

**ISOLATION AND CHARACTERIZATION OF ACTIVE
BACTERIA DEGRADING CORONENE,
AN OVERLY COMPLEX POLYCYCLIC
AROMATIC HYDROCARBON**

BY

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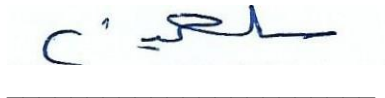
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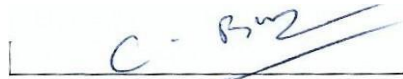
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Dedication

This thesis is dedicated to Almighty Allah for the successful completion of this program. |

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All praises and adoration belong to Allah, the Omnipotent, the Most Merciful, the Most Benevolent, the Giver of Knowledge, the Provider of sustenance and the Most encompassing to all His creations. I am wholeheartedly grateful and honoured by His infinite mercies for sparing my life to complete this program. It gives me great pleasure to express my unreserved gratitude for the encouragement, guidance and unflinching support of my revered teacher and advisor, Dr Alexis Nzila. The research would not have been excellently executed without your massive contribution. I am highly indebted to my thesis committee in persons of Dr Assad Ahmed Al-Thukair and Dr Basheer Chanbasha for their essential and scholarly contributions to this accomplishment. I appreciate the Dean of the College of Chemicals and Materials, the Chairman of the Bioengineering department, and all the faculties and staff in the College for their support. I greatly appreciate the faculties: Dr Alexis, Dr Thukair, Dr Amjad, Dr Irshad, Dr Tahir, and others for imparting their knowledge and expertise to me during the program. My undiluted and most sincere appreciation also goes to the laboratory technicians, Mr Saravanan and Mr Deepak for their unrelenting technical support and guidance in successfully completing this research. I am eternally grateful to my ever-supportive parents, Mr and Mrs Okeyode, my caring siblings and my friends for their ceaseless prayers, love, and encouragement.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	V
TABLE OF CONTENTS	VI
LIST OF FIGURES.....	VIII
LIST OF ABBREVIATIONS.....	IX
ABSTRACT	X
ملخص الرسالة	XII
CHAPTER 1 INTRODUCTION.....	1
1.1 Polycyclic Aromatic Hydrocarbons.....	2
1.2 Sources of PAHs	3
1.3 Toxicity of PAHs	4
1.4 Removal of PAHs and the Project Rational	5
CHAPTER 2 LITERATURE REVIEW	6
2.1 Biodegradation of HMW-PAHs	7
2.2 Biodegradation of Coronene.....	7
CHAPTER 3 METHODOLOGY.....	9
3.1 Materials	9
3.2 Sample collection	9
3.3 Enrichment Cultures	10
3.4 Microbial Isolation.....	10
3.5 Bacterial enumeration	11
3.6 Scanning electron microscopy (SEM)	11

3.7	Bacteria Species identification by 16S rRNA Gene Sequencing and Phylogeny	12
3.8	Assessment of bacterial degradation of coronene and the effects of Temperature, pH, and salinity 14	
3.9	Coronene quantification and substrate utilization	15
3.10	Effects of concentration, pH, temperature, and salinity on bacterial growth.....	15
CHAPTER 4 RESULTS AND CONCLUSION		16
4.1	Bacterial Growth Rate as a Function of Coronene Concentration.....	16
4.2	Bacterial Growth Rate as a Function of Temperature Variation	17
4.3	Bacterial Growth as a Function of Salinity Variation	18
4.4	Quantification of coronene degradation using Gas chromatography	20
4.5	Effect of temperature on Quantification of coronene degradation using Gas chromatography	22
4.6	Effect of salinity on Quantification of coronene degradation using Gas chromatography	23
4.7	Effect of time variation on Quantification of coronene degradation using Gas chromatography ..	24
4.8	Discussion	26
4.9	Conclusion	30
REFERENCES.....		31
VITAE.....		41

LIST OF FIGURES

Figure 1: Chemical structure of the major HMW-PAHs.	3
Figure 2: SEM images of 10CRN4D	12
Figure 3: Bacterial Growth Rate as a Function of Coronene Concentration	17
Figure 4: Bacterial Growth Rate as a Function of Temperature	18
Figure 5: Bacterial Growth Rate as a Function of Salinity	20
Figure 6: Quantification of coronene degradation at (pH 7, temp. 30 °C and salinity 10% NaCl w/v).....	21
Figure 7: Effect of temperature on Quantification of coronene degradation.....	23
Figure 8: Effect of salinity on Quantification of coronene degradation	24
Figure 9: Effect of time variation on Quantification of coronene degradation.....	25

LIST OF ABBREVIATIONS

BaP	:	Benzo[a]pyrene
BH	:	Bushnell Haas
BHM	:	Bushnell Haas Media
CFU	:	Colony Forming Units
CRN	:	Coronene
EESI	:	Environmental and Energy Study Institute
FESEM	:	Field Emission Scanning Electron Microscopy
FTIR	:	Fourier Transform Infrared Spectroscopy
HMW-PAHs	:	High molecular Weight Polycyclic Aromatic Hydrocarbons
LMW-PAHs	:	Low molecular Weight Polycyclic Aromatic Hydrocarbons
OD	:	Optical Density
PAHs	:	Polycyclic Aromatic Hydrocarbons
GC	:	Gas Chromatography

ABSTRACT

Full Name : Ajibola Hakeem Okeyode

Thesis Title : Isolation and characterization of active bacteria degrading coronene, an overly complex polycyclic aromatic hydrocarbon

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Petroleum pollutants have been a major concern in recent years. Among the most important petroleum pollutants are polycyclic aromatic hydrocarbons (PAH). Generally, PAHs are cyclic aromatic hydrocarbons with fused benzene rings ranging from 2 rings and above. Coronene, one of the most complex PAHs is the least studied probably due to its high recalcitrance. In this study, we focus on isolating bacteria that can actively degrade coronene using bioremediation. A bacterial strain, capable of degrading coronene as the only source of carbon was isolated by enrichment of soil-contained inoculum, in a mineral culture medium, in the presence of coronene as the only source of carbon, and salinity at 10% NaCl. This enrichment led to the isolation of the 10SCRN4D strain, and 16S RNA gene sequencing and homology analyses have indicated that this strain belongs to the *Halomonas caseinilytica* species. The growth rate of this bacterial strain in the presence of coronene was carried out at varying temperatures (30°C, 37 °C and 50 °C), concentrations (5ppm, 25ppm and 50ppm) and pH (3.0, 7.0 and 10.0), salinity (0.5, 10, 20% w/v of NaCl), and the data presented as doubling time (dt). The results indicate pH 7, 30°C and 0.5 % NaCl is the optimum growth condition, characterized by the lowest dt (thus highest growth) of 8.78 hrs. As expected, an increase in coronene concentration led to an increase in doubling time (decrease in growth), from 11.13hrs at 10ppm to 15.71 hrs at 50 ppm. The

effect of time variation on the bacterial degradation of coronene was also investigated for a period of 80 days. More than 45% of coronene was degraded on day 40 while 75% of coronene was degraded on day 80, which attests to the fact that the degradation rate increases with the increased duration of the experiment. Thus, the 10SCRN4D strain is a unique halotolerant halophilic bacterium that can grow and degrade in the presence of 7-ring PAH coronene with up to 75% degradation as demonstrated by our study

ملخص الرسالة

الاسم الكامل: أجيبولا حكيم اوكيودي

عنوان الرسالة: عزل وتحديد صفات البكتيريا النشطة المحطمة للكورونين، وهو هيدروكربون أروماتي شديد التعقيد

