrnensis*. Several tests have been conducted to establish the best condition of growth of these bacteria in presence of pyrene. : The variation of temperature (10, 25, 37, 50) ºC, variation of pH (3, 5, 7, 9), variation of salinity (0, 5, 10, 15, 20) % w/v NaCl, variation of initial pyrene concentration (1, 5, 50, 100, 1000) mg/L, and also the several aromatic compounds variety have been tested (Phenanthrene, Anthracene, Naphthalene, Salicylic acid).

In addition to the growth profile, the doubling time was used to compare the growth of bacteria in various condition. Overall the results show that these three isolates were non-acidophilic since no growth observed at pH 3 and pH 5, yet, they grew better at an alkaline condition, pH 7 and pH 9. The isolated strains also survived at low temperature (10ºC) and no growth monitored at high temperature (50ºC). Furthermore, the best

temperature for all isolated strains to grow was around 25-37ºC. In addition, no isolated strains survived in the absence of NaCl (0% w/v). The optimum salinity of each strain to grow was varied: *Idiomarina* sp. 10PY1A at 5%, *Halomonas shengliensis* 10PY2B and *Halomonas smyrnensis* 20PY1A at 10%. The initial pyrene concentration also affected the growth kinetic, the lower the initial pyrene concentration, the faster the growth of strains. On the contrary, no growth was observed at a high concentration of pyrene, 1000 ppm.

Finally, the fastest degradation rate was represented by *Halomonas smyrnensis* 20PY1A with the degradation constant at 0.0495/day. Moreover, these isolated strains exhibited a wide range of biodegradation capability of aromatic compounds, from 1 ring (salicylic acid), 2 rings (Naphthalene), 3 rings (Phenanthrene and Anthracene) up to 4 rings (pyrene).

6.2 RECOMMENDATION

The writer recommended some future experiments might be done:

1. To investigate the biochemical pathways of pyrene biodegradation in these strains.
2. To analyze the production of bio-surfactant by these halophilic bacteria or to add a synthetic surfactant to increase the availability of pyrene and other PAHs.
3. To carry out various pilot laboratory studies to investigate the ability of these strain to biodegrade pyrene in high saline conditions as produced water.

References

[1] S. F. Erdogmus, B. Mutlu, S. E. Korcan, K. Guven, and M. Konuk, "Aromatic Hydrocarbon Degradation by Halophilic Archaea Isolated from Çamalt ı Saltern , Turkey," *Water. Air. Soil Pollut.*, vol. 224, pp. 1–9, 2013.

[2] J. Yan, L. Wang, P. P. Fu, and H. Yu, "Photomutagenicity of 16 polycyclic aromatic hydrocarbons from the US EPA priority pollutant list," *Mutat Res*, vol. 4, no. 164, pp. 99–108, 2011.

[3] P. Arulazhagan, C. Sivaraman, S. A. Kumar, M. Aslam, and J. R. Banu, "Co-metabolic degradation of benzo (e) pyrene by halophilic bacterial consortium at different saline conditions," *J. Environ. Biol.*, vol. 35, pp. 1–8, 2014.

[4] J. a. Simon and J. a. Sobieraj, "Contributions of common sources of polycyclic aromatic hydrocarbons to soil contamination," *Remediat. J.*, vol. 16, no. 3, pp. 25–35, 2006.

[5] S. Alagić, B. S. Maluckov, and V. B. Radojičić, "How can plants manage polycyclic aromatic hydrocarbons? May these effects represent a useful tool for an effective soil remediation? A review," *Clean Technol. Environ. Policy*, vol. 17, no. 3, pp. 597–614, 2015.

[6] USEPA, "APPENDIX B: Documentation of 7-PAHs and 16-PAHs national emission extimates," *USEPA website*, 2013. [Online]. Available: https://www3.epa.gov/airtoxics/112c6/app_b.pdf. [Accessed: 08-Sep-2016].

[7] G. Zhang, Z. Pan, X. Wang, X. Mo, and X. Li, "Distribution and accumulation of polycyclic aromatic hydrocarbons (PAHs) in the food web of Nansi Lake, China," *Environ. Monit. Assess.*, vol. 187, no. 4, pp. 1–12, 2015.

[8] E. O. Nwaichi and S. A. Ntorgbo, "Assessment of PAHs levels in some fish and seafood from different coastal waters in the Niger Delta," *Toxicol. Reports*, vol. 3, pp. 167–172, 2016.

[9] P. Arulazhagan and N. Vasudevan, "Role of a moderately halophilic bacterial consortium in the biodegradation of polyaromatic hydrocarbons.," *Mar. Pollut. Bull.*, vol. 58, no. 2, pp. 256–262, Feb. 2009.

[10] M. Ligaray, S. S. Baek, H.-O. Kwon, S.-D. Choi, and K. H. Cho, "Watershed-scale modeling on the fate and transport of polycyclic aromatic hydrocarbons (PAHs)," *J. Hazard. Mater.*, vol. 320, pp. 442–457, 2016.

[11] D. M. Al-Mailem, N. a Sorkhoh, H. Al-Awadhi, M. Eliyas, and S. S. Radwan, "Biodegradation of crude oil and pure hydrocarbons by extreme halophilic archaea from hypersaline coasts of the Arabian Gulf.," *Extremophiles*, vol. 14, no. 3, pp. 321–8, May 2010.

[12] S. Llado, S. Covino, A. M. Solanas, M. Vi??as, M. Petruccioli, and A. D'annibale, "Comparative assessment of bioremediation approaches to highly recalcitrant PAH degradation in a real industrial polluted soil," *J. Hazard. Mater.*, vol. 248–249, no. 1, pp. 407–414, 2013.

[13] B. Biswas, B. Sarkar, R. Rusmin, and R. Naidu, "Bioremediation of PAHs and VOCs: Advances in clay mineral-microbial interaction," *Environ. Int.*, vol. 85, pp. 168–181, 2015.

[14] A. Sharma, S. B. Singh, R. Sharma, P. Chaudhary, A. K. Pandey, R. Ansari, V. Vasudevan, A. Arora, S. Singh, S. Saha, and L. Nain, "Enhanced biodegradation of PAHs by microbial consortium with different amendment and their fate in in-situ condition," *J. Environ. Manage.*, vol. 181, pp. 728–736, 2016.

[15] G. Guo, F. He, F. Tian, Y. Huang, and H. Wang, "Effect of salt contents on enzymatic activities and halophilic microbial community structure during phenanthrene degradation," *Int. Biodeterior. Biodegrad.*, vol. 110, pp. 8–15, 2016.

[16] B. Balamurugan, M. Thirumarimurugan, and T. Kannadasan, "Anaerobic degradation of textile dye bath effluent using Halomonas sp," *Bioresour. Technol.*, vol. 102, no. 10, pp. 6365–6369, 2011.

[17] L. F. Martins and R. S. Peixoto, "Biodegradation of petroleum hydrocarbons in hypersaline environements," *Brazilian J. Microbiol.*, pp. 865–872, 2012.

[18] S. A. Cantrell, L. Casillas-Martínez, and M. Molina, "Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques.," *Mycol. Res.*, vol. 110, no. Pt 8, pp. 962–970, Aug. 2006.

[19] B. Z. Fathepure, "Recent studies in microbial degradation of petroleum hydrocarbons in hypersaline environments.," *Front. Microbiol.*, vol. 5, no. April, pp. 1–16, Jan. 2014.

[20] H. Al-Madfa, M. A. R. Abdel-Moati, and A. Al-Naama, "Beach tar contamination on the Qatari coastline of the gulf," *Environ. Int.*, vol. 25, no. 4, pp. 505–513, 1999.

[21] Y. Gao, J. Wang, S. Guo, Y.-L. Hu, T. Li, R. Mao, and D.-H. Zeng, "Effects of salinization and crude oil contamination on soil bacterial community structure in the Yellow River Delta region, China," *Appl. Soil Ecol.*, vol. 86, pp. 165–173, 2015.

[22] M. Sadiq, "Metal contamination in sediments from a desalination plant effluent outfall area," *Sci. Total Environ.*, vol. 287, no. 1–2, pp. 37–44, 2002.

