Microbial Biodegradation of Hydrocarbons

A Dissertation Report for

Course code and Course Title: MMD 412 Dissertation

Credits: 8

Submitted in partial fulfillment of

Master's Degree's

Master of Sciences in Marine Microbiology

by

SONALI D. PHADTE

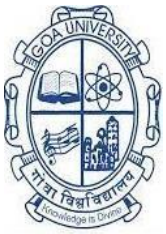
210P039020

Under the Supervision of

Dr. VARADA S. DAMARE

School of Earth, Ocean and Atmospheric Sciences

Marine Microbiology



Goa University

APRIL 2023

Examined by: Seal of the School

DECLARATION

I hereby declare that the data presented in this Dissertation report entitled, "Microbial Biodegradation of Hydrocarbons " is based on the results of investigations carried out by me in the M.sc in Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University under the Supervision/Mentorship of Dr. Varada Samir Damare and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

Sonali Devanand Phadte
21P0390020
M. Sc. in Marine Microbiology School of Earth, Ocean and Atmospheric Sciences, Goa University

Date:

Place: Goa University

CERTIFICATE

This is to certify that the dissertation "Microbial Biodegradation of Hydrocarbons" is a bonafide work carried out by Ms Sonali Devanand Phadte under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Master of Science in the Discipline M.Sc. Marine Microbiology at the School of Earth , Ocean and Atmospheric Sciences, Goa University.

Dr. Varada Samir Damare, Assistant Professor
Marine Microbiology
School of Earth, Ocean and Atmospheric sciences,
Goa University
Goa-403206

Date: 27/04/2023

School Stamp

Prof. C.U. Rivonker
M.Sc. Marine Microbiology
School of Earth, Ocean and Atmospheric Sciences,
Goa University
Date:
Place: Goa University

Dr. C. U. Rivonker
Senior Professor and Dean,
School of Earth, Ocean
& Atmospheric Sciences,
Goa University,
Goa - 403 206.

3

CERTIFICATE

This is to certify that the dissertation "Microbial Biodegradation of Hydrocarbons" is a bonafide work carried out by Ms Sonali Devanand Phadte under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Master of Science in the Discipline M.Sc. Marine Microbiology at the School of Earth , Ocean and Atmospheric Sciences, Goa University.

Dr. Varada Samir Damare, Assistant Professor
Marine Microbiology
School of Earth, Ocean and Atmospheric sciences,
Goa University
Goa-403206

Date:

Prof. C.U. Rivonker School Stamp
M.Sc. Marine Microbiology
School of Earth, Ocean and Atmospheric Sciences,
Goa University Date:
Place: Goa University

Acknowledgement

Firstly, I would like to express my immense gratitude and would like to thank my guide,Dr.Varada Samir Damare,Marine Microbiology,School of Earth, Ocean and Atmospheric Sciences, for her valuable guidance,support and untiring effort during the course of the dissertation. She has been a great source of motivation and encouragement for me to take up this topic as my study.Also, I am grateful to all the faculties of the School of Earth,Ocean and Atmospheric Sciences,for their willingness to help me whenever approached. I extend my sincere thanks to and all my other non-teaching and laboratory staff members.I am grateful to all my friends and classmates for helping me and morally supporting me throughout the course of the dissertation. Also, I would like to thank my senior and juniors in the course for helping and encouraging me.

Lastly,I would like to express my profound gratitude to my parents,my sister and all my friends for their encouragement and support.

Sonali Devanand Phadte

CHAPTER I

INTRODUCTION

1.1 Introduction

What are hydrocarbon?

Hydrocarbon are classified as pollutants and recalcitrant compound (Sunita et al 2017). Hydrocarbon are in two forms; Aliphatic saturated and unsaturated hydrocarbon which can be degraded by bacteria.(“A comprehensive of Aliphatic Hydrocarbon Biodegradation by bacteria”, 2015) crude oil are defined as a heterogeneous mixtures of alkanes, alkenes, cycloalkanes, and aromatics (Haque et al,2022; Wang et al 2022).