التخصص: علوم الحياة

تاريخ الدرجة العلمية: تشرين الثاني – 2022

تشكل الملوثات البترولية مخاوف كبيرة في السنوات الحالية. تعد الهيدروكربونات الأروماتية (PAH) متعددة الحلقات واحدة من أهم هذه الملوثات. عمومًا، الهيدروكربونات الأروماتية متعددة الحلقات عبارة عن هيدروكربونات أروماتية حلقية، تتكون من حلقتين أو أكثر من حلقات البنزين الملتحمة. يعد الكورونين، أحد هذه المركبات الأكثر تعقيدًا، ويمتاز بقدرته العالية على المقاومة؛ لذلك هو من أقل المركبات الحلقية الأروماتية دراسةً. هذه الدراسة تركز على عزل بكتيريا لديها القدرة على تحطيم الكورونين من خلال المعالجة الحيوية. تم عزل العزلات البكتيرية، القادرة على تحطيم الكورونين، من التربة عن طريق إثراء اللقاح (inoculum) الموجود فيها. بوجود الكورونين، كمصدر لا بديل عنه للكربون، و10% نسبة ملحوظة كلوريد الصوديوم. أدى هذا الإثراء إلى عزل سلالة SCRN4D10، وقد أشار تسلسل الجين 16S RNA وتحليلات التماثل إلى أن هذه السلالة تنتمي إلى أنواع *Halomonas caseinilytica*. تم زراعة هذه السلالة على تراكيز مختلفة من مادة الكورونين (5 جزء في المليون، 25 جزء في المليون وكذلك 50 جزء في المليون)، وجرت ظروف الزراعة على درجات حرارة متفاوتة (30 درجة مئوية، 37 درجة مئوية و 50 درجة مئوية)، وإختلاف في درجة الحموضة (3.0 ، 7.0 و 10.0) ، وكذلك تراكيز ملحوظة متنوعة من كلوريد الصوديوم (0.5 ، 10 ، 20% وزن/حجم)، وعرضت البيانات بالنسبة للوقت الذي تحتاجه البكتيريا حتى يتضاعف عددها (dt). تشير النتائج إلى أن الرقم الهيدروجيني 7 ودرجة الحرارة 30 مئوية و التركيز الملحي 0.5% من كلوريد الصوديوم هي ظروف النمو المثلى، حيث يتميز بأقل dt (أي أعلى نمو) يبلغ 8.78 ساعة. كما هو متوقع، أدت الزيادة في تركيز الكورونين إلى زيادة وقت التضاعف، مما يعني الانخفاض في النمو البكتيري (من 11.13 ساعة عند 10 جزء في المليون إلى 15.71 ساعة عند 50 جزء في المليون). تأثير اختلاف الوقت على التحلل الكورونين بواسطة البكتيريا تم رصده كذلك، لمدة 80 يومًا. تم تحطيم أكثر من 45% من الكورونين في اليوم الأربعين بينما تحطم 75% من الكورونين في اليوم الثمانين، مما يدل على أن معدل التحطيم يزداد مع زيادة المدة الزمنية للتجربة. بناءً على ما تقدم، فإن سلالة

SCRN4D10 تعتبر بكتيريا فريدة من نوعها، يمكن أن تنمو بوجود بيئة ملحية أو غيابها. كما يمكن أن تنمو في وجود الكورونين المكون من 7 حلقات ملتزمة مع تحطيمه بنسبة تصل إلى 75% كما هو موضح في هذه الدراسة.

CHAPTER 1

INTRODUCTION

Petroleum pollutants have been a major concern in recent years. Among the most important petroleum pollutants are the polycyclic aromatic hydrocarbons (PAH) that have originated from both anthropogenic and non-anthropogenic sources in recent years. They have been subjects of global health and environmental concerns due to their high acute and chronic toxicity and their carcinogenic, mutagenic, and teratogenic effects in humans. (Ashraf & Salam, n.d.; López et al., 2020; Sazykin et al., 2015; Sojinu et al., 2010). Generally, PAHs are cyclic aromatic hydrocarbons with fused benzene rings ranging from 2 rings and above. As a rule of thumb, the more the number of rings, the more complex the PAH. Although many studies have focused on removing these PAHs using several methods, bioremediation remains one of the most influential and proven methods. Several studies have reported degradation of PAHs with fewer rings due to their amenable ability to degradation while PAHs with higher number have received less attention over the years.

1.1 Polycyclic Aromatic Hydrocarbons

Polycyclic Aromatic Hydrocarbons are aromatic organic compounds that occur in the environment from incomplete combustion of organic matter. They contain one or more benzene rings in their chemical structure (Brandt et al., 2017). They are hydrophobic; hence, they do not dissolve in water. More than 400 PAHs have been reported but only 16 of them are categorized as priority pollutants by the US Environmental Protection Agency (EPA) based on their persistence and toxicity (Bojes & Pope, 2007). Generally, PAHs are classified based on their molecular weight, low and high-molecular weight polycyclic aromatic hydrocarbons. The low molecular weight polycyclic aromatic hydrocarbons (LMW-PAHs) comprise less than four aromatic rings, and they include phenanthrene, acenaphthene, naphthalene, and fluorene. The high molecular weight polycyclic aromatic hydrocarbons (HMW-PAHs) contain at least four aromatic rings. They including dibenz[a, h]anthracene, chrysene, pyrene, coronene, benzo[a]pyrene (BaP), etc. Fig 1 summarizes the chemical structure of the major HMW-PAHs. The HMW-PAHs have lower vapour pressure, high chemical stability, and less solubility in water than the LMW-PAHs (Van Hamme et al., 2003) (Peng et al., 2008). Also, the molecular weight of the PAHs is proportional to their lipophilicity and recalcitrance nature hence, increased bioaccumulation in the environment of HMW-PAHs (Okere, 2011).

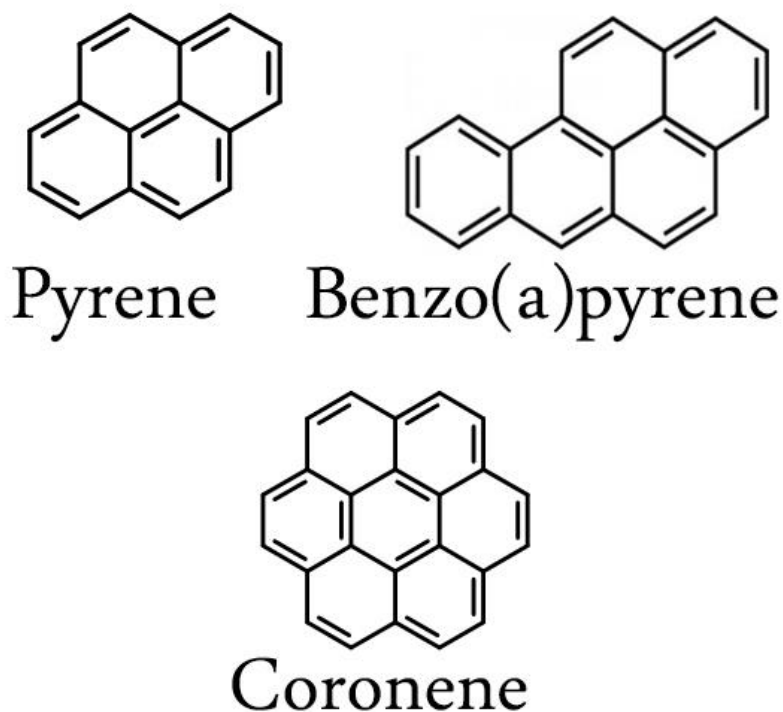


Figure 1: Chemical structure of the major HMW-PAHs.

1.2 Sources of PAHs

Generally, PAHs are ubiquitous in nature and can be introduced into the environment via two major processes namely; anthropogenic and non-anthropogenic processes (Adeniji et al., 2019)(Abdel-Shafy & Mansour, 2016). The occurrence of PAHs can also either be from pyrogenic or petrogenic sources(Abdel-Shafy & Mansour, 2016). While petrogenic production of PAHs occurs within a long period with temperatures ranging from (100–300°C), pyrogenic PAHs are formed easily at high thermal combustion of (>700°C) in automobiles, the thermal destructive distillation of coal and power plants(Lima et al., 2007). Other sources of petrogenic PAHs include oil spills on aquatic and terrestrial environments via transportation, storage, or usage. Most of these PAHs are products of incomplete combustion of fossil fuels.

Furthermore, these PAHs are readily available in the environment by improper treatment or disposal of produced water used during the oil extraction process. Produced water contains dissolved salts, organic compounds, emulsified oil, hydrocarbons, dissolved gases and minerals(A et al., 2009; *About Produced Water (Produced Water 101)*, n.d.; Bostick et al., n.d.; dos Santos et al., 2014; Li et al., 2016). On average, for every 1L of oil displaced from its reservoir, 3L of produced water is generated(A et al., 2009). With a daily estimate of 12 million barrels of oil produced per day in the KSA, 36 million of produced water is generated(*Saudi Arabia Oil Reserves, Production and Consumption Statistics - Worldometer*, n.d.)

1.3 Toxicity of PAHs

The majority of PAHs are widely recognised to be carcinogenic, mutagenic, teratogenic, and toxic to living cells- microorganisms inclusive(Y. Chen et al., 2011; Guo et al., 2012; Niu et al., 2017). Not only do they bioaccumulate in the environment and living cells, but they also pose perilous ecotoxic effects which lead to magnification in living organisms as the food chain grows (Abdel-Shafy & Mansour, 2016). Studies have also linked the exposure of PAHs to non-cancerous diseases including embryo toxicity, egg viability, testicular malfunction, sperm quality problems and viability, and ovarian and oocyte damage(Abdel-Shafy & Mansour, 2016; Bolden et al., 2017; Patel et al., 2020; Rengarajan et al., 2015). According to the Centre for Children's Environmental Health, exposure to PAHs during pregnancy is connected to anomalies such as low weight, premature delivery,

low IQ, childhood asthma, heart malfunctions and increased behavioural problems in birth outcomes(Perera et al., 2014; Rengarajan et al., 2015).

The constant and growing introduction of PAHs via various routes of entry into living tissues has heightened the risk of toxicity, making them a subject of global health and environmental concerns thus, the pressing need for their removal from the environment.