[23] P. S. Bray, C. M. Jones, S. J. Fallon, J. J. Brocks, and S. C. George, "Radiocarbon analysis of halophilic microbial lipids from an Australian salt lake," *Quat. Res.*, vol. 77, no. 1, pp. 104–109, 2012.

[24] R. Golan, I. Gavrieli, J. Ganor, and B. Lazar, "Controls on the pH of hyper-saline

lakes - A lesson from the Dead Sea," *Earth Planet. Sci. Lett.*, vol. 434, pp. 289–297, 2016.

[25] A. Ventosa, A. Oren, and Y. Ma, *Halophiles and hypersaline environments: Current research and future Treands*. Berlin, Heidelberg: Springer-Verlag, 2011.

[26] N. Gunde-Cimerman, P. Zalar, S. Hoog, and A. PlemenitaÅ¡, "Hypersaline waters in salterns â□" natural ecological niches for halophilic black yeasts," *FEMS Microbiol. Ecol.*, vol. 32, no. 3, pp. 235–240, Jun. 2000.

[27] P. K. Abdul Azis, I. Al-Tisan, M. Al-Daili, T. N. Green, A. G. I. Dalvi, and M. A. Javeed, "Effects of environment on source water for desalination plants on the eastern coast of Saudi Arabia," *Desalination*, vol. 132, no. 1–3, pp. 29–40, 2000.

[28] A. Purnama, H. H. Al-Barwani, and R. Smith, "Calculating the environmental cost of seawater desalination in the Arabian marginal seas," *Desalination*, vol. 185, no. 1–3, pp. 79–86, 2005.

[29] G. L. Meerganz von Medeazza, "'Direct' and socially-induced environmental impacts of desalination," *Desalination*, vol. 185, no. 1–3, pp. 57–70, 2005.

[30] R. Smith, A. Purnama, and H. H. Al-Barwani, "Sensitivity of hypersaline Arabian Gulf to seawater desalination plants," *Appl. Math. Model.*, vol. 31, no. 10, pp. 2347–2354, Oct. 2007.

[31] C. Cui, L. Ma, J. Shi, K. Lin, Q. Luo, and Y. Liu, "Metabolic pathway for degradation of anthracene by halophilic Martelella sp. AD-3," *Int. Biodeterior. Biodegradation*, vol. 89, pp. 67–73, 2014.

[32] M. R. L. Bonfá, M. J. Grossman, E. Mellado, and L. R. Durrant, "Biodegradation of aromatic hydrocarbons by Haloarchaea and their use for the reduction of the chemical oxygen demand of hypersaline petroleum produced water," *Chemosphere*, vol. 84, pp. 1671–1676, 2011.

[33] OPEC, "OPEC: Annual statistical Bulletin 2016," *OPEC*, p. 128, 2016.

[34] N. Taher and B. Hajjar, "Chapter 2: Environmental Concerns and Policies in Saudi Arabia," in *Energy and Environment in Saudi Arabia: Concern and Opportunities*, 2014, pp. 1–203.

[35] M. Ahmad, A. S. Bajahlan, and W. S. Hammad, "Industrial effluent quality, pollution monitoring and environmental management," *Environ. Monit. Assess.*, vol. 147, no. 1–3, pp. 297–306, 2008.

[36] Z. Wang, F. Pan, A. E. L. Hesham, Y. Gao, Y. Zhang, and M. Yang, "Impacts of produced water origin on bacterial community structures of activated sludge," *J. Environ. Sci. (China)*, vol. 37, pp. 192–199, 2015.

[37] J. D. Arthur, B. G. Langhus, and D. Ph, "WATER TREATMENT TECHNOLOGIES," no. March 2005, pp. 1–53.

[38] C. A. Nicholson, “BIODEGRADATION OF PETROLEUM HYDROCARBONS BY,” Oklahoma State University, 2005.

[39] Z. Ghasemi Naraghi, S. Yaghmaei, M. M. Mardanpour, and M. Hasany, “Produced Water Treatment with Simultaneous Bioenergy Production Using Novel Bioelectrochemical Systems,” *Electrochim. Acta*, vol. 180, pp. 535–544, 2015.

[40] H. Zhou, H. Wang, Y. Huang, and T. Fang, “Characterization of pyrene degradation by halophilic Thalassospira sp . strain TSL5-1 isolated from the coastal soil of Yellow Sea , China,” *Int. Biodeterior. Biodegradation*, vol. 107, pp. 62–69, 2016.

[41] A. Elhakeem and W. Elshorbagy, “Hydrodynamic evaluation of long term impacts of climate change and coastal effluents in the Arabian Gulf,” *Mar. Pollut. Bull.*, vol. 101, no. 2, pp. 667–685, 2015.

[42] S. de Mora, S. W. Fowler, E. Wyse, and S. Azemard, “Distribution of heavy metals in marine bivalves, fish and coastal sediments in the Gulf and Gulf of Oman.,” *Mar. Pollut. Bull.*, vol. 49, no. 5–6, pp. 410–424, Sep. 2004.

[43] A. Elhakeem, W. Elshorbagy, and T. Bleninger, “Long-term hydrodynamic modeling of the Arabian Gulf,” *Mar. Pollut. Bull.*, vol. 94, no. 1–2, pp. 19–36, 2015.

[44] A. Elhakeem and W. Elshorbagy, “Evaluation of the long-term variability of seawater salinity and temperature in response to natural and anthropogenic stressors in the Arabian Gulf.,” *Mar. Pollut. Bull.*, vol. 76, no. 1–2, pp. 355–9, Nov. 2013.

[45] N. Mezhoud, M. Temimi, J. Zhao, M. R. Al Shehhi, and H. Ghedira, “Analysis of the spatio-temporal variability of seawater quality in the southeastern Arabian Gulf,” *Mar. Pollut. Bull.*, vol. 106, no. 1–2, pp. 127–138, 2016.

[46] C. V John, S. L. Coles, and A. Abozed, “Seasonal cycles of temperature, salinity and water masses of the Western Arabian Gulf ,” *Oceanol. Acta*, vol. 13, no. 3, pp. 273–281, 1990.

[47] J. Kämpf and M. Sadrinasab, “The circulation of the Persian Gulf: a numerical study,” *Ocean Sci. Discuss.*, vol. 2, no. 3, pp. 129–164, 2005.

[48] A. M. Freije, “Heavy metal, trace element and petroleum hydrocarbon pollution in the Arabian Gulf: Review,” *J. Assoc. Arab Univ. Basic Appl. Sci.*, Mar. 2014.

[49] A. Basaham, “Distribution and Partitioning of Heavy Metals in Subtidal Sediments of the Arabian Gulf Coast of Saudi Arabia,” *J. King Abdulaziz Univ. Sci.*, vol. 21, no. 1, pp. 201–222, 2010.

[50] A. a Al-Taani, A. Batayneh, Y. Nazzal, H. Ghrefat, E. Elawadi, and H. Zaman, “Status of trace metals in surface seawater of the Gulf of Aqaba, Saudi Arabia.,” *Mar. Pollut. Bull.*, vol. 86, no. 1–2, pp. 582–90, Sep. 2014.

[51] M. A. . Zyadah and M. G. . Almoteiry, “Evaluation of Environmental Pollution in the Arabian Gulf Coast at the Eastern Province , SA,” *Asian Trans. Basic Appl. Sci.*, vol. 2, no. 3, pp. 14–21, 2012.

[52] I. Al-But’hie and M. Eben Saleh, “Urban and industrial development planning as an approach for Saudi Arabia: the case study of Jubail and Yanbu,” *Habitat Int.*, vol. 26, no. 1, pp. 1–20, 2002.

[53] A. S. El-Sorogy, M. Youssef, K. Al-Kahtany, and N. Al-Otaiby, “Assessment of arsenic in coastal sediments, seawaters and molluscs in the Tarut Island, Arabian Gulf, Saudi Arabia,” *J. African Earth Sci.*, vol. 113, pp. 65–72, 2016.

[54] J. Michel, “Chapter 37: 1991 Gulf War Oil Spill,” in *Oil Spill Science and Technology*, Elsevier Inc., 2011, pp. 1127–1132.