Oceans cover 70% of the Earth crust (Bollmann et at,2010). About 47% of the crude oil enters the marine environment through natural seeps. The remaining 53% result from leakage and spillage during extraction, transportation, refining, storage, and utilization of petroleum (Kvenvolden & Cooper,2003)

1.2 TYPE AND CHARACTEISTIC OF HYDROCARBONS

Although hydrocarbons arise from number of sources, crude oil is a complex mixture of many hydrocarbons which are directly release into waterway (Chaerun et al, 2004; Chen et al, 2015; Wang et al, 2018). The aromatic compounds consist of lower molecular weight hence their solubility in water decrease therefore oil appear in smaller droplet.(Robert et al.1951).The aromatic ring arrangement can be in form of linear, angular or cluster (Abdelshafy and Mansour,2016). Polyaromatic hydrocarbons (PAHs) depend on number of ring in compound which are than classified into low molecular weight and high molecular weight. PAHs are either emitted as gaseous phase or in particulate form since they depend on their molecular weight(lee and Vu,2010). PAHs further been classified as alternant PAHs (fused with six carbon Benzene ring) and non alternant PAHs like fluorene (fused with six carbon benzene rings along with an additional ring of less than six carbon) (Gupte et al., 2016). The biochemical persistence of PAHs that make PAHs more resistant to nucleophilic attack due to the existence of dense π electrons on aromatic rings (Haritash and Kaushik, 2009).

PAHs have low water solubility, low vapour pressure, and high melting and boiling points, rely on their structures (Lee and Vu, 2010). Decrease water solubility with increased molecular weight and increase lipophilicity which make them more recalcitrant compounds (Okere and Semple, 2012).

In the marine environment this natural seepage of crude oil(liquid petroleum:crude oil and tar) seeps can have a long-term presence (k.A.Kvenvolden & C.K Cooper,2003).

1.3 Review of literature

Gaseous and liquid petroleum hydrocarbons are ubiquitous in the world marine ecosystem both as a consequence of natural inputs or by way of anthropogenic contamination. Specific microorganisms have the capacity to utilize hydrocarbons as carbon and power sources under each oxic and anoxic conditions. Hydrocarbon biodegradation is beneficial as this process impacts the destiny of hydrocarbons and moderates dangers posed to humans and the environment via hydrocarbon exposure. Hydrocarbon-degrading microorganisms react to slow diffusive natural discharges at natural fuel and oil seeps and to massive anthropogenic air pollution events such as during the Deepwater Horizon blowout and the Prestige vessel disaster (Sara et al 2019). Assessing aerobic microbial degradation on a world scale through improved techniques or strategies remains challenging (Sara & Samantha 2019). Polycyclic aromatic hydrocarbons (PAHs) in the aquatic surroundings are in most cases regarded to be of 4 types: derived from fuels (petrogenic), derived from an incomplete combustion method (pyrogenic), generated by way of natural metabolism (biogenic), and generated by way of the transformation manner in sediment (diagenetic) (Hylland et at.,2006). Of these four types of sources, petrogenic and pyrogenic sources are broadly speaking artificial. PAHs can enter marine surroundings through more than a few ways, such as wastewater discharge, atmospheric deposition, floor runoff and crude oil leakage (Heemken et al., 2000). PAHs are highly lipid soluble and can be absorbed by the lungs, gut, skin of the mammals. The marine organisms close to the shore are affected by the use of coal power plants, petroleum refineries and petrochemical plants in surrounding cities, industrialized harbor and sea areas (Bandowe et al., 2014a; Benali et al., 2017). Marine pollution can be monitored by recording PAHs in the coastal environment and accumulated. PAHs cause damage to the tissues of coastal marine organisms that give an indication of the bioavailable portion of environmental PAH contamination (Zuloag et al., 2009).

The exploration and extraction of oil and gasoline produce waste materials such as used drilling fluids and drilling cuttings like complex combos of clays and chemicals. Usually the waste is discharged directly from the systems into the surrounding marine water (Lodungi et al., 2016). Most of the drilling wastes discharged in offshore and onshore is essentially similar. Offshore

petroleum drilling waste includes drilling fluids and drill stable cuttings (Melton, et al. ,2000). Drilling fluids consist of remnants of drill mud. Meanwhile drilling cuttings materials consists of crushed rock cuttings from borehole and again to the floor with drilling fluid (Khondaker, 2000). Drilling waste is produced when exploration drilling is conducted to obtain crude oil and the waste generated during the exploratory drillings is mostly non-hazardous. The next and the most common solid waste, that all the oil and gas industry have to overcome is to process the produced waste (Lodungi et al., 2016,). Offshore production accounts for 30% of the world's oil and gasoline manufacturing and is expected to extend in the future and it is similarly moving to deeper waters and harsher environments such as the Arctic which is a lot more challenging for a safe and environmentally sound operations. Produced water is the largest waste stream from oil and gasoline manufacturing unit (Zheng et al 2016).