1.4 Removal of PAHs and the Project Rational

Being an onerous global health concern, many physical and chemical methods have been employed toward the removal of PAH from the environment. Although these approaches recorded some success, there are several limitations including generation of secondary pollutants, inefficiency, and expensive cost of operation among others. However, the use of biological means to degrade these pollutants especially through bioremediation has recorded efficient and consistent results over time (Al-Thukair et al., 2020; Al-Thukair & Malik, 2016; BudiyoFitri et al., 2018; Nzila, 2013, 2018; TA & AA, 2017). Bioremediation exploits the use of microbes to break down complex compounds into smaller and less toxic substances. Majority of the reports on bioremediation of PAHs have targeted the few ringed (2-5 rings) compounds since they are amenable to degradation by microorganisms. However, more complex ringed structures including coronene, which contains 7 benzene rings have not been studied extensively due to its recalcitrance to biodegradation and complex structure. To that effect, we have focused our experimental research on using biological measures to degrade one of the most recalcitrant PAH, coronene.

CHAPTER 2

LITERATURE REVIEW

Over the past decades, a few research have targeted the PAHs degradation with the majority of them summarizing and focusing on the LMW-PAHs(Annweiler et al., 2000; Ghosal et al., 2016; Hwang et al., 2007; Liu et al., 2010; Niepceron et al., 2010; Nzila, 2013; Peng et al., 2008; Seo et al., 2010). Investigations have shown that the order of biodegradability follows this sequence: linear alkanes > branched alkanes > monoaromatics > cyclic alkanes > polyaromatics > asphaltenes(Seo et al., 2010; Varjani & Upasani, 2017). The higher the complexity or number of aromatic rings present in the compound, the more complex and recalcitrant it proves to degradation. These studies, among others, have isolated active PAHs degrading bacteria, which have been exploited on a commercial scale as a bioremediation product(Tyagi et al., n.d.). These bacteria belong to different genera but are not limited to *Pseudomonas*, *Pseudoalteromonas*, *Burkholderia*, *Acinetobacter*, *Streptococcus*, *Marinobacter*, *Mycolicibacterium*, *Mycobacterium*, *Rhodococcus*, *Enterobacter*, *Cyclocasticus*, *Glaciacola* and *Streptobacillus*(Gutiérrez et al., 2020; Liu et al., 2010; Niepceron et al., 2010; Sarkar et al., 2017; Steliga et al., 2020; Tremblay et al., 2017).

2.1 Biodegradation of HMW-PAHs

While most of the available reports have investigated and concentrated on the biodegradation of LMW-PAHs because they are generally amenable to degradation, few studies have targeted the recalcitrant HMW-PAHs. The recalcitrant nature of the HMW-PAHs is subject to their reduced responsiveness to oxidative attack, which contributes to their low solubility and accessibility to biodegradation (Madsen, 2011). Most work has focused on the 4 rings (Pyrene, Naphthacene, Chrysene, Benzo[c]fluorene, Fluoranthene, Acephenanthrylene, Benz[a]anthracene), and the 5 ring PAHs (Benzo[a]pyrene and benzo[a]anthracene), with a few on 6-7 rings PAHs (Juhasz et al., 1996, 2000). Although positive results have been reported from the 4 and 5-ring PAHs (importantly pyrene) (TA & AA, 2017) (Al-Thukair et al., 2020; Al-Thukair & Malik, 2016), the 7-ring PAH (coronene), being a larger compound with more complexities, has received little attention so far. Studies on the biodegradation of this compound are discussed in the section below.

2.2 Biodegradation of Coronene

As far as we know, 3 bacterial strains that belongs to the same species, *Pseudomonas cepacia* have been reported to break down CRN. In the first report, *Burkholderia cepacia* which was initially isolated with pyrene was later discovered to degrade CRN, but this was done using high-density cells as inoculates and in the presence of other substrates (Juhasz et al., 1996) (Juhasz et al., 1997). Later in another report, the *Burkholderia cepacia* strain was reclassified as *Stenotrophomonas maltophilia* and was reported to degrade CRN with

associated mutagenicity against *Salmonella typhimurium* strains. To the best of our knowledge, this is the only study that has reported on the biodegradation of coronene using *Burkholderia cepacia* or *Stenotrophomonas maltophilia*, strains, and this biodegradation was carried out with high bacteria cell density in the presence of another substrate, known as a growth substrate and low salinity level (0.5 % NaCl (w/v)).

In the study with *Stenotrophomonas maltophilia* strain VUN 10,003, about 55% of coronene was reported to be degraded after 42 days of incubation which is similar to their previous work in 1996, where about 65% of coronene was degraded after 63 days (Juhasz et al., 1996) (Juhasz et al., 2000). The research of Juhasz and his team has been the first of its kind, however, coronene was not used for the enrichment and isolation of the bacterial strain. Also, the experiment was carried out with a mixture of other LMW-PAHs which served as co-substrate for the HMW-PAHs during the experimental work. More so, they failed to explore different laboratory conditions and report the best growth conditions for effective results. In this study, we have isolated and characterised a bacterial strain capable of degrading COR as the only source of carbon in high salinity. More specifically, the optimum growth condition of temperature, salinity and pH were investigated, and the degradation rate was assessed.

CHAPTER 3

METHODOLOGY

3.1 Materials

Coronene with $\geq 98\%$ purity, Magnesium sulphate (MgSO_4), calcium chloride (CaCl_2), Monopotassium Phosphate (KH_2PO_4), Dipotassium Phosphate (K_2HPO_4), Ammonium nitrate (NH_4NO_3), Ferric chloride (FeCl_3), and Agar used for the culture media preparation were all procured from the Sigma-Aldrich (St. Louis, MO, USA). The percentage purity of other chemicals is $\geq 96\%$. The chemicals and reagents used for gas chromatography (GC) and liquid chromatography were of analytical grade. Luria–Bertani (LB) medium, tryptone, yeast extract, agar, and common salt (NaCl) was acquired from Difco in Detroit, MI, USA.

3.2 Sample collection

Soil samples already contaminated were fetched from a filling station in King Fahd University of Petroleum and Minerals (KFUPM) KSA. The filling station serves as a significant fuel depot for school buses within the KFUPM campus, and it is known for its oil-contaminated soils, which is an ideal environment to isolate bacteria capable of degrading PAH and oil samples

3.3 Enrichment Cultures

About 1.0 g of the soil sample was enriched in a 100ml Bushnell–Hass (BH) culture medium. The BH medium contains 0.2g MgSO₄, 0.02g CaCl₂, 1.0g KH₂PO₄, 1.0g K₂HPO₄, 1.0g NH₄NO₃, 0.05g FeCl₃, 15% w/v NaCl per litre and final pH 7.0±0.2 (at 25°C). 1ml containing a mixture of trace elements was added to the BH media to provide essential cofactors needed by the microorganisms. The medium was then supplemented with coronene (5ppm ≈ 0.5mg/L) as the only source of carbon in a 250ml flask containing 100ml of the culture medium incubated for 4 weeks at a temperature of 37°C and 120 rpm. The protocol for the enrichment process was carried out as described in some previous studies (Nzila, 2013; Nzila et al., 2016)

Bacterial strains isolated during the enrichment process were confirmed using the LB solid agar plates. Out of the 4 isolates, 10CRN4D which is a gram-negative bacterium was the most actively growing bacteria in the BH-COR medium. Light microscopy (×40–1000) confirms that 10CRN4D colonies are rod-shaped, have convex elevation, are spherical, light yellow in colour, and have an entire margin and punctiform. SEM confirmed that 10CRN4D is rod-shaped with an average size 2.1×0.5 µm (Length x Width) as shown in Fig 2.

3.4 Microbial Isolation

Once turbidity was observed in the culture after week 3, 1.0ml of the BH-COR culture was repeatedly pipetted into a fresh medium (1/10, v/v) until bacterial colonies were observed by measuring the turbidity with a spectrophotometer. To get separate individual bacterial

colonies, each of the colonies was transferred to separate solidified agar plates by spreading 1.0ml of each bacterial culture on the agar plate and incubated overnight at 37°C. The agar plates were prepared from Luria–Bertani together with agar (1%, w/v) and NaCl (15%, w/v). Thereafter, the resulting bacterial colonies were transferred to a 15ml LB medium, incubated overnight at 37°C and later centrifuged at $3500 \times g$ for 5 min at 4°C to obtain the bacterial pellets which were cryopreserved in an aqueous solution of glycerol (15%, v/v) at –80 °C for further analysis.

3.5 Bacterial enumeration

After a series of serial dilution by 10, 100, and 1000-folds in distil water containing NaCl (15%, w/v), 0.1ml of the dilution folds were pipetted and spread onto LB solid agar plates (with NaCl (15%, w/v), and incubated for 24 h at 37°C. The bacterial colonies were counted and recorded as colony-forming units per ml (CFU·ml⁻¹).