[55] L. Berthe-Corti and T. Höpner, “Geo-biological aspects of coastal oil pollution,” *Palaeogeogr. Palaeoclimatol. Palaeoecol.*, vol. 219, no. 1–2, pp. 171–189, 2005.

[56] A. R. G. Price, “Impact of the 1991 Gulf War on the coastal environment and ecosystems: Current status and future prospects,” *Environ. Int.*, vol. 24, no. 1–2, pp. 91–96, 1998.

[57] T. V. Joydas, M. A. Qurban, A. Al-Suwailem, P. K. Krishnakumar, Z. Nazeer, and N. A. Cali, “Macrobenthic community structure in the northern Saudi waters of the Gulf, 14years after the 1991 oil spill,” *Mar. Pollut. Bull.*, vol. 64, no. 2, pp. 325–335, 2012.

[58] A. C. Bejarano and J. Michel, “Large-scale risk assessment of polycyclic aromatic hydrocarbons in shoreline sediments from Saudi Arabia: Environmental legacy after twelve years of the Gulf war oil spill,” *Environ. Pollut.*, vol. 158, no. 5, pp. 1561–1569, 2010.

[59] M. A. Vazquez, K. W. Allen, and Y. M. Kattan, “Long-term effects of the 1991 Gulf War on the hydrocarbon levels in clams at selected areas of the Saudi Arabian Gulf coastline,” *Mar. Pollut. Bull.*, vol. 40, no. 5, pp. 440–448, 2000.

[60] A. A. Al-Thukair, R. M. M. Abed, and L. Mohamed, “Microbial community of cyanobacteria mats in the intertidal zone of oil-polluted coast of Saudi Arabia,” *Mar. Pollut. Bull.*, vol. 54, no. 2, pp. 173–179, 2007.

[61] N. A. Sorkhoh, H. Al-Awadhi, D. M. Al-Mailem, M. K. Kansour, M. Khanafer, and S. S. Radwan, “Agarolytic bacteria with hydrocarbon-utilization potential in fouling material from the Arabian Gulf coast,” *Int. Biodeterior. Biodegrad.*, vol. 64, no. 7, pp. 554–559, 2010.

[62] A. Fakhru’l-Razi, A. Pendashteh, L. C. Abdullah, D. R. A. Biak, S. S. Madaeni, and Z. Z. Abidin, “Review of technologies for oil and gas produced water treatment.,” *J. Hazard. Mater.*, vol. 170, no. 2–3, pp. 530–551, Oct. 2009.

[63] J. M. Younker and M. E. Walsh, “Bench-scale investigation of an integrated

adsorption-coagulation-dissolved air flotation process for produced water treatment," *J. Environ. Chem. Eng.*, vol. 2, no. 1, pp. 692–697, 2014.

[64] R. Hosny, M. Fathy, M. Ramzi, T. Abdel Moghny, S. E. M. Desouky, and S. A. Shama, "Treatment of the oily produced water (OPW) using coagulant mixtures," *Egypt. J. Pet.*, pp. 0–5, 2015.

[65] J. M. Ranck, R. S. Bowman, J. L. Weeber, L. E. Katz, and E. J. Sullivan, "BTEX Removal from Produced Water Using Surfactant-Modified Zeolite," no. March, pp. 434–442, 2005.

[66] J. a Simpson and R. S. Bowman, "Nonequilibrium sorption and transport of volatile petroleum hydrocarbons in surfactant-modified zeolite.," *J. Contam. Hydrol.*, vol. 108, no. 1–2, pp. 1–11, Aug. 2009.

[67] G. R. Alther, "Organically modified clay removes oil from water," *Waste Manag.*, vol. 15, no. 8, pp. 623–628, 1996.

[68] C. R. Altare, R. S. Bowman, L. E. Katz, K. a. Kinney, and E. J. Sullivan, "Regeneration and long-term stability of surfactant-modified zeolite for removal of volatile organic compounds from produced water," *Microporous Mesoporous Mater.*, vol. 105, no. 3, pp. 305–316, Oct. 2007.

[69] Y. Qiao, Y. Yang, J. Gu, and J. Zhao, "Distribution and geochemical speciation of heavy metals in sediments from coastal area suffered rapid urbanization, a case study of Shantou Bay, China.," *Mar. Pollut. Bull.*, vol. 68, no. 1–2, pp. 140–146, Mar. 2013.

[70] G. Beall, "The use of organo-clays in water treatment," *Appl. Clay Sci.*, vol. 24, no. 1–2, pp. 11–20, Nov. 2003.

[71] G. T. Tellez, N. Nirmalakhandan, and J. L. Gardea-Torresdey, "Performance evaluation of an activated sludge system for removing petroleum hydrocarbons from oilfield produced water," *Adv. Environ. Res.*, vol. 6, no. 4, pp. 455–470, Oct. 2002.

[72] E. Attorney, "United States Patent: Patent no 5,135,656 (Process for removing water soluble organic compounds from produced water," 1992.

[73] I. E. Mousa, "Total petroleum hydrocarbon degradation by hybrid electrobiochemical reactor in oilfield produced water," *Mar. Pollut. Bull.*, vol. 109, no. 1, pp. 356–360, 2016.

[74] S. Mondal and S. R. Wickramasinghe, "Produced water treatment by nanofiltration and reverse osmosis membranes," *J. Memb. Sci.*, vol. 322, no. 1, pp. 162–170, Sep. 2008.

[75] P. Xu, J. E. Drewes, D. Heil, and G. Wang, "Treatment of brackish produced water using carbon aerogel-based capacitive deionization technology.," *Water Res.*, vol. 42, no. 10–11, pp. 2605–17, May 2008.

[76] D. T. Meijer and C. Madin, “Removal of dissolved and dispersed hydrocarbons from oil and gas produced water with MPPE technology to reduce toxicity and allow water reuse,” *Aust. Pet. Prod. Explor. Assoc. J.*, no. 50, pp. 1–12, 2010.

[77] T. Sirivedhin, J. McCue, and L. Dallbauman, “Reclaiming produced water for beneficial use: salt removal by electrodialysis,” *J. Memb. Sci.*, vol. 243, no. 1–2, pp. 335–343, Nov. 2004.

[78] A. M. Z. Ramalho, C. A. Martínez-Huitle, and D. R. Da Silva, “Application of electrochemical technology for removing petroleum hydrocarbons from produced water using a DSA-type anode at different flow rates,” *Fuel*, vol. 89, no. 2, pp. 531–534, Feb. 2010.

[79] R. Shpiner, G. Liu, and D. C. Stuckey, “Treatment of oilfield produced water by waste stabilization ponds: biodegradation of petroleum-derived materials.,” *Bioresour. Technol.*, vol. 100, no. 24, pp. 6229–6235, Dec. 2009.

[80] Q. Li, C. Kang, and C. Zhang, “Waste water produced from an oilfield and continuous treatment with an oil-degrading bacterium,” *Process Biochem.*, vol. 40, no. 2, pp. 873–877, Feb. 2005.

[81] M. Lu, Z. Zhang, W. Yu, and W. Zhu, “Biological treatment of oilfield-produced water: A field pilot study,” *Int. Biodeterior. Biodegradation*, vol. 63, no. 3, pp. 316–321, Apr. 2009.

[82] M. Sherafatmand and H. Y. Ng, “Using sediment microbial fuel cells (SMFCs) for bioremediation of polycyclic aromatic hydrocarbons (PAHs),” *Bioresour. Technol.*, vol. 195, pp. 122–132, 2015.

[83] ATSDR, “Toxicity of Polycyclic Aromatic Hydrocarbons (PAHs): What Are Polycyclic Aromatic Hydrocarbons (PAHs)? | ATSDR - Environmental Medicine & Environmental Health Education - CSEM,” 2015. [Online]. Available: http://www.atsdr.cdc.gov/csem/csem.asp?csem=13&po=4. [Accessed: 07-Mar-2015].

[84] R. Peng, A. Xiong, Y. Xue, X. Fu, F. Gao, W. Zhao, and Y. Tian, “Microbial biodegradation of polyaromatic hydrocarbons,” *FEMS Microbiol. Rev.*, vol. 32, pp. 927–955, 2008.