The presence of sea ice is a hazard to the marine transport and petroleum industries and an obstruction to oil spill responders. Recent detection of spilled oil in ice (Bassett et al. 2016), Oil spilled under sea ice, as would maybe appear from a well blowout or damaged vessel, will upward jostle through the water column and pool at the underside of the ice, collecting in recessed undulations (Glaeser and Vance 1972).

Biosurfactants are one of the microbial bioproducts that are in most demand from microbialenhanced oil restoration (MEOR).

CHAPTER II

MATERIALS AND METHODS

2. MATERIAL AND METHODS

2.1 Collection of sample

2.2 The water sample was collected form Arambol beach and Aldona mangrove area,located in North Goa.Sample was collected on 23rd in 100ml plastic bottle rinsed with the same water before collection.

2.3 Ten ml of water sample was enriched in sterile Bushell Haas medium and supplemented with 0.5ml of crude oil and Kept for incubation at room temperature for 5 days.After incubation,this was streaked on Malt Extract Agar for isolation of microorganism.

2.4 Biochemical test

Biochemical tests were conducted on freshly growing bacterial culture for tentative identification of the isolates.The biochemical test were based on Bergey's manual(Bergey,1974) and these were Gram staining, indole test, Methyl red test, Voges Proskauer test,Citrate test,Triple sugar iron test,Urease test,and Catalase test(Details in Appendix).

2.5 EXTRACELLULAR ENZYMATIC ACTIVITY

The isolates were tested for production of extracellular enzymes such as protease,amylase and lipase.This was carried out by growing the isolates on Malt Extract Agar supplemented with 1% of different substrates substrates such as milk for protease,soluble starch for amylase,andTween 80 with 0.01%$Cacl_2$ .The substrates were autoclaved separately and added to the media when molten. Medium used for lipase activity contained 10g/L peptone,5g/L Nacl and 20g/L agar agar

2.6 SCREENING FOR BIO-SURFACTANT PRODUCTION

2.6.1 Hydrocarbon overlay test: ZMA plates were coated individually with 40ul of benzene, xylene, kerosene. Freshly grown bacterial isolates were spotted on the coated plates and incubated at room temperature.

2.6.2 Drop collapse test: Twenty ul of distilled water was taken on slide and 20ul of benzene was added . Ten ul of culture broth was added to the benzene surface.reduce in surface tension indicate positive surfactant activity.

2.6.3 Calculation of emulsification index: Pure culture were suspended in test tubes contain

2ml of BH and incubated for 48h. Later 2ml petroleum was added to each tube and vortexed.

Emulsification index was calculated as fellows;

Emulsification index=(height of the emulsion layer/total height)x100

2.7 EFFECT OF VARIOUS CONDITIONS ON THE GROWTH OF ISOLATES

2.7.1 Inoculum preparation for the experiments: Bacterial isolates were grown separately in Nutrient broth (NB) and sub-cultured in NB after 1day.The freshly growing culture from this flask was used as inoculum for final experiment.

2.7.2 Salinity: Different flasks of Nutrient broth were prepared using seawater of different salinities prepared by diluting natural seawater with distilled water. Salinity was checked using refractometer. Salinities tested were 30, 15and 0 (distilled water).

2.7.3 pH: BH media with different pH such as 5,6,7,8and 9 were prepared. The pH was adjusted using 1NHCL or1NNaOH. Benzene was added to 1% and the media flasks were inoculated and incubated at room temperature for 2 days after which biomass was measured.

2.7.4 Temperature: BH media with 1% benzene and inoculated separately with bacterial isolates were incubated at four different temperatures i.e. 37^{o}C,

4°C, room temperature (31°C) and 60°C. After incubation for 5 days, biomass was measured.

2.7.5 Nitrogen and Carbon source: Nitrogen sources tested were ammonium sulphate and potassium nitrate.Carbon sources were yeast extract and peptone.BH media with and without 1% benzene were prepared.After inoculating the cultures,flasks were incubated at room temperature for 2 days and then the biomass in each flask was measured.

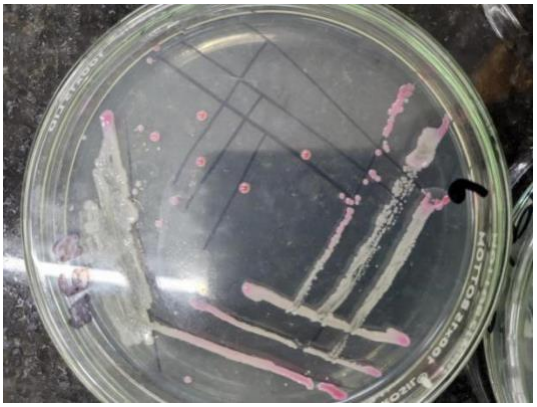
CHAPTER III

RESULTS AND DISCUSSION

3. RESULTS

3.1 Isolation and biochemical characterization:

One isolate each were obtained from Arambol beach and Aldona mangrove on MEA plate. These were named as AR_P(Fig.1A) and ALD_W(Fig.1B). The colony characteristics of the isolates is given in Table 1. Biochemical tests showed that isolate AR_P belonged to *Salmonella* sp. and ALD_W belonged to *Klebsiella* sp. using Bergey's Manual of Differentiation as reference (Table 2).