3.6 Scanning electron microscopy (SEM)

Around 1.0ml of the aliquot of bacterial cells were cultured in a 10.0ml of LB medium in a shaker incubator at 37°C and 120 rpm for 12 hours. The bacterial cells were then centrifuged at $3500 \times g$ for 5 min at 4°C to harvest the cells. The harvested bacterial cells are then suspended in 1.0 ml of phosphate buffer (PBS, pH7) and then incubated for 12H at 25°C after 20µl glutaraldehyde solution (fixating agent) was added to the suspension. Then, 100µl of the suspension was pipetted and spread on microscopic glass slides and incubated at 37°C for another 12 hours. Once the bacterial cells are fixed on the glass slides, they were embedded in a serial dilution of ethanol in the sequence of 10%, 25%, 50%,

75%, 96%, and 100% v/v to dehydrate the bacterial cells. The embedded slips were left for 90 hours before they were coated with gold for 5 minutes and finally observed using a JSM-T300 scanning electron microscope (JEOL, Japan). The image generated from the SEM is represented in figure 2 below

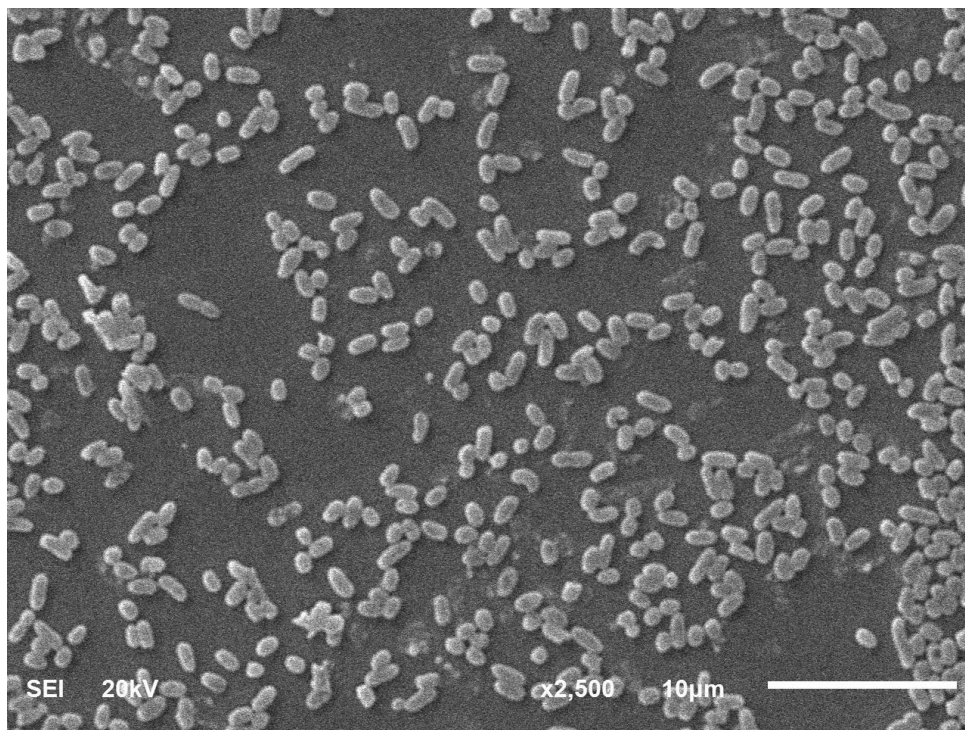


Figure 2: SEM images of 10CRN4D

3.7 Bacteria Species identification by 16S rRNA Gene Sequencing and Phylogeny

For bacterial specie identification, the cryopreserved bacterial cells were pre-cultured in a 100ml LB medium and centrifugation at $6000 \times g$ for 5 minutes at 4°C to harvest the bacterial cells. The cells were lysed followed by DNA extraction and purification using a Qiagen Power fecal Kit (Qiagen, Hilden, Germany). Then, 16S rRNA gene

amplification was carried out as reported by Nzila(Nzila et al., 2016) and Budiyanto (BudiyantoFitri et al., 2018) followed by sequencing as described elsewhere(Nzila et al., 2016).

After the 16S rRNA gene amplification was carried out, fragments of an approximate size of 1200bp were generated which was followed by sequencing and sequence comparison using Basic Local Alignment Search Tool (BLAST) to identify 10CRN4D as *Halomonas caseinilytica* (NCBI, accession number, GI: OP815347). Bacteria strains that belong to the genus *Halomonas* have been studied extensively and have been reported to have degraded several aromatic pollutants^{51–53}, however, so far, no publication has been reported on PAH degradation by a *H. caseinilytica* strain. To the best of our knowledge, this is the first report on *H. caseinilytica* biodegrading polycyclic aromatic hydrocarbon in general, and coronene (a highly recalcitrant high molecular weight PAH) in particular.

3.8 Assessment of bacterial degradation of coronene and the effects of Temperature, pH, and salinity

A bacterial biodegradation experiment of coronene was conducted using the most actively growing bacterial stain that was isolated during the microbial isolation. This bacterial labelled 10CRN4D was cultured in a plastic tube containing 10ml of rich LB medium overnight. The culture was centrifuged at $6000 \times g$ for 5 minutes at 4°C to harvest the bacterial cells. The bacterial pellet was adjusted using a spectrophotometer to get an optical density of 1.0 at 600nm. Thereafter, a BH-COR medium was prepared by dissolving coronene in DMSO prior to adding to BH culture together with 1.0ml of trace elements. An inoculum of approximately $5 \times 10^5 \text{ CFU} \cdot \text{ml}^{-1}$ of the bacterial load was introduced into the culture. The effects of coronene concentration, temperature, pH, and salinity on the capability of 10CRN4D to grow in coronene were also assessed at temperatures of 30°C , 37°C , 50°C ; concentrations of 5ppm, 10ppm, and 560ppm; salinity levels of 0.5, 10, and 20% NaCl (w/v); and pH values of 3, 7, and 10. All experiments were duplicated during the investigation.

To quantify the bacterial growth, the doubling time (dt) was assessed by using the equation $Q_t = Q_0 e^{-kt}$, where Q_t represent the bacterial count at time t while Q_0 at time 0 and the growth rate is denoted by k . The formular $dt = \ln(2)/k$. was then used to calculate the dt.

$$Q_t = Q_0 e^{-kt} \dots\dots\dots (3.1)$$

3.9 Coronene quantification and substrate utilization

COR was quantified by using the plate counting method to count the number of bacterial colonies for 45 days at a different temperature, concentrations, salinity, and pH. The plate count method was further corroborated by using GC to quantify the amount of coronene consumed by the 10CRN4D strain at different laboratory conditions.

3.10 Effects of concentration, pH, temperature, and salinity on bacterial growth

Before commencing the study, the effect of DMSO (0.01% v/v) on the bacteria growth was assessed. A culture of the around 10^5 CFU/ml was incubated in DMSO, and no bacteria growth was observed after 30 days of incubation. To establish the optimal bacterial growth conditions in the presence of COR as the unique carbon source, the doubling time (dt) was assessed the presence of COR as the only carbon source, the doubling time (dt) was assessed following the variation of concentration, temperature, pH, and salinity.

CHAPTER 4

RESULTS AND CONCLUSION

4.1 Bacterial Growth Rate as a Function of Coronene Concentration

Since PAHs are generally known to be noxious to both living organisms including bacteria, we extended our research to investigate the toxicity tolerance of 10CRN4D in the presence of increased CRN concentration. The experiment was carried out at increasing concentrations (5, 10, and 50ppm) while the pH, temperature and salinity were kept at 7, 37°C and 10w/v NaCl respectively. Before commencing the study, the effect of DMSO (0.1% v/v) on the bacteria growth was assessed as explained in section 5.0. As represented in figure 3, the bacterial growth was highest at the lowest concentration, 5ppm with maximum bacterial count of around 7^{11} CFU/ml within 9 days after starting the experiment from starting from 5.10^5 CFU/ml. On the contrary, 10 and 50ppm reached the peak around 8.90^{10} and 9.90^8 CFU/ml respectively also within 9days. However, the growth decreased generally for all concentration from day 20. When the growth profile was compared with the doubling time, the dt increases with increasing concentration such that 8.78hrs, 11.15 hrs and 15.71 hrs was recorded for 5ppm, 10ppm, and 50ppm respectively. Similarly, the quantification results of the remaining coronene supports data from the dt results as shown in figure 6.