[85] PubChem, “Pyrene | C16H10 - PubChem,” 2015. [Online]. Available: http://pubchem.ncbi.nlm.nih.gov/compound/31423#section=Top. [Accessed: 07-Mar-2015].

[86] U. EPA, “Pyrene Fact Sheet - pyrene.pdf,” *CAS Number: 129-00-0*, 2012. [Online]. Available: http://www.epa.gov/epawaste/hazard/wastemin/minimize/factshts/pyrene.pdf. [Accessed: 10-Mar-2015].

[87] Canadian Council of Ministers of the Environment, *Canadian Soil Quality*

Guidelines: Carcinogenic and Other PAHs. Quebec, 2010.

[88] P. Shivanand and G. Mugeraya, "Halophilic bacteria and their compatible solutes – osmoregulation and potential applications," *Curr. Sci.*, vol. 100, no. 10, pp. 1516–1521, 2011.

[89] A. Najjari, M. S. Elshahed, A. Cherif, and N. H. Youssef, "Patterns and Determinants of Halophilic Archaea (Class Halobacteria) Diversity in Tunisian Endorheic Salt Lakes and Sebkhet Systems," *Appl. Environ. Microbiol.*, vol. 81, no. 13, pp. 4432–4441, 2015.

[90] A. Oren, "Molecular ecology of extremely halophilic Archaea and Bacteria," *FEMS Microbiol. Ecol.*, vol. 39, no. 1, pp. 1–7, Jan. 2002.

[91] M. K. Sarwar, I. Azam, and T. Iqbal, "Biology and Applications of Halophilic Bacteria and Archaea : A Review," vol. 11, no. 3, pp. 98–103, 2015.

[92] M. Kamekura, "Diversity of extremely halophilic bacteria," *Extremophiles*, vol. 2, no. 3, pp. 289–295, Aug. 1998.

[93] W. Liesack and K. A. I. Finster, "Phylogenetic Analysis of Five Strains of Gram-Negative , Obligately Anaerobic , Sulfur-Reducing Bacteria and Description of Desulfirornusa gen . nov ., Including Desulfuromusa kysingii sp . nov ., Desulfurornusa bakii sp . nov ., and Desulfuromusa succino," *Int. J. Syst. Bacteriol.*, no. 9, pp. 753–758, 1994.

[94] H. K. Gupta, A. Singh, and R. Sharma, "Genome sequence of Idiomarina sp. Strain A28L, Isolated from Pangong Lake, India," *J. Bacteriol.*, vol. 193, no. 20, pp. 5875–5876, 2011.

[95] A. Oren, "Microbial life at high salt concentrations: phylogenetic and metabolic diversity.," *Saline Systems*, vol. 4, no. 1, p. 2, 2008.

[96] K. P. Williams, J. J. Gillespie, B. W. S. Sobral, E. K. Nordberg, E. E. Snyder, J. M. Shallom, and A. W. Dickerman, "Phylogeny of gammaproteobacteria," *J. Bacteriol.*, vol. 192, no. 9, pp. 2305–2314, 2010.

[97] M. Niepceron, F. Martin-Laurent, M. Crampon, F. Portet-Koltalo, M. Akpa-Vinceslas, M. Legras, D. Bru, F. Bureau, and J. Bodilis, "GammaProteobacteria as a potential bioindicator of a multiple contamination by polycyclic aromatic hydrocarbons (PAHs) in agricultural soils," *Environ. Pollut.*, vol. 180, pp. 199–205, 2013.

[98] A. T. Kivistö and M. T. Karp, "Halophilic anaerobic fermentative bacteria.," *J. Biotechnol.*, vol. 152, no. 4, pp. 114–124, Apr. 2011.

[99] B. Z. Fathepure, "Recent studies in microbial degradation of petroleum hydrocarbons in hypersaline environments.," *Front. Microbiol.*, vol. 5, p. 173, Jan. 2014.

[100] R. A. Kanaly and S. Harayama, “Review article Advances in the field of high-molecular-weight polycyclic aromatic hydrocarbon biodegradation,” vol. 3, no. April 2009, pp. 136–164, 2010.

[101] A. D. G. Wright, “Phylogenetic relationships within the order Halobacteriales inferred from 16S rRNA gene sequences,” *Int. J. Syst. Evol. Microbiol.*, vol. 56, no. 6, pp. 1223–1227, 2006.

[102] X. Zhuang, Z. Han, Z. Bai, G. Zhuang, and H. Shim, “Progress in decontamination by halophilic microorganisms in saline wastewater and soil,” *Environ. Pollut.*, vol. 158, no. 5, pp. 1119–1126, 2010.

[103] B. O. Aljohny, “Halophilic Bacterium - A Review of New Studies,” *Biosci. Biotechnol. Res. Asia*, vol. 12, no. December, pp. 2061–2069, 2015.

[104] J. Yin, J. C. Chen, Q. Wu, and G. Q. Chen, “Halophiles, coming stars for industrial biotechnology,” *Biotechnol. Adv.*, vol. 33, no. 7, pp. 1433–1442, 2015.

[105] M. Putri and R. Hertadi, “Effect of Glycerol as Carbon Source for Biosurfactant Production by Halophilic Bacteria Pseudomonas Stutzeri BK-AB12,” *Procedia Chem.*, vol. 16, pp. 321–327, 2015.

[106] M. Koller, P. Hesse, R. Bona, C. Kutschera, A. Atlić, and G. Braunegg, “Potential of various archae- and eubacterial strains as industrial polyhydroxyalkanoate producers from whey,” *Macromol. Biosci.*, vol. 7, no. 2, pp. 218–226, 2007.

[107] A. K. Paul and A. Biswas, “Production of poly(3-hydroxyalkanoic acids) by moder- ately halophilic bacteria isolated from solar salterns,” *N. Biotechnol.*, vol. 25, no. September, pp. S91–S92, 2009.

[108] J. Chuprom, P. Bovornreungroj, M. Ahmad, D. Kantachote, and S. Dueramae, “Approach toward enhancement of halophilic protease production by Halobacterium sp. strain LBU50301 using statistical design response surface methodology,” *Biotechnol. Reports*, vol. 10, pp. 17–28, 2016.

[109] N. M. Mesbah and J. Wiegel, “Halophilic alkali- and thermostable amylase from a novel polyextremophilic Amphibacillus sp. NM-Ra2,” *Int. J. Biol. Macromol.*, vol. 70, pp. 222–229, 2014.

[110] N. Udomsil, S. Rodtong, S. Tanasupawat, and J. Yongsawatdigul, “Proteinase-producing halophilic lactic acid bacteria isolated from fish sauce fermentation and their ability to produce volatile compounds,” *Int. J. Food Microbiol.*, vol. 141, no. 3, pp. 186–194, 2010.

[111] R. Sathish Kumar, G. Ananthan, and A. Selva Prabhu, “Optimization of medium composition for alkaline protease production by Marinobacter sp. GA CAS9 using response surface methodology - A statistical approach,” *Biocatal. Agric. Biotechnol.*, vol. 3, no. 2, pp. 191–197, 2014.

[112] P. Harvey, D. Bailey, A. Ben-Amotz, V. Verdelho, G. Harris, D. Rooke, H.

Hoekstra, P. Goacher, J. Crespi, G. Reinhardt, L. Martinelli, D. Schroder, R. Pipe, N. Igl-Schmid, A. Kokossis, and K. Perrson, “The CO2 microalgae biorefinery: high value products and biofuels using halophilic microalgae in the ‘D-Factory,’” *N. Biotechnol.*, vol. 31, no. July, pp. S14–S15, 2014.

[113] J. Duan, H. Fang, B. Su, J. Chen, and J. Lin, “Characterization of a halophilic heterotrophic nitrification-aerobic denitrification bacterium and its application on treatment of saline wastewater.,” *Bioresour. Technol.*, vol. 179, pp. 421–428, Mar. 2015.

[114] K. Shi, W. Zhou, H. Zhao, and Y. Zhang, “Performance of halophilic marine bacteria inocula on nutrient removal from hypersaline wastewater in an intermittently aerated biological filter.,” *Bioresour. Technol.*, vol. 113, pp. 280–287, Jun. 2012.