A 

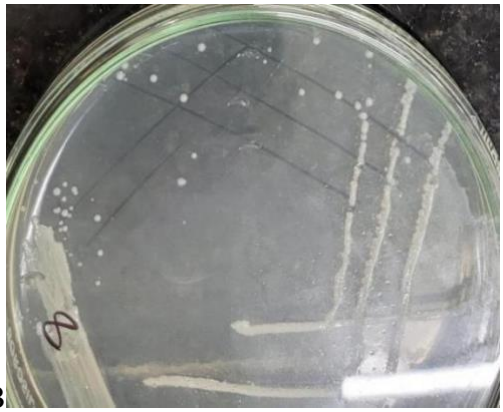
B 

Fig. 1: (A) AR_P and (B) ALD_W isolates obtained after purification.

Table 1 Colony characteristics of AR_P and ALD_W isolates.

S.No.	Colony Characteristics	AR_P	ALD_W
1	Time	24hour	24hour
2	Temperature	Room temperature	Room temperature
3	Colour	Pink	White
4	Sharp	Circular	Circular

5	Margin	Entire	Entire
6	Surface texture	Smooth	Smooth
7	Mac Conkeys Agar	White	Pink
8	Consistency	Buttery	Buttery
9	Opacity	opaque	opaque
10	Elevation	Rised	Rised
11	Size	0.5cm	0.1cm
12	pigment	Pink pigment	No pigment
13	microscopic	Rod shaped	Rod shaped
14	Gram's staining	Gram Negative	Gram Negative

Table 2 Biochemical tests of AR_P and ALD_W isolates.

	Tests	AR_P	ALD_W
6	Fermatation of lactose	Negative	Positive
7	Mac conkey agar	white colony	Pink colony
8	Starch hydrolysis	positive	negative
9	Lipase hydrolysis	positive	Negative
10	Protease hydrolysis	positive	Negative

11	Indole production	negative	negative
12	Methyl red test	negative	negative
13	Voges prokeur	negative	negative
14	Citrate utilization	prositive	positive
15	Catalase activity	positive	positive
16	urease	prositive	prositive
17	TSI agar	Alkaline slant, Acid butt, Gas, H_2S	Acid butt, Acid slant, Gas, No H_2S
18	spores	negative	negative
19	Tentative identification	*Salmonella* sp.	*Klebsiella* sp.

EXTRACELLULAR ENZYMATIC ACTIVITY

AR_P isolate produced lipase (Fig. 2), amylase (Fig. 3) and protease enzymes as seen by the zone of clearance of the substrate (Table 3, Fig. 4). Isolate ALD_W did not produce any enzyme.

Table 3 Diameter of zone of clearance (mm) of isolate AR_P.

	Average	Std. Dev.
lipase	3	1.290994449
amylase	1.45	0.238047614
protease	2.15	0.238047614

Fig. 2 Lipase test of isolate AR_P.