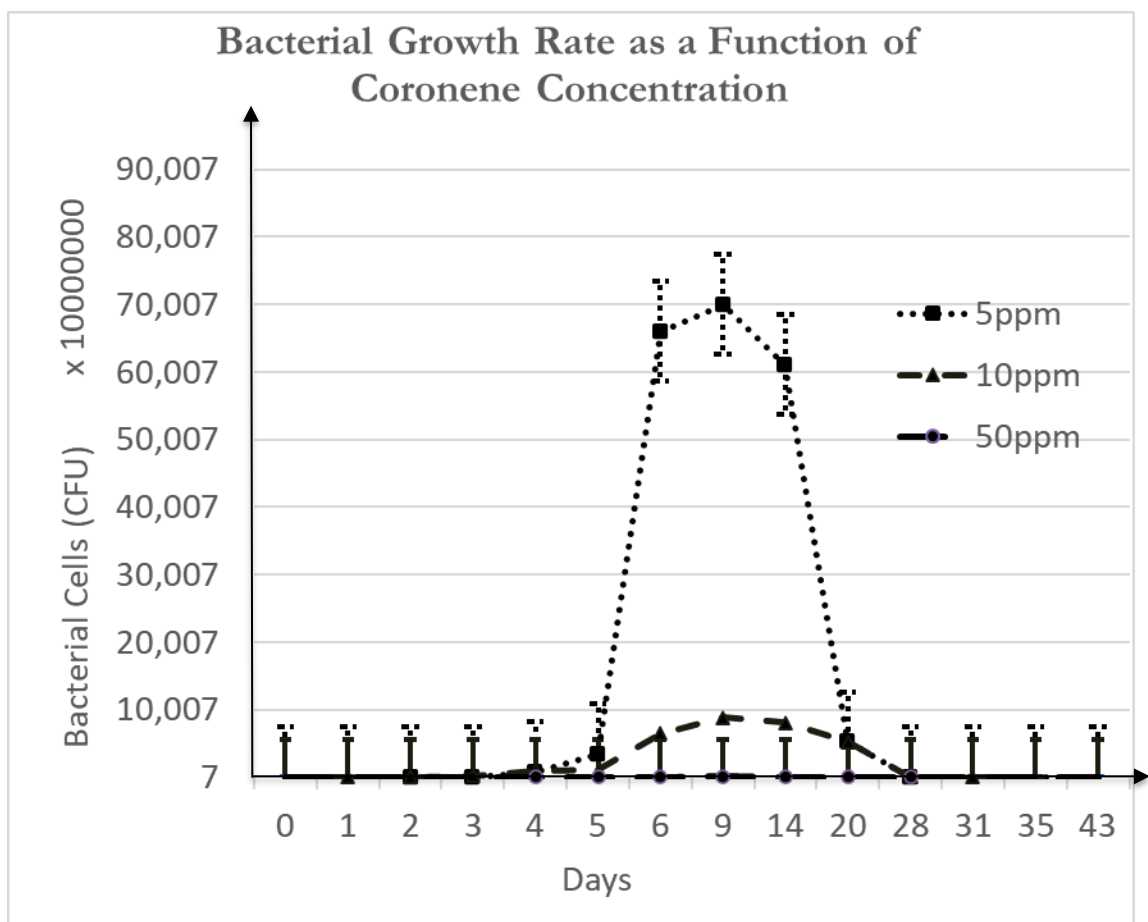


Figure 3: Bacterial Growth Rate as a Function of Coronene Concentration

4.2 Bacterial Growth Rate as a Function of Temperature Variation

In relation with the temperature, bacterial growth was monitored at 30, 37, 50 °C, while the concentration, pH and salinity fixed at 7, 5ppm and 10% NaCl (w/v) respectively. The best growth was recorded at 30°C, with a maximum growth of 9.80×10^{11} CFU/ml attained at day 28. As the temperature increases, bacterial growth decreases, as illustrated with a maximum count of 9.7×10^{11} CFU/ml on day 28 and temperature 37°C; while at temperature 50°C, the values peaked to 8.5×10^9 CFU/ml at day 9 as represented in figure

4 below. The dt values at these temperatures are 9.81, 10.51 and 14.73 hrs for 30, 37, 50°C, respectively which corresponds to the difference to the growth rate at each temperature.

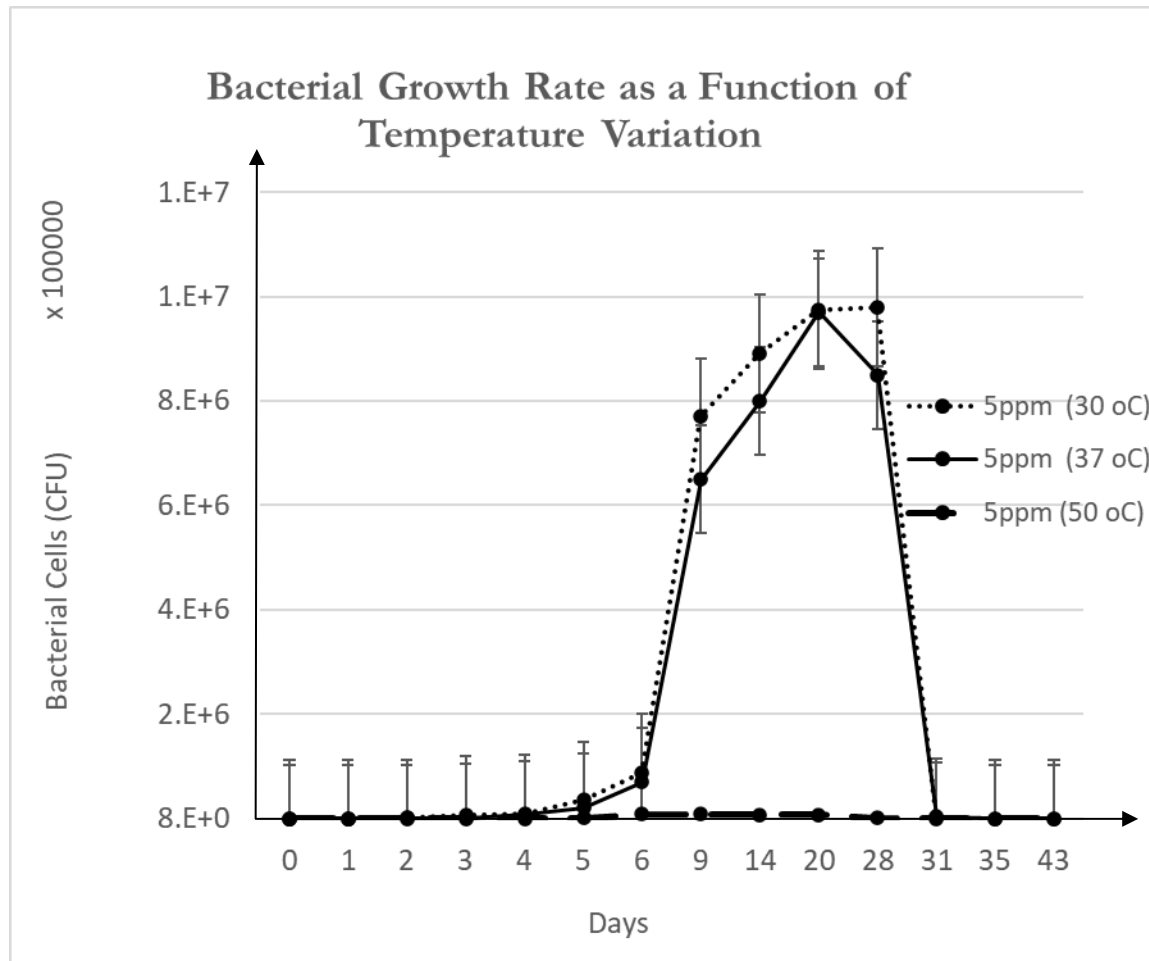


Figure 4: Bacterial Growth Rate as a Function of Temperature

4.3 Bacterial Growth as a Function of Salinity Variation

Fig 5 represents the growth profile of 10CRN4D strain at different salinity levels ranging between 0.5 to 20% w/v NaCl, corresponding to its high tolerance to low and high salinity levels respectively. Although the 10CRN4D strain was isolated at 10% w/v NaCl, the

growth profile at 0.5% and 10% NaCl showed similar results. On day 20, the bacterial count of 0.5% was slightly higher than 10% showing 7^{11} CFU/ml and 6.5^{11} CFUml⁻¹ respectively. In contrast, the bacterial growth decreased at 20% salinity with maximum growth 4.86^{10} CFU/ml only, within 9 days with dt values of 9.86, 10.42 and 11.78hrs correlating to 0.5%, 10% and 20% salinity w/v NaCl. These results show that this strain is halotolerant which is similar to the results obtained from another study by Wu et., al(Wu et al., 2008) although the *H. caseinilytica* strain was tested on a nutrient media.

The quantification result of the remaining CRN shown in Fig 8 during in vitro degradation experiment confirmed the high growth profile of 10CRN4D strain in CRN. It demonstrates a 57% degradation rate at 0.5% w/v NaCl which is followed by a gradual decrease as the salinity increases to 5, 10, 15% and 20% NaCl. Surprisingly, this strain shows a 19% degradation rate at 20% NaCl- an indication that this bacterium can actively grow up to 20% salinity. Overall, these data show that 10CRN4D strain is halotolerant and can grow and degrade COR within a salinity range of 0.5-20% w/v NaCl.