[115] F. Piubeli, M. James, F. Fantinatti-garboggini, and L. Regina, “International Biodeterioration & Biodegradation Enhanced reduction of COD and aromatics in petroleum-produced water using indigenous microorganisms and nutrient addition,” *Int. Biodeterior. Biodegradation*, vol. 68, pp. 78–84, 2012.

[116] E. Koch, “Biodegradation of Polyaromaric Hydrocarbons by Arthrobacter sp. UG50 isolated from petroleum refibery waste,” The University of Guelph, Ontario, Canada, 2011.

[117] N. Mangwani, S. K. Shukla, S. Kumari, T. S. Rao, and S. Das, “Characterization of Stenotrophomonas acidaminiphila NCW-702 biofilm for implication in the degradation of polycyclic aromatic hydrocarbons,” *J. Appl. Microbiol.*, vol. 117, pp. 1012–1024, 2014.

[118] C. B. Smith, C. N. Johnson, and G. M. King, “Assessment of polyaromatic hydrocarbon degradation by potentially pathogenic environmental Vibrio parahaemolyticus isolates from coastal Louisiana, USA,” *Mar. Pollut. Bull.*, vol. 64, no. 1, pp. 138–143, 2012.

[119] B. P. Hedlund, A. D. Geiselbrecht, and J. T. Staley, “Marinobacter strain NCE312 has a Pseudomonas -like naphthalene dioxygenase,” *FEMS Microbiol. Lett.*, vol. 201, pp. 47–51, 2001.

[120] S. Kuppusamy, P. Thavamani, M. Megharaj, and R. Naidu, “Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by novel bacterial consortia tolerant to diverse physical settings - Assessments in liquid- and slurry-phase systems,” *Int. Biodeterior. Biodegrad.*, vol. 108, pp. 149–157, 2016.

[121] A. A. Al-Thukair and K. Malik, “Pyrene metabolism by the novel bacterial strains Burkholderia fungorum (T3A13001) and Caulobacter sp (T2A12002) isolated from an oil-polluted site in the Arabian Gulf,” *Int. Biodeterior. Biodegrad.*, vol. 110, pp. 32–37, 2016.

[122] H. Pactao and C. Inoue, “Polycyclic aromatic hydrocarbons (PAHs)

biodegradation potential and diversity of microbial consortia enriched from tsunami sediments in Miyagi , Japan," *J. Hazard. Mater.*, vol. 283, pp. 689–697, 2015.

[123] C. Guo, Z. Dang, Y. Wong, and N. F. Tam, "Biodegradation ability and dioxgenase genes of PAH-degrading Sphingomonas and Mycobacterium strains isolated from mangrove sediments," *Int. Biodeterior. Biodegradation*, vol. 64, no. 6, pp. 419–426, Sep. 2010.

[124] S. Luo, B. Chen, L. Lin, X. Wang, N. F. Tam, and T. Luan, "Pyrene Degradation Accelerated by Constructed Consortium of Bacterium and Microalga: Effects of Degradation Products on the Microalgal Growth," *Environ. Sci. Technol.*, vol. 48, pp. 13917–13924, 2014.

[125] S. Kim, O. Kweon, R. C. Jones, J. P. Freeman, R. D. Edmondson, and C. E. Cerniglia, "Complete and Integrated Pyrene Degradation Pathway in Mycobacterium vanbaalenii PYR-1 Based on Systems Biology," *J. Bacteriol.*, vol. 189, no. 2, pp. 464–472, 2007.

[126] R. D. Hunter, S. I. N. Ekunwe, D. E. Dodor, H.-M. Hwang, and L. Ekunwe, "Bacillus subtilis is a Potential Degrader of Pyrene and Benzo[a]pyrene," *Int. J. Environ. Res. Public Health*, vol. 2, no. 2, pp. 267–271, Aug. 2005.

[127] H. Yuan, J. Yao, K. Masakorala, and F. Wang, "Isolation and characterization of a newly isolated pyrene-degrading Acinetobacter strain USTB-X," *Environ. Sci. Pollut. Res.*, vol. 21, pp. 2724–2732, 2014.

[128] R. R. De La Haba, D. R. Arahal, M. C. Márquez, and A. Ventosa, "Phylogenetic relationships within the family Halomonadaceae based on comparative 23S and 16S rRNA gene sequence analysis," *Int. J. Syst. Evol. Microbiol.*, vol. 60, no. 4, pp. 737–748, 2010.

[129] E. P. Ivanova, S. Flavier, and R. Christen, "Phylogenetic relationships among marine Alteromonas-like proteobacteria: Emended description of the family Alteromonadaceae and proposal of Pseudoalteromonadaceae fam. nov., Colwelliaceae fam. nov., Shewanellaceae fam. nov., Moritellaceae fam. nov., Ferri," *Int. J. Syst. Evol. Microbiol.*, vol. 54, no. 5, pp. 1773–1788, 2004.

[130] M. B. Gomes, E. E. Gonzales-Limache, S. T. P. Sousa, B. M. Dellagnezze, A. Sartoratto, L. C. F. Silva, L. M. Gieg, E. Valoni, R. S. Souza, A. P. R. Torres, M. P. Sousa, S. O. De Paula, C. C. Silva, and V. M. Oliveira, "Exploring the potential of halophilic bacteria from oil terminal environments for biosurfactant production and hydrocarbon degradation under high-salinity conditions," *Int. Biodeterior. Biodegradation*, vol. in press a, pp. 1–12, 2016.

[131] S. W. Roh, Y. Do Nam, H. W. Chang, Y. Sung, K. H. Kim, H. M. Oh, and J. W. Bae, "Halalkalicoccus jeotgali sp. nov., a halophilic archaeon from shrimp jeotgal, a traditional Korean fermented seafood," *Int. J. Syst. Evol. Microbiol.*, vol. 57, no. 10, pp. 2296–2298, 2007.

[132] Z. Huang, P. Wang, H. Li, K. Lin, Z. Lu, X. Guo, and Y. Liu, "Community analysis and metabolic pathway of halophilic bacteria for phenol degradation in saline environment," *Int. Biodeterior. Biodegradation*, vol. 94, pp. 115–120, Oct. 2014.

[133] M. T. García, E. Mellado, J. C. Ostos, and A. Ventosa, "Halomonas organivorans sp. nov., a moderate halophile able to degrade aromatic compounds.," *Int. J. Syst. Evol. Microbiol.*, vol. 54, no. Pt 5, pp. 1723–1728, Sep. 2004.

[134] E. Acikgoz and B. Ozcan, "International Biodeterioration & Biodegradation Phenol biodegradation by halophilic archaea," *Int. Biodeterior. Biodegradation*, vol. 107, pp. 140–146, 2016.

[135] S. Dalvi, S. Azetsu, M. A. Patrauchan, D. F. Aktas, and B. Z. Fathepure, "Proteogenomic Elucidation of the Initial Steps in the Benzene Degradation Pathway of a Novel Halophile , Arhodomonas sp . Strain Rozel , Isolated from a Hypersaline Environment," vol. 78, no. 20, pp. 7309–7316, 2012.

[136] T. A. Oyehan and A. A. Al-Thukair, "Isolation and characterization of PAH-degrading bacteria from the Eastern Province, Saudi Arabia," *Mar. Pollut. Bull.*, 2016.

[137] T. Feng, C. Cui, F. Dong, Y. Feng, Y. Liu, and X. Yang, "Phenanthrene biodegradation by halophilic Martelella sp .," *J. Appl. Mycrobiology*, vol. 113, pp. 779–789, 2012.

[138] M. Lin, X. Hu, W. Chen, H. Wang, and C. Wang, "International Biodeterioration & Biodegradation Biodegradation of phenanthrene by Pseudomonas sp . BZ-3 , isolated from crude oil contaminated soil," *Int. Biodeterior. Biodegradation*, vol. 94, pp. 176–181, 2014.

[139] S. Cuadros-orellana, M. Pohlschro, and L. R. Durrant, "Isolation and characterization of halophilic archaea able to grow in aromatic compounds," vol. 57, pp. 151–154, 2006.