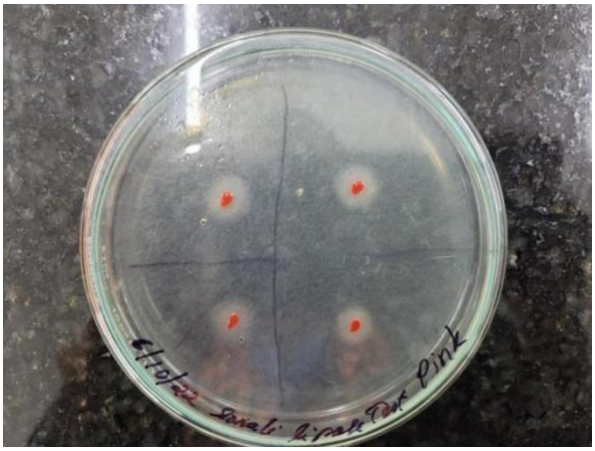


Fig. 3 Amylase test of isolate AR_P

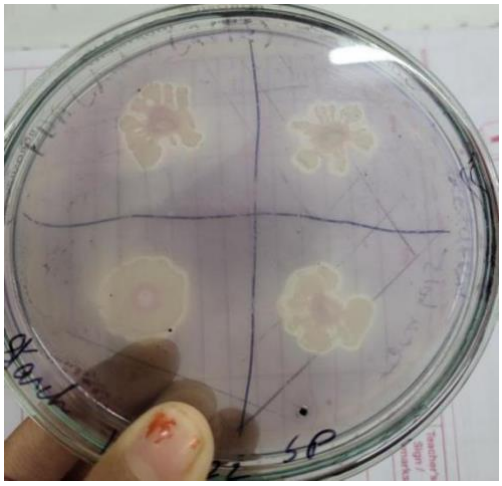
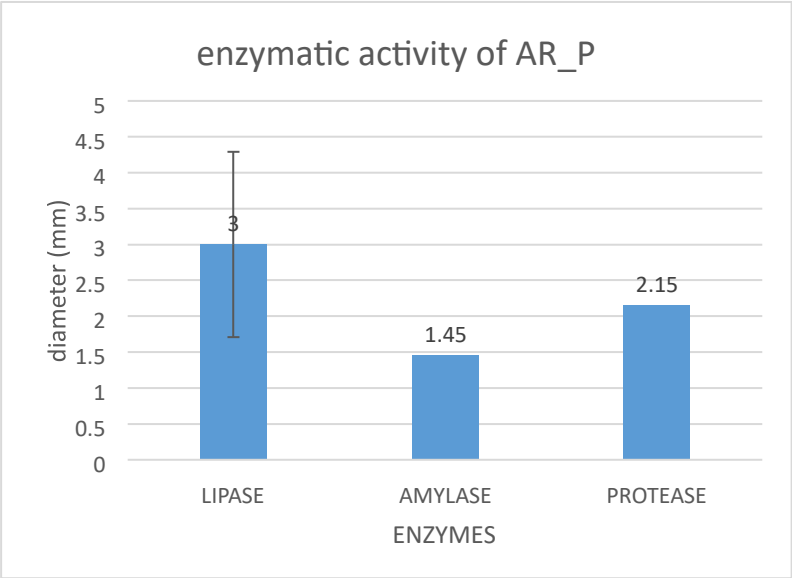


Fig. 4. Enzymatic activity of AR_P isolate.



SCREENING FOR BIO-SURFACTANT PRODUCTION

Hydrocarbon overlay test: Bacterial growth was seen in benzene but no zone of clearance.

They did not grow on kerosene and xylene plated medium. Thus, AR_P and ALD_W gave negative results for hydrocarbon overlay agar plate.

Oil collapse test: Islate AR_P showed positive result for this by collapsing in the oil drop.

Emulsification index: This was found to be 20 % for isolate ALD_W and 30 % for isolate AR_P.

Table 4. Results for oil drop collapse assay, hydrocarbon overlay assay and assay for emulsification index

Isolate	Oil drop collapse assay	Hydrocarbon overlay	Emulsification assay*
AR_P	+	Nil	30%
ALD_W	-	Nil	20%

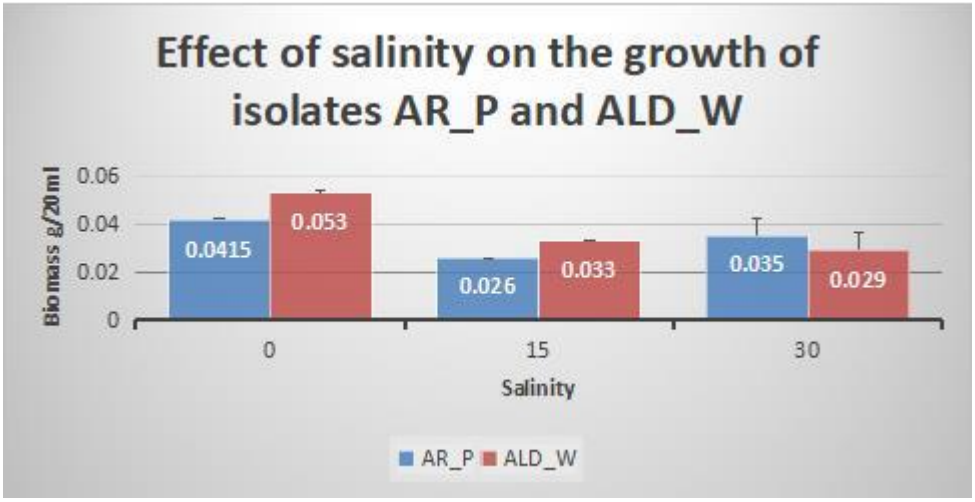
'+' raised surface,'-'unraised surface.