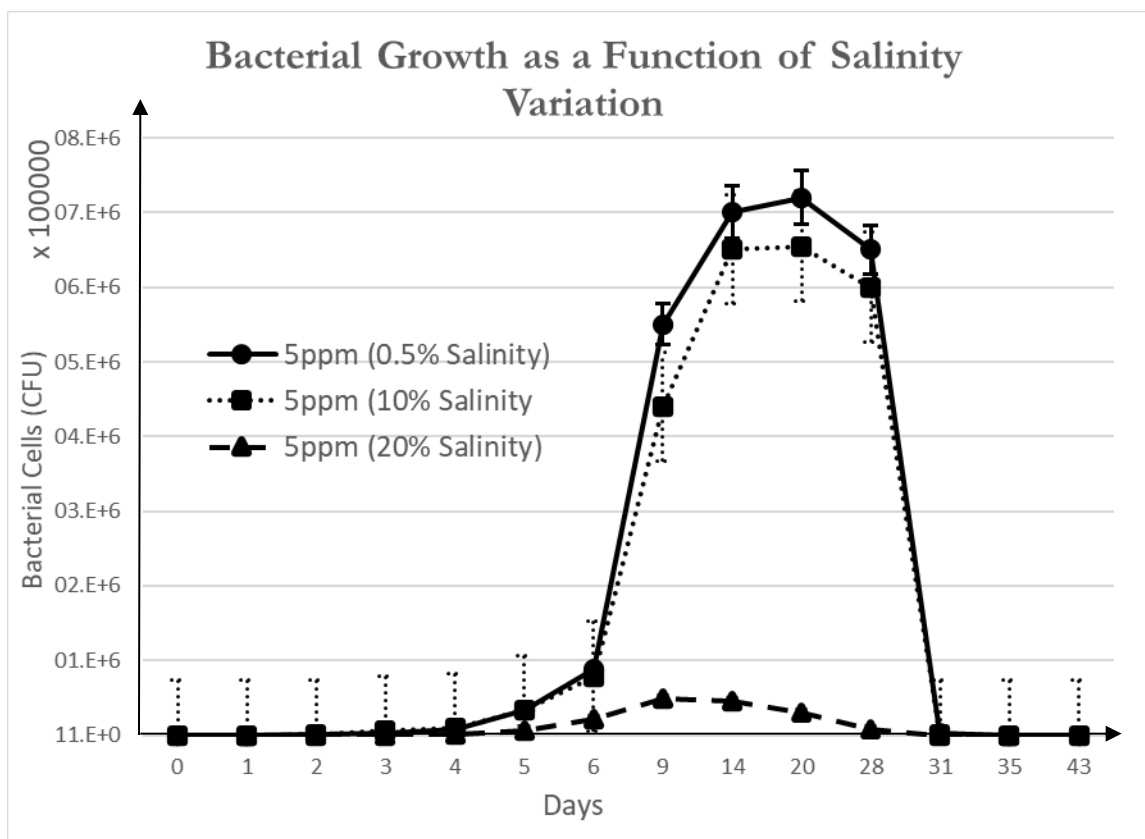


Figure 5: Bacterial Growth Rate as a Function of Salinity

For the pH investigation, pH was varied from 3 to 10 with concentration, temperature and salinity fixed at 5ppm, 37°C and 10% NaCl (w/v) respectively. The optimal growth was recorded at a pH of 7, corresponding to maximum count of 9.80×10^{11} CFU/ml, while growth reduced drastically after day 2 for both pH 3 and 10.

4.4 Quantification of coronene degradation using Gas chromatography

Using GC technique to quantify the amount of coronene degraded for a period of 30 days at 5, 10 and 50 ppm of COR by applying the conditions (pH 7, temperature 30 °C and

salinity 10% NaCl w/v). After 30 days of incubation, the results indicate a rate of degradation (of around 48%, 38% and 25 % respectively at 5, 25 and 50ppm COR, a clear indication that high COR concentration is associated with decrease bacterial activity, thus COR toxicity. The relationship of degradation rate and COR concentration follows a linear trend of $y = -11.255x + 59.637$ ($R^2 = 0.99$)

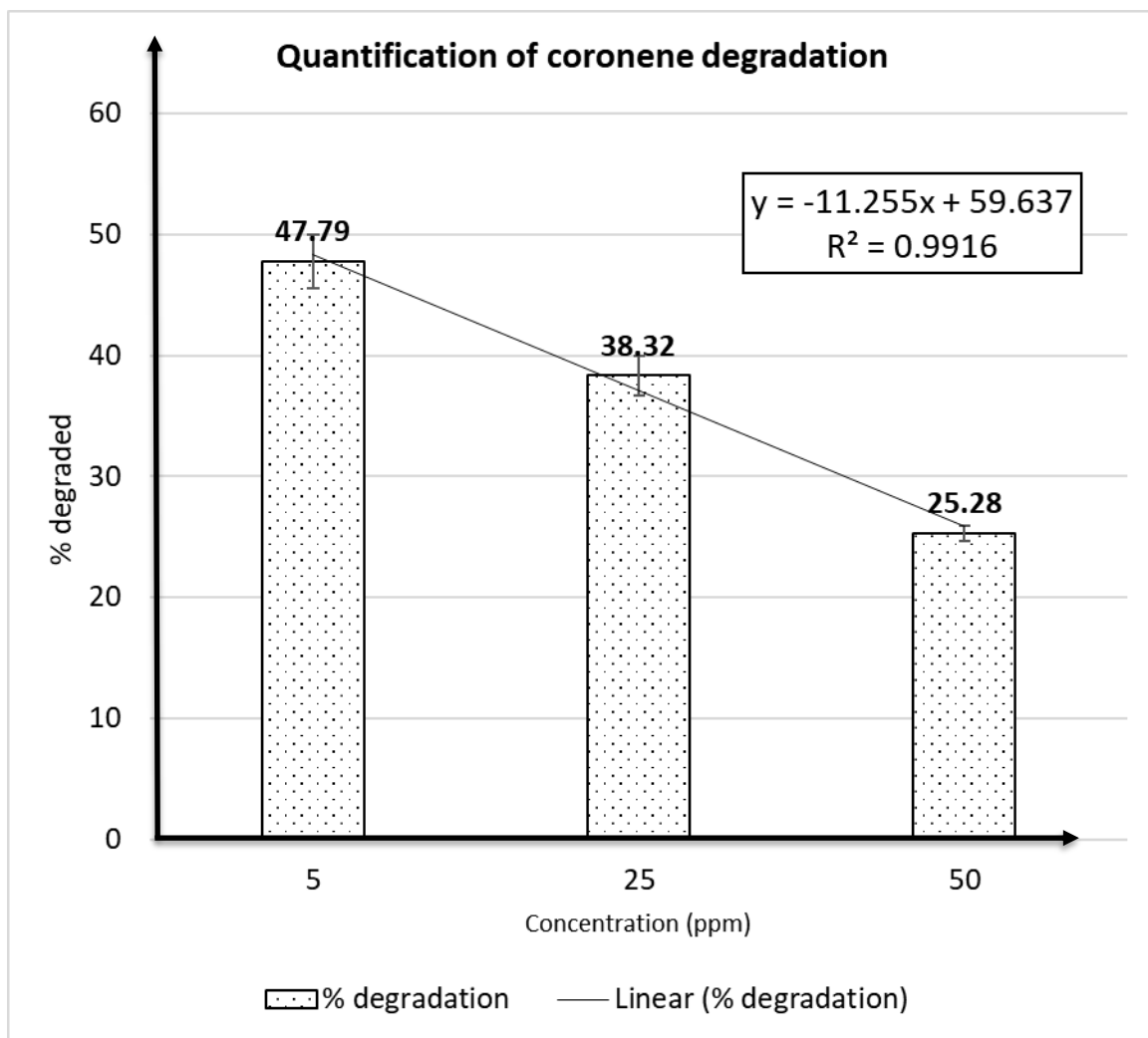


Figure 6: Quantification of coronene degradation at (pH 7, temp. 30 °C and salinity 10% NaCl w/v)

4.5 Effect of temperature on Quantification of coronene degradation using Gas chromatography

Following the quantification of in vitro investigation of coronene degradation where temperature was varied at 30, 37 and 50°C, and salinity, COR concentration and pH were maintained at 10% w/v NaCl, 5ppm and 7 respectively, the highest percentage of coronene degraded was recorded at 30 °C followed by 37 and 50 °C with percentage of degradation of approximately 50, 34 and 33% respectively as shown in figure 7. However, the efficiency reduced as the temperature increases as shown below. A comparison between the bacterial growth profile and quantification experiment shows no wide margin between temperatures 30 °C and 37 °C. However, the 50 °C associated with low growth profile surprising degraded about 33% of the coronene at the end of the investigation. Hence, this 10CRN4D strain has a relatively wide range tolerance for temperature.