[140] Y. H. Tapilatu, V. Grossi, M. Acquaviva, C. Militon, J.-C. Bertrand, and P. Cuny, "Isolation of hydrocarbon-degrading extremely halophilic archaea from an uncontaminated hypersaline pond (Camargue, France).," *Extremophiles*, vol. 14, no. 2, pp. 225–31, Mar. 2010.

[141] W. Gao, Z. Cui, Q. Li, G. Xu, X. Jia, and L. Zheng, "Marinobacter nanhaiticus sp. nov., polycyclic aromatic hydrocarbon-degrading bacterium isolated from the sediment of the South China Sea.," *Antonie Van Leeuwenhoek*, vol. 103, no. 3, pp. 485–91, Mar. 2013.

[142] B. Zhao, H. Wang, X. Mao, and R. Li, "Biodegradation of phenanthrene by a halophilic bacterial consortium under aerobic conditions.," *Curr. Microbiol.*, vol. 58, no. 3, pp. 205–210, Mar. 2009.

[143] D. L. McNally, J. R. Mihelcic, and D. R. Lueking, “Biodegradation of three and four ring polycyclic aromatic hydrocarbons under aerobic and denitrifying conditions,” *Environ. Technol.*, vol. 32, no. 98, pp. 2633–2639, 1998.

[144] A. Louvado, N. C. M. Gomes, M. M. Q. Sim??es, A. Almeida, D. F. R. Cleary, and A. Cunha, “Polycyclic aromatic hydrocarbons in deep sea sediments: Microbe-pollutant interactions in a remote environment,” *Sci. Total Environ.*, vol. 526, pp. 312–328, 2015.

[145] L. C. Castillo-carvajal, J. L. Sanz-martín, and B. E. Barragán-huerta, “Biodegradation of organic pollutants in saline wastewater by halophilic microorganisms : a review,” pp. 9578–9588, 2014.

[146] M. Hassanshahian and N. A. Boroujeni, “Enrichment and identification of naphthalene-degrading bacteria from the Persian Gulf,” *Mar. Pollut. Bull.*, vol. 107, no. 1, pp. 59–65, 2016.

[147] M. Kumar, V. León, A. De Sisto Materano, and O. A. Ilzins, “A halotolerant and thermotolerant Bacillus sp. degrades hydrocarbons and produces tensio-active emulsifying agent,” *World J. Microbiol. Biotechnol.*, vol. 23, no. 2, pp. 211–220, 2007.

[148] P. Isaac, F. L. Mart??nez, N. Bourguignon, L. A. S??nchez, and M. A. Ferrero, “Improved PAHs removal performance by a defined bacterial consortium of indigenous Pseudomonas and actinobacteria fromPatagonia, Argentina,” *Int. Biodeterior. Biodegrad.*, vol. 101, pp. 23–31, 2015.

[149] N. González, R. Simarro, M. C. Molina, L. F. Bautista, L. Delgado, and J. A. Villa, “Effect of surfactants on PAH biodegradation by a bacterial consortium and on the dynamics of the bacterial community during the process,” *Bioresour. Technol.*, vol. 102, no. 20, pp. 9438–9446, 2011.

[150] B. Prakash and M. Irfan, “Pseudomonas aeruginosa is Present in Crude Oil Contaminated Sites of Barmer Region (India),” *J. Bioremediation Biodegrad.*, vol. 2, no. 5, pp. 1–2, 2011.

[151] A. M. Kashi, K. Tahemanesh, S. Chaichian, M. T. Joghataei, F. Moradi, S. M. Tavangar, A. S. M. Najafabadi, N. Lotfibakhshaiesh, S. P. Beyranvand, A. F. Anvari-Yazdi, and S. M. Abed, “How to Prepare Biological Samples and Live Tissues for Scanning Electron Microscopy (SEM),” *Galen Med. J.*, vol. 3, no. 2, pp. 63–80, 2014.

[152] J. M. Cervantes-Uc, J. Catzin, I. Vargas, W. Herrera-Kao, F. Moguel, E. Ramirez, S. Rincón-Arriaga, and G. Lizama-Uc, “Biosynthesis and characterization of polyhydroxyalkanoates produced by an extreme halophilic bacterium, Halomonas nitroreducens, isolated from hypersaline ponds.,” *J. Appl. Microbiol.*, vol. 117, no. 4, pp. 1056–1065, Oct. 2014.

[153] K. V. Gayathri and N. Vasudevan, “Enrichment of Phenol Degrading Moderately

Halophilic Bacterial Consortium from Saline Environment," *J. Bioremediation Biodegrad.*, vol. 1, no. 1, pp. 1–6, 2010.

[154] G. Nkulu, S. Gaydardzhiev, E. Mwema, and P. Compere, "SEM and EDS observations of carrollite bioleaching with a mixed culture of acidophilic bacteria," *Miner. Eng.*, no. 75, pp. 70–76, May 2015.

[155] A. Nzila, A. Thukair, S. Sankara, B. Chanbasha, and M. M. Musa, "Isolation and characterization of naphthalene biodegrading Methylobacterium radiotolerans bacterium from the eastern coastline of the Kingdom of Saudi Arabia," *Arch. Environ. Prot.*, vol. 42, no. 3, pp. 25–32, 2016.

[156] A. Poli, H. Kazak, B. Gürleyendağ, G. Tommonaro, G. Pieretti, E. T. Öner, and B. Nicolaus, "High level synthesis of levan by a novel Halomonas species growing on defined media," *Carbohydr. Polym.*, vol. 78, no. 4, pp. 651–657, 2009.

[157] A. P. J, G. Melchias, A. Edward, and P. Kumaravel, "Phylogenetic and functional characterization of halophilic bacterial isolates of upper cretaceous fossils in Ariyalur Peninsula," *World J. Pharm. Pharm. Sci.*, vol. 3, no. 5, pp. 794–812, 2014.

[158] A. Roohi, I. Ahmed, N. Khalid, M. Iqbal, and M. Jamil, "Isolation and Phylogenetic Identification of Halotolerant / Halophilic Bacteria from the Salt Mines of Karak , Pakistan," *Int. J. Agric. Biol.*, no. 16, pp. 564–570, 2014.

[159] K. Song, T. Suenaga, A. Hamamoto, K. Satou, S. Riya, M. Hosomi, and A. Terada, "Abundance, transcription levels and phylogeny of bacteria capable of nitrous oxide reduction in a municipal wastewater treatment plant.," *J. Biosci. Bioeng.*, vol. 118, no. 3, pp. 289–297, Sep. 2014.

[160] L. Fernando Bautista, R. Sanz, M. Carmen Molina, N. González, and D. Sánchez, "Effect of different non-ionic surfactants on the biodegradation of PAHs by diverse aerobic bacteria," *Int. Biodeterior. Biodegrad.*, vol. 63, no. 7, pp. 913–922, 2009.

[161] D. R. Absolom, F. V Lamberti, Z. Policova, W. Zingg, C. J. Van Oss, and a W. Neumann, "Surface thermodynamics of bacterial adhesion . Surface Thermodynamics of Bacterial Adhesion," *Appl. Environ. Microbiol.*, vol. 46, no. 1, pp. 90–97, 1983.

[162] A. Nzila, A. Thukair, S. Sankara, and S. A. Razzak, "Characterization of aerobic oil and grease-degrading bacteria in wastewater," *Environ. Technol.*, vol. 0, no. 0, pp. 1–10, 2016.

[163] S. Thiele-Bruhn and G. W. Brümmer, "Kinetics of polycyclic aromatic hydrocarbon (PAH) degradation in long-term polluted soils during bioremediation," *Plant Soil*, vol. 275, no. 1–2, pp. 31–42, 2005.

[164] J. Sitdhipol, W. Visessanguan, S. Benjakul, and P. Yukphan, "Idiomarina piscisalsi

sp . nov ., from fermented fish (pla-ra) in Thailand," *J. Gen. Appl. Microbiol*, no. 59, pp. 385–391, 2013.