*Emulsification index of >30% indicates high activity of biosurfactant.

EFFECT OF VARIOUS CONDITIONS ON THE GROWTH OF ISOLATES

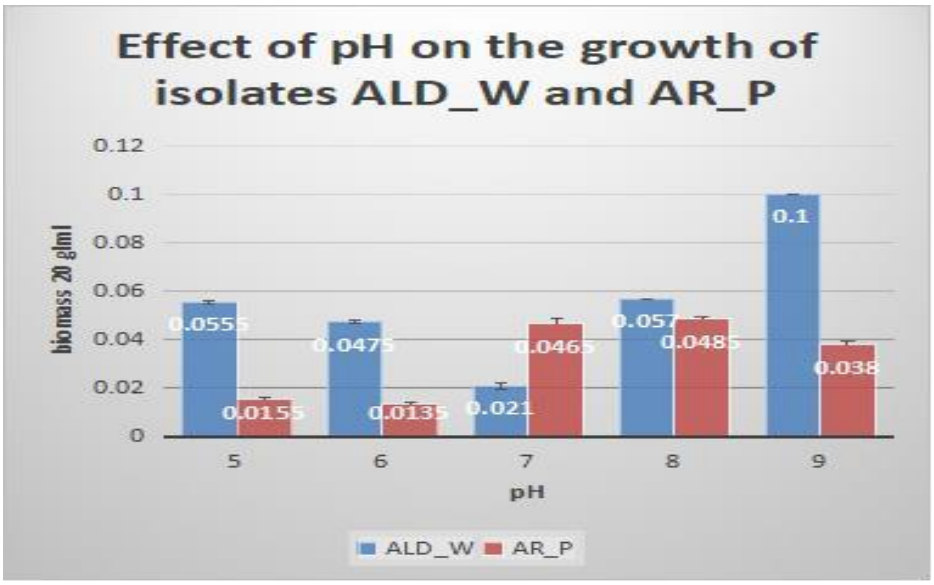
Salinity: It was seen that both the isolates AR_P and ALD_W grew well in distilled water and their growth stunted as the salinity increased (Fig. 5).

Fig. 5 Effect of salinity on the growth of isolates AR_P and ALD_W.



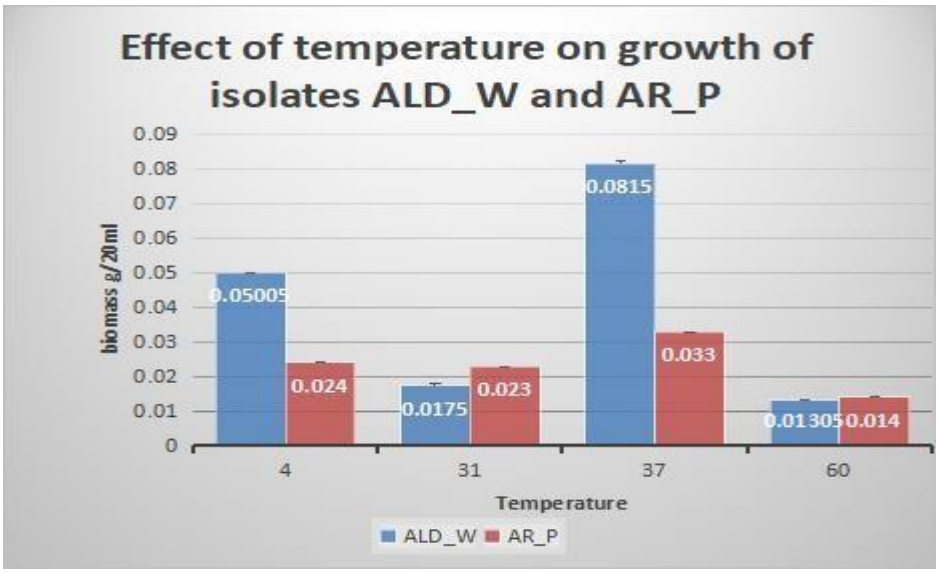
pH: With the increase in pH of the medium, the biomass obtained also increased for both the isolates (Fig. 6). ALD_W showed very less growth at pH 7 whereas AR_P showed very good growth.

Fig. 6 Effect of pH on the growth of isolates AR_P and ALD_W.



Temperature: Isolate ALD_W showed good growth at room temperature i.e. 31ºC and isolate AR_P showed good growth at 37ºC (Fig. 7).