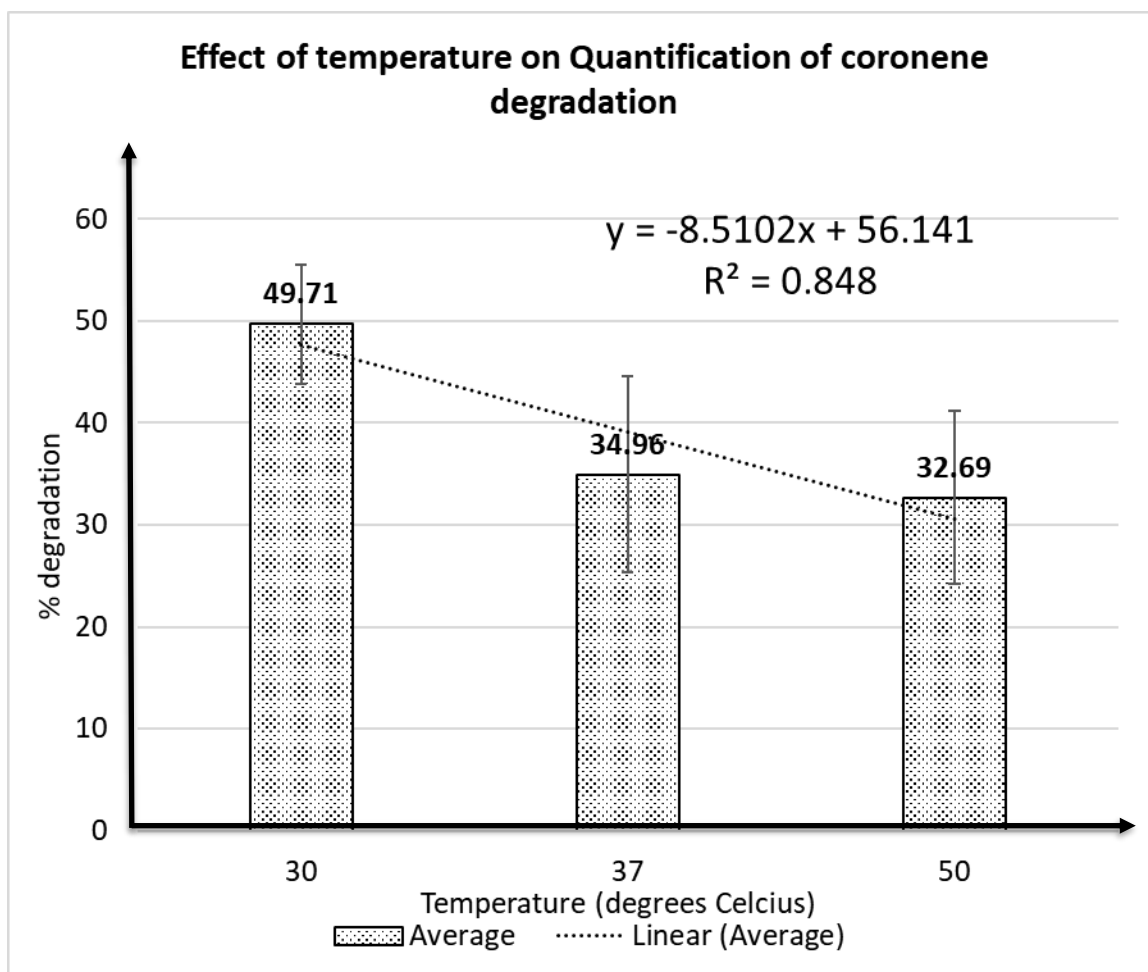


Figure 7: Effect of temperature on Quantification of coronene degradation

4.6 Effect of salinity on Quantification of coronene degradation using Gas chromatography

For this test, concentration remains at 5ppm while pH, temperature and time duration were maintained at 7, 30 °C and 30 days respectively. The result is represented in the graph below.

The experiment on the effect of salinity on coronene degradation proves that *H. caseinilytica* is most efficient in degrading coronene at low salinity conditions.

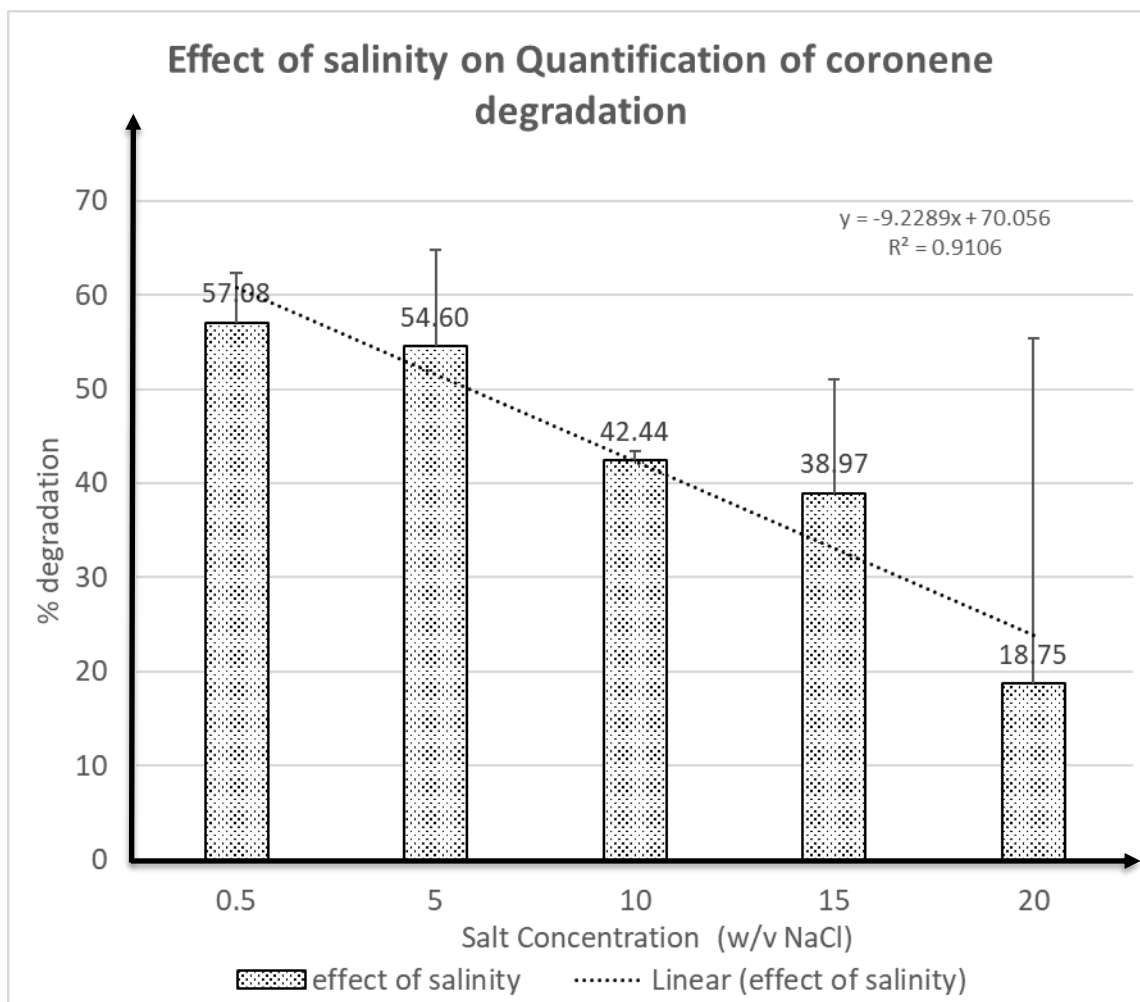


Figure 8: Effect of salinity on Quantification of coronene degradation

4.7 Effect of time variation on Quantification of coronene degradation using Gas chromatography

In the previous experiment, incubation was carried out within 30 days and since degradation rate increases with time, we sought to evaluate the degradation rate over a period of 80 days and the results are summarised fig 9. The effect of time variation was tested on coronene degradation and analysed using GC. The concentration was kept at 5ppm while salinity, temperature and pH were maintained at 10% w/v NaCl, 30 °C and 7

respectively. The concentration was kept at 5ppm while salinity, temperature and pH were maintained at 10% w/v NaCl, 30 °C and 7 respectively.

As shown in the figure 9 below, the highest degradation percentage of coronene (75%) was recorded at day 80, overall, this degradation increases as the time increases. In essence, the efficiency of this bacteria strain in degrading coronene increases as the time duration increases. The rate of degradation was $0.11\mu\text{mol}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$, similar to the rate of degradation of complex PAHs reported elsewhere(Nzila et al., 2022)