[165] E. P. Ivanova, L. A. Romanenko, J. Chun, M. H. Matte, G. R. Matte, V. V. Mikhailov, V. I. Svetashev, A. Huq, T. Maugel, and R. R. Colwell, "Idiomarina gen. nov., comprising novel indigenous deep-sea bacteria from the Pacific Ocean, including descriptions of two species, Idiomarina abyssalis sp. nov. and Idiomarina zobellii sp. nov.," *Int. J. Syst. Evol. Microbiol.*, vol. 50, no. 2, pp. 901–907, 2000.

[166] L. Wang, Q. Lai, Y. Fu, H. Chen, W. Wang, J. Wang, F. Sun, and Z. Shao, "Idiomarina xiamenensis sp. nov., isolated from surface seawater, and proposal to transfer Pseudidiomarina aestuarii to the genus idiomarina as Idiomarina aestuarii comb. nov," *Int. J. Syst. Evol. Microbiol.*, vol. 61, no. 4, pp. 969–973, 2011.

[167] M. H. Chen, S. Y. Sheu, C. A. Chen, J. T. Wang, and W. M. Chen, "Idiomarina aquimaris sp. nov., isolated from the reef-building coral Isopora palifera," *Int. J. Syst. Evol. Microbiol.*, vol. 62, no. 7, pp. 1536–1542, 2012.

[168] I. Brettar, R. Christen, M. G. Ho, M. Weg, and D.- Braunschweig, "Idiomarina baltica sp . nov ., a marine bacterium with a high optimum growth temperature isolated from surface water of the central Baltic Sea," *Int. J. Syst. Evol. Microbiol.*, no. 53, pp. 407–413, 2003.

[169] F. Nobre and S. Milton, "Description of Idiomarina insulisalsae sp . nov ., isolated from the soil of a sea salt evaporation pond , proposal to transfer the species of the genus Pseudidiomarina to the genus Idiomarina and emended description of the," *Syst. Appl. Microbiol.*, vol. 32, pp. 371–378, 2009.

[170] J. Du, Q. Lai, Y. Liu, Y. Du, X. Liu, F. Sun, and Z. Shao, "Idiomarina atlantica sp. nov., a marine bacterium isolated from the deep sea sediment of the North Atlantic Ocean," *Antonie Van Leeuwenhoek*, vol. 107, no. 2, pp. 393–401, 2015.

[171] D. H. Choi and B. C. Cho, "Idiomarina seosinensis sp . nov ., isolated from hypersaline water of a solar saltern in Korea," *Int. J. Syst. Evol. Microbiol.*, vol. 55, pp. 379–383, 2005.

[172] M. J. Martínez-Cánovas, V. Béjar, F. Martínez-Checa, R. Páez, and E. Quesada, "Idiomarina fontislapidosi sp. nov. and Idiomarina ramblicola sp. nov., isolated from inland hypersaline habitats in Spain," *Int. J. Syst. Evol. Microbiol.*, vol. 54, no. 5, pp. 1793–1797, 2004.

[173] B.-S. Yun, J.-C. Lee, Y.-S. Kim, and K.-S. Whang, "Idiomarina halophila sp. nov., isolated from a solar saltern sediment," *Int. J. Syst. Evol. Microbiol.*, vol. 65, no. 4, pp. 1268–1273, 2015.

[174] L. Song, F. Ren, Y. Huang, X. Dai, and Y. Zhou, "Idiomarina indica sp. nov., isolated from seawater," *Int. J. Syst. Evol. Microbiol.*, vol. 63, pp. 2497–2500, 2013.

[175] S. P. Donachie, S. Hou, T. S. Gregory, A. Malahoff, and M. Alam, “Idiomarina loihiensis sp. nov., a halophilic alphaproteobacterium from the Lo’ihi submarine volcano, Hawai’i,” *Int. J. Syst. Evol. Microbiol.*, vol. 53, no. 6, pp. 1873–1879, 2003.

[176] Y. J. Zhang, X. Y. Zhang, H. L. Zhao, M. Y. Zhou, H. J. Li, Z. M. Gao, X. L. Chen, H. Y. Dang, and Y. Z. Zhang, “Idiomarina maris sp. nov., a marine bacterium isolated from sediment,” *Int. J. Syst. Evol. Microbiol.*, vol. 62, no. 2, pp. 370–375, 2012.

[177] Z. Zhong, Y. Liu, H. Liu, F. Wang, L. Song, and Z. Liu, “Idiomarina planktonica sp . nov ., isolated from a saline lake,” *Int. J. Syst. Evol. Microbiol.*, vol. 64, pp. 3411–3416, 2014.

[178] A. Poddar, R. T. Lepcha, D. Mukherjee, and D. Bhattacharyya, “Comparative analysis of 16S rRNA signature sequences of the genus Idiomarina and Idiomarina woesei sp . nov ., a novel marine bacterium isolated from the Andaman Sea,” *Res. Microbiol.*, vol. 165, no. 7, pp. 501–507, 2014.

[179] S. Kwon, B. Kim, H. Weon, Y. Baek, B. Koo, S. Go, and B. Kim, “Idiomarina homiensis sp . nov ., isolated from seashore sand in Korea,” no. 2006, pp. 2229–2233, 2016.

[180] S.-I. Aizawa, “Idiomarina loihiensis — A Habitat of Deep-Sea Volcano,” in *The Flagellar World*, 2014, pp. 48–49.

[181] A. Poli, B. Nicolaus, A. A. Denizci, and B. Yavuzturk, “Halomonas smyrnensis sp . nov ., a moderately halophilic , exopolysaccharide-producing bacterium,” *Int. J. Syst. Evol. Microbiol.*, vol. 63, pp. 10–18, 2013.

[182] Y. Wang, H. Cai, C. Chi, A. Lu, X. Lin, Z. Jiang, and X. Wu, “Halomonas shengliensis sp . nov ., a moderately halophilic , denitrifying , crude-oil-utilizing bacterium,” *Int. J. Syst. Evol. Microbiol.*, vol. 57, pp. 1222–1226, 2007.

[183] M. R. L. Bonfá, M. J. Grossman, F. Piubeli, E. Mellado, and L. R. Durrant, “Phenol degradation by halophilic bacteria isolated from hypersaline environments.,” *Biodegradation*, vol. 24, no. 5, pp. 699–709, Sep. 2013.

[184] J. A. Mata, J. Martínez-Cánovas, E. Quesada, and V. Béjar, “A detailed phenotypic characterisation of the type strains of Halomonas species.,” *Syst. Appl. Microbiol.*, vol. 25, no. 3, pp. 360–375, 2002.

[185] D. R. Arahal, A. M. Castillo, W. Ludwig, K. H. Schleifer, and A. Ventosa, “Proposal of Cobetia marina gen. nov., comb. nov., within the family Halomonadaceae, to include the species Halomonas marina.,” *Syst. Appl. Microbiol.*, vol. 25, no. 2, pp. 207–211, 2002.

[186] H. Amjres, V. B??jar, E. Quesada, D. Carranza, J. Abrini, C. Sinquin, J. Ratiskol, S. Colliec-Jouault, and I. Llamas, “Characterization of haloglycan, an

exopolysaccharide produced by Halomonas stenophila HK30," *Int. J. Biol. Macromol.*, vol. 72, pp. 117–124, 2015.

[187] J. Antonio, V. Béjar, I. Llamas, and S. Arias, "Exopolysaccharides produced by the recently described halophilic bacteria Halomonas ventosae and Halomonas anticariensis," *Res. Microbiol.*, vol. 157, pp. 827–835, 2006.

[188] E. Sogutcu, Z. Emerence, M. Arikan, A. Cakiris, N. Abaci, E. T. Oner, D. Ustek, and K. Y. Arga, "Draft Genome Sequence of Halomonas smyrnensis AAD6 T," *J. Bacteriol.*, vol. 194, no. 20, pp. 5690–5691, 2012.

[189] H. K. Sarilmiser, O. Ates, G. Ozdemir, K. Y. Arga, and E. T. Oner, "Effective stimulating factors for microbial levan production by Halomonas smyrnensis AAD6 T," *J. Biosci. Bioeng.*, vol. 119, no. 4, pp. 455–463, 2015.