Fig. 7 Effect of temperature on the growth of isolates AR_P and ALD_W.



Nitrogen and Carbon source: Out of all the substrates tested, isolate ALD_W showed the best growth in the presence of yeast extract followed by potassium nitrate (Fig. 8). Isolate AR_P showed best growth in the presence of potassium nitrate (Fig. 9).

Fig. 8 The Effect of Nitrogen and Carbon Source on the growth of ALD_W.

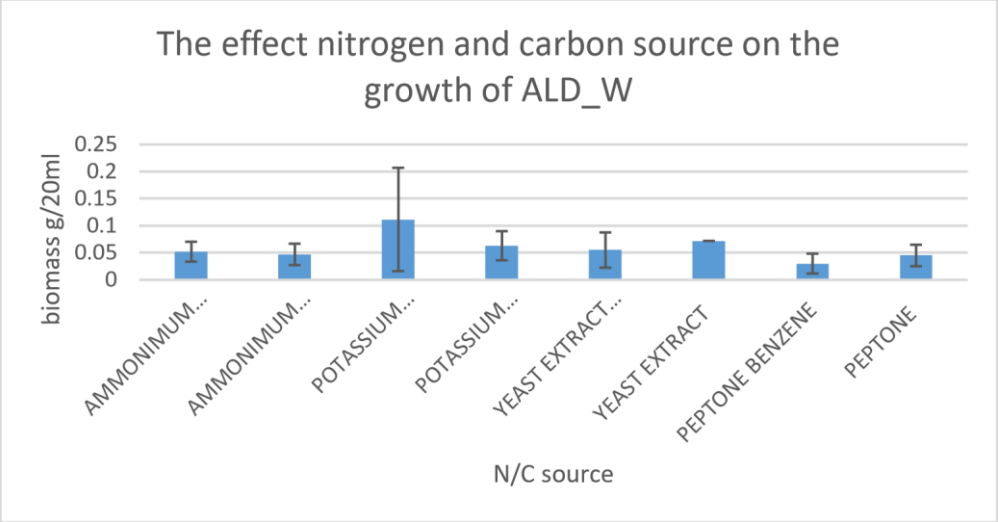
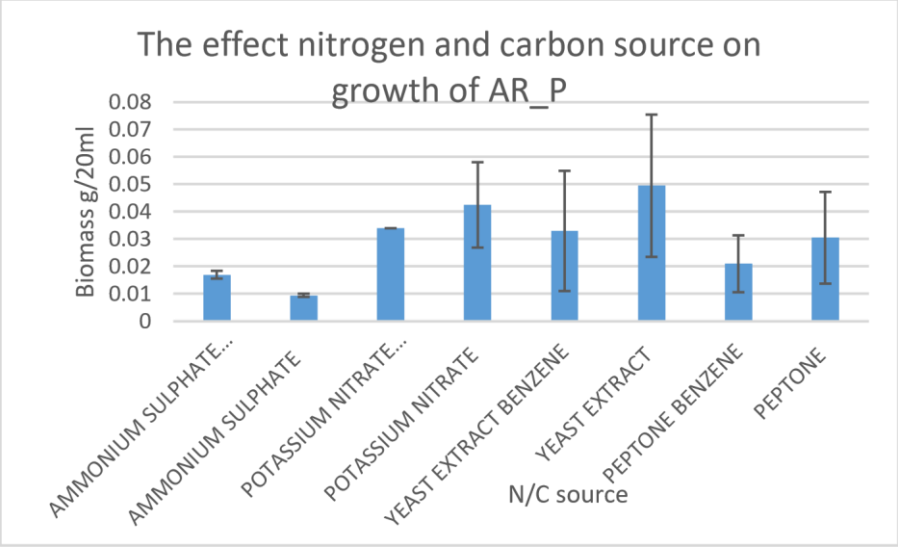


Fig. 9 The Effect of Nitrogen and Carbon Source on the growth of AR_P.



3.2 DISCUSSION

Petroleum, crude oil, Polyaromatic hydrocarbons (PAHs),benzene, xylene, kerosene are toxic and hazardous to marine environment and marine organisms.

The production, transportation, refining, disposal of hydrocarbon is increasing day by day and hydrocarbons have become main energy source for industrial line. Biodegradation by microorganism helps in reducing oil contamination from marine sites (Bartha,1996).