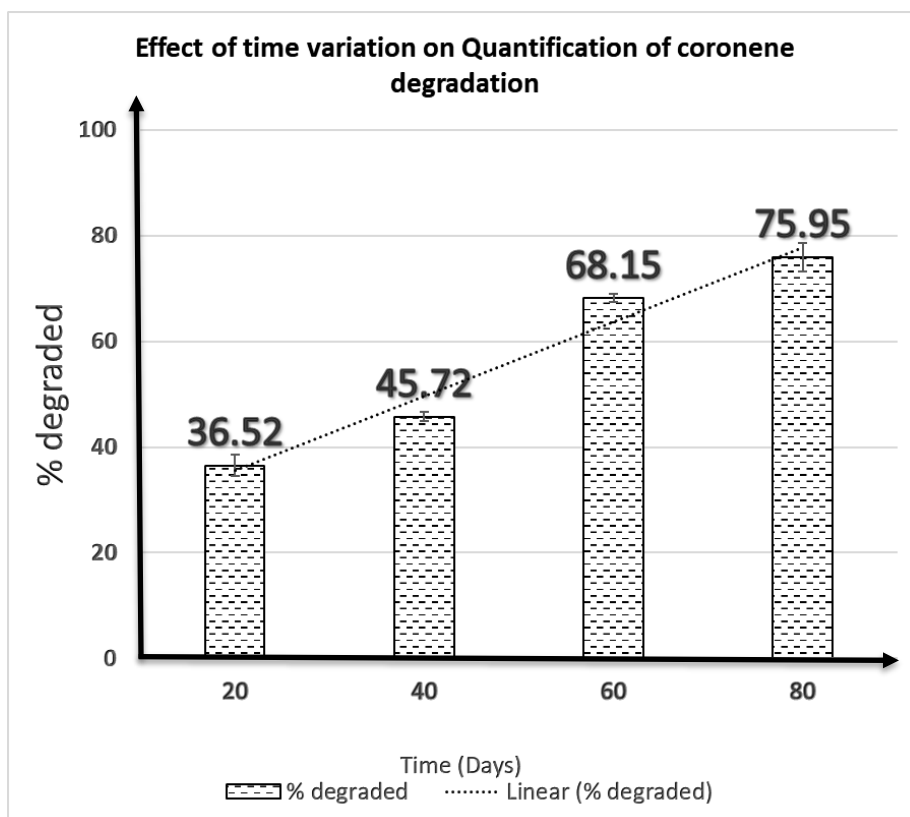


Figure 9: Effect of time variation on Quantification of coronene degradation

4.8 Discussion

This study has led to the isolation of *H. caseinilytica* bacterium, a unique specie that has surprising capabilities to survive under stress and has a wide substrate adaptability. *H. caseinilytica* belongs to the genera *Halomonas*. that are well known for their halophilic properties(Gan et al., 2018; Jiang et al., 2014; Kazak Sarilmiser et al., 2015; Lee et al., 2016; Pleissner et al., 2014) and effectiveness in degrading PAHs. For instance, Pleissner et al, Chaohua et al, Longzhan et al, Jae-Chan et al, and Juquan et al, have reported *H. boliviensis*, *H. smyrnensis* AAD6T, *H. huangheensis*, *H. saliphila*, *H. sediminicola* and *H. songnenensis* respectively which belong to the bacteria genus *Halomonas* to be halophilic(Gan et al., 2018; Jiang et al., 2014; Lee et al., 2016; Pleissner et al., 2014). Likewise, several bacteria from this genus have been reported to grow and degrade a wide range of LM-PAHs(Anbu, 2013; Cheffi et al., 2020; Corti Monzón et al., 2018; Govarthanan et al., 2020a; Mnif et al., 2009) and few HW-PAHs(Govarthanan et al., 2020a). This includes *Halomonas* sp. RM which was reported to biodegrade phenanthrene, pyrene, naphthalene and benzo [a] pyrene(Govarthanan et al., 2020b), *H. pacifica* that degrades naphthalene(Cheffi et al., 2020), *H. koreensis* that degrades fluorene(Nanca et al., 2018), and *Halomonas* sp. strain C2SS100 that degrades crude oil(Mnif et al., 2009). As much as we know, majority of these studies on *Halomonas* sp. and other bacteria generally focus on monoaromatics and LM-PAHs rather than HW-PAHs and heterocyclic compounds. In this current work, we have demonstrated that *H. caseinilytica* can grow and degrade HW coronene as the only carbon source.

Generally, bacteria of the genus *Halomonas* are widely known to be halotolerant growing within a salinity range between 0.5-30% w/v NaCl⁶⁴⁻⁶⁶. For instance, *H. socia* grows within 0.5-25% NaCl(Cao et al., 2013), *H. aestuarii* within 3.0-10.0 % (w/v) NaCl(Koh et al., 2017), *H. ramblicola* within 1 - 30% (w/v) NaCl(Luque et al., 2012) and *H. qiaohouensis* between 0.5-20 % (w/v) NaCl(Wang et al., 2014). In addition, *H. populi*(Xu et al., 2021), *H. endophytica*(C. Chen et al., 2018), *H. bachuensis*(Xiao et al., 2021), *H. lactosivorans*(Ming et al., 2020), *H. azerica*(Wenting et al., 2021) actively grow at 0.5–18.0%, 0.5-6.0 %, 1-15%, 1-12% and 1-17% w/v NaCl respectively. Similarly, *H. caseinolytica* can also grow and degrade PAHs within this range and even up to 20% w/v NaCl. Thus, the growth in a wide salinity range is a common feature in *Halomonas* bacteria. However, no growth was observed for 10CRN4D strain in the absence of salt which is comparably the same result reported for majority of the bacteria strains belonging to this genus. For example, *H. neptunia*, *H. neptunia*, *H. sulfidaeris*, *H. axialensis* and *H. hydrothermalis* did not grow in the absence of salt(Kaye et al., 2004).

As stated earlier, several species of halophilic bacteria including the genus *Halomonas* have been reported to degrade LW-PAHs such as naphthalene, phenanthrene, fluorene, (Anbu, 2013; Cheffi et al., 2020; Corti Monzón et al., 2018; Govarthan et al., 2020a; Mnif et al., 2009)(Fathepure, 2014; Le Borgne et al., 2008; Margesin & Schinner, 2001; Martins & Peixoto, 2012) and even a few HW-PAHs especially pyrene(Al Farraj et al., 2019; Rathour et al., 2018; Zhou et al., 2016) and benzo[a]pyrene(Govarthan et al., 2020a; Nzila et al., 2021a). For instance, *Halomonas* strains were reported to degrade a 4 ringed PAH -pyrene. In this study by Budiyanto, two strains of *H. shengliensis* and *H. smyrnensis* were reported to degrade pyrene within a range of 5-15% and 5-20% w/c NaCl

respectively(BudiyantoFitri et al., 2018). However, no studies have reported halophilic degradation of coronene. Presumably, this is because of the recalcitrant nature of coronene.

Furthermore, the general optimum pH for degrading PAHs have ranged from pH 6 and 8 with most results at pH 7(Leahy & Colwell, 1990; Margesin & Schinner, 2001). In contrast, some studies have reported degradation of PAHs at extreme pH levels. For example, an alkaliphilic *Mycobacterium* sp. strain MHP-1 has been reported to degrade pyrene at pH 9(Habe et al., 2004) while naphthalene and phenanthrene have been removed at acidic conditions as low as pH 3(Stapleton et al., 1998). Similar, several bacteria strains that belongs to the genera *Arthrobacter*, *Acidocella* and *Clavibacter* have been demonstrated to grow in naphthalene at pH 3.5 or lower(Dore et al., 2003). In respect to the genus *Halomonas*, some strains (*H. shengliensis* and *H. smyrnensis*) have been reported to degrade pyrene at pH 7 and 9(BudiyantoFitri et al., 2018).

In addition, several strains of *Halomonas* species have been reported to grow within a wide range of temperature ranging from 25-60 °C. For instance, *H. nitroreducens* strain WB1 was reported to have been isolated at 60 °C and its growth was tested at lower temperatures down to 25 °C(Chikkanna et al., n.d.). Other strains belonging to the genus *Halomonas* that grows within a wide range of temperature include *H. axialensis*, *H. hydrothermalis*, *H. neptunia*, and *H. sulfidaeris*(Harrison et al., 2015; Kaye et al., 2004; Yin et al., 2015). Similar to other bacteria belonging to the genus *Halomonas*, this 10CRN4D strain also exhibits the ability to grow within a range of temperatures.

With the knowledge that PAHs are generally known to be noxious to both living organisms including bacteria, results showing declined growth as PAH concentration increases is

never a surprising expectation because toxicity tends to increase as concentration. Our result as expected did nothing different to previous studies on PAH degradation. For example, degradation of pyrene by *Ochrobactrum sp.*, *Achromobacter xylosoxidans*, and halophiles such as *H. shengliensis* and *H. smyrnensis* (Arulazhagan & Vasudevan, 2011; BudiyanitoFitri et al., 2018; Nzila, 2018). Likewise, anthracene with phenanthrene on a co-culture of *Pseudomonas citronellolis* and *S. maltophilia* (Nzila et al., 2018)

Lastly, degradation rate of PAHs especially benzo[a]pyrene, a 5-ringed PAH has been evaluated by several bacterial strains using it as the only carbon source and the results indicate that the degradation rate falls within 0.04–0.3 $\mu\text{mol l}^{-1} \text{ day}^{-1}$ (Nzila et al., 2021b). This correlates with the results (0.11 $\mu\text{mole/L/day}$) in this current study though it falls on the lower side which is not surprising since it is 7-ringed and complex compound than BaP.

4.9 Conclusion

Having isolated the *Halomonas caseinilytica* bacterium in coronene and found to actively degrade coronene within a wide range of salinity levels (0.5-20% NaCl w/v) and temperature range of 30-50°C, it is a proven halotolerant and eurytherm microorganism. Its halotolerant nature is similar to other bacteria belonging to the genus *Halomonas* (Cao et al., 2013; Fathepure, 2014; Gan et al., 2018; Jiang et al., 2014; Lee et al., 2016; Miao et al., 2014; Ming et al., 2020; Nanca et al., 2018; Wenting et al., 2021; Xiao et al., 2021; Xu et al., 2021) that are also capable of degrading PAHs. To the best of our knowledge, this is the first report of a halophilic bacterial strain capable of degrading COR as the sole source of carbon. The optimal growth condition on coronene was assessed and tested which are pH 7, temperature 30°C and, salinity 0.5 w/v NaCl. This strain was effective and could degrade up to 75% of coronene within 80 days of incubation. However, it would be exciting to improve and expand the study on the PAHs degradative ability of *H. caseinilytica*. This expansion will determine its potential to be employed as a key component of programs designed to biodegrade environments contaminated with these PAHs.

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