[190] P. Arulazhagan and N. Vasudevan, "Biodegradation of polycyclic aromatic hydrocarbons by a halotolerant bacterial strain Ochrobactrum sp. VA1," *Mar. Pollut. Bull.*, vol. 62, no. 2, pp. 388–394, 2011.

[191] V. Patel, J. Patel, and D. Madamwar, "Biodegradation of phenanthrene in bioaugmented microcosm by consortium ASP developed from coastal sediment of Alang-Sosiya ship breaking yard," *Mar. Pollut. Bull.*, vol. 74, no. 1, pp. 199–207, 2013.

[192] Y. H. Kim, J. P. Freeman, J. D. Moody, K. H. Engesser, and C. E. Cerniglia, "Effects of pH on the degradation of phenanthrene and pyrene by Mycobacterium vanbaalenii PYR-1," *Appl. Microbiol. Biotechnol.*, vol. 67, no. 2, pp. 275–285, 2005.

[193] D. B. Nedwell, "Effect of low temperature on microbial growth: lowered affinity for substrates limits growth at low temperature," *FEMS Microbiol. Ecol.*, vol. 30, no. 2, pp. 101–111, 1999.

[194] M. Eriksson, G. Dalhammar, and A. K. Borg-Karlson, "Aerobic degradation of a hydrocarbon mixture in natural uncontaminated potting soil by indigenous microorganisms at 20 ??C and 6 ??C," *Appl. Microbiol. Biotechnol.*, vol. 51, no. 4, pp. 532–535, 1999.

[195] M. Eriksson, E. Sodersten, Z. Yu, G. Dalhammar, and W. W. Mohn, "Degradation of polycyclic aromatic hydrocarbons at low temperature under aerobic and nitrate-reducing conditions in enrichment cultures from northern soils," *Appl. Environ. Microbiol.*, vol. 69, no. 1, pp. 275–284, 2003.

[196] M. Eriksson, J. Ka, and W. W. Mohn, "Effects of Low Temperature and Freeze-Thaw Cycles on Hydrocarbon Biodegradation in Arctic Tundra Soil Effects of Low Temperature and Freeze-Thaw Cycles on Hydrocarbon Biodegradation in Arctic Tundra Soil," *Appl. Environ. Microbiol.*, vol. 67, no. 11, pp. 5107–5112, 2001.

[197] F. Coulon, E. Pelletier, L. Gourhant, and D. Delille, “Effects of nutrient and temperature on degradation of petroleum hydrocarbons in contaminated sub-Antarctic soil,” *Chemosphere*, vol. 58, no. 10, pp. 1439–1448, 2005.

[198] C. Sartoros, L. Yerushalmi, P. Béron, and S. R. Guiot, “Effects of surfactant and temperature on biotransformation kinetics of anthracene and pyrene,” *Chemosphere*, vol. 61, no. 7, pp. 1042–1050, 2005.

[199] S. A. Mohammed, D. L. Sorensen, R. C. Sims, and J. L. Sims, “Pentachlorophenol and phenanthrene biodegradation in creosote contaminated aquifer material,” *Chemosphere*, vol. 37, no. 1, pp. 103–111, 1998.

[200] S. Feyza and B. Mutlu, “Aromatic Hydrocarbon Degradation by Halophilic Archaea Isolated from Çamalt ı Saltern , Turkey,” *Water, Air, Soil Pollut.*, vol. 224, pp. 1449–9, 2013.

[201] N. A. S. Mohd Radzi, K. S. Tay, N. K. Abu Bakar, C. Uche Emenike, S. Krishnan, F. Shahul Hamid, and M. R. Abas, “Degradation of polycyclic aromatic hydrocarbons (pyrene and fluoranthene) by bacterial consortium isolated from contaminated road side soil and soil termite fungal comb,” *Environ. Earth Sci.*, vol. 74, no. 6, pp. 5383–5391, 2015.

[202] K. Rehmann, H. P. Noll, C. E. W. Steinberg, and A. A. Kettrup, “Pyrene degradation by Mycobacterium sp. strain KR2,” *Chemosphere*, vol. 36, no. 14, pp. 2977–2992, 1998.

[203] P. Arulazhagan and N. Vasudevan, “Role of a moderately halophilic bacterial consortium in the biodegradation of polyaromatic hydrocarbons.,” *Mar. Pollut. Bull.*, vol. 58, no. 2, pp. 256–62, Feb. 2009.

[204] I. Llamas, V. Béjar, F. Martínez-Checa, M. J. Martínez-Cánovas, I. Molina, and E. Quesada, “Halomonas stenophila sp. nov., a halophilic bacterium that produces sulphate exopolysaccharides with biological activity,” *Int. J. Syst. Evol. Microbiol.*, vol. 61, no. 10, pp. 2508–2514, 2011.

[205] C. S. I. Oie, C. E. Albaugh, and B. M. Peyton, “Benzoate and salicylate degradation by Halomonas campisalis, an alkaliphilic and moderately halophilic microorganism,” *Water Res.*, vol. 41, no. 6, pp. 1235–1242, 2007.

[206] A. Haddadi and M. Shavandi, “Biodegradation of phenol in hypersaline conditions by Halomonas sp. strain PH2-2 isolated from saline soil,” *Int. Biodeterior. Biodegradation*, vol. 85, pp. 29–34, Nov. 2013.

[207] S. Le Borgne, D. Paniagua, and R. Vazquez-Duhalt, “Biodegradation of organic pollutants by halophilic bacteria and archaea.,” *J. Mol. Microbiol. Biotechnol.*, vol. 15, no. 2–3, pp. 74–92, Jan. 2008.

Vitae

Name :Fitri Budiyanto

Nationality :Indonesian

Date of Birth :5/27/1987

Email :fitri.budiyanto@gmail.com

Address :Research Center for Oceanography

Indonesian Institute of Sciences

Jl. Pasir Putih 1, No. 1, Ancol Timur, Jakarta, Indonesia,

14430

E-mail : fitr019@lipi.go.id

Telp. : 62-21-64713850 Ext 313

Fax : 62-21-64711948

Academic Background

2005-2009	:	Dept. of Chemical Engineering, Gadjah Mada University
2014-2017	:	Dept. of Earth Science, King Fahd University of Petroleum and Minerals

RESEARCH EXPERIENCE

2008	:	Purification of bioethanol at azeotropic point using adsorption distillation
2011-2012	:	Study of geochemistry of heavy metal in shoreline of west Kalimantan (Borneo Island)
2012	:	Anthropogenic impact of environmental condition in Lombak Island, Indonesia

2012-2014	:	Study of antropogenic effect on rayfish distribution in Jakarta Bay, Indonesia: Impact of heavy metals pollution
2015-2017	:	Study of biodegradation of pyrene by halophilic bacteria under hypersaline condition

RESEARCH CRUISES AND EXPEDITION

R/V Baruna Jaya VIII, Enggano Environmental Baseline Assessment: Research Center for Oceanography, Indonesian Institute of Sciences-Total E&P Indonesie joint research program, April 2013.

RESEARCH CONFERENCE

Budiyanto, F. 2012. Distribution of heavy metal in Cisanggarung Estuarine, West Java, Indonesia. Indonesian Institute of Sciences-Japan Society for the Promotion of Science, Jakarta-Indonesia.

Budiyanto, F, and Lestari. 2012. Spatial distribution of heavy metal in Natuna, Indonesia. Indonesian Association of Oceanologist, Lombok-Indonesia.

Budiyanto. F. 2016. Phenol removal by domestic waste. PetroEnvironment, Dammam-Kingdom of Saudi Arabia.

RESEARCH PAPER

Fitri Budiyanto, Assad Thukair, Saravanan Sankara, Musu M. Musa, Alexis Nzila. Characterisation of halophilic bacteria capable of biodegrading the highly molecular weight poly-aromatic pyrene. Under preparation.

Budiyanto, F., and Lestari, 2013. Study of Metal Contaminant Level In The Mahakam Delta: Sediment And Dissolved Metal Perspectives. *Journal of Coastal Development*, *16*(2), pp.147-157.

Budiyanto, F., 2015. Distribution of Metals in Cisanggarung Estuary Sediment, West Java, Indonesia. *Marine Research in Indonesia*, *39*(1), pp.23-30.