Hydrocarbon degrading bacteria are widely spread all over marine environment (Bartha,1996). Bacteria can survive in oil because they utilize hydrocarbon as carbon source (Bagga,2015). in the present study isolates grw in the presence of crude oil and benzene. The study was carried out by collecting water sample form Arambol beach and Aldona mangrove area and enriching in Bushell Haas broth with supplement of crude oil as carbon source. The objective of this study was to isolate and identify hydrocarbon-degradative ability of marine microorganism.The isolated strain of bacteria were tested for biosurfactant production and extracellular enzymatic activity.

The Biosurfactant and extracellular enzymes are secreted form bacterial cell surface (Karanthv et al,1999). The microbial biodegradation of hydrocarbon may occur in following stages i.e., 1) microorganism associating with hydrocarbon, 2) secretion of extracellular enzyme, 3) cleavage of hydrocarbon chain and 4) biodegradation. Biosurfactant help in bioremediation of hydrocarbon pollutant (Nayarisseri et al 2018). Screening for biosurfactant production using hydrocarbon overlay agar plate (HOA) which shows degrading hydrocarbon ability by bacteria that gave a zone of clearance with kerosene,hexadecane and benzene the bacteria *staphylococcus* were observed for positive result and negative for *Ecoli* (Nayarisseri et al 2018) . In the present study negative result was obtained for hydrocarbon overlay test but AR_P isolate showed positive biosurfactant production with oil collapse test. AR_P showed growth in hydrocarbon overlay plate with benzene but no zone of clearance and no growth with kerosene, xylene and ALD_W also gave negative result.

Oil spreading assay result were positive for *Staphylococcus* and *Bacillus* but negative result in *E.coli* (Nayarisseri et al 2018).

Emulsification index of >30% indicates that high biosurfactant activity. However since both the isolates showed 20 and 30 % index, it indicated less or negligible biosurfactant activity. Petroleum was used as the hydrophobic substrate. In the present study .The observation shown with *bacillus*(87%) and *E.coli* (15%) (Nayarisseri et al 2018) comparatively high emulsifier than current study.

Nitrogen source plays important role in biosurfactant production (silva et al 2010). Our study also showed good growth in the presence of potassium nitrate and yeast extract. The latter is also a carbon source.

CHAPTER IV

SUMMARY AND CONCULSIONS

4.SUMMARY AND CONCLUSION

In this study of microbial biodegradation of hydrocarbon was successfully carried out. Hydrocarbon degrading bacteria named AR_P was isolated form Arambol beach and ALD_W from Aldona mangrove water and these were tentatively identified as *Salmonella* sp. and *Klebsiella* sp. respectively. AR_P was able to secrete extracellular enzymes which shows its potential in bioremediation and biodegradation . the bio-surfactant produced in strain AR_P and was not found in ALD_W. Alkaline pH produced more biomass than neutral or acidic. Room temperature and 37^{o}C were favourable for their growth.

BIBLIOGRAPHY

Bibliography

A.K.Haritash,C.P. Kaushik2009 Biodegradation ascepts of Polycyclic Aromatic Hydrocarbon (PAHs): A review 169.(1-3):1-15.doi:10.1016/j.jhazmat.2009.03.137

A.N.Khondaker.2000 Modeling the fate of drilling waste in marine environment. Vol 26.(5):531-540.doi:10.1016/S0098-3004(99)00135-1

Abdel-shafy,H.I and Mansour, M.S.M(2016)A Review on Polycyclic Aromatic Hydrocarbons: Source, Environmention Impact,Effect on Human Health and Remediation.Egyptian Journal of petroleum,25,107-123.doi:10.1016/ejpe.2015.03.011

Aksaya Gupte,Archana Tripathi,Helina Patel,Darshan Rudakiya and Shilpa Gupte. Bioremediation of Polycyclic Aromatic Hydrocarbon(PAHs).*The open Biotechnology Journal,*2016,10(Suppl-2,M9)363-378.WWW.benthamopen.com/TOBIOTJ/ doi:10.2174/18740701610010363

Anuraj Nayarisseri,Poonam Singh,Sanjeev Kumar Singh.2018. Screening, isolation and Characterization of Bio-surfactant producing *Bacillus Subtilis* strain ANSKLAB03.Vol14(6):304-314doi:10.6026/97320630014304

Bandowe,B.A.M.,Bigalke,M.,Boamah,L.,Nyarko,E.,saalia,F.K.,&Wilcke,W.(2014).polycylic aromatic compounds(PAHs and oxygenated PAHs) and trace metalsin fish species from Ghana(West Africa):Bioaccumulation and health risk assessment.*Environment international,65,*135-146.

R. Bartha,''Biotechnology of Petrolum pollutant Biodegradation", Microbiol.Ecol.,12:155172,1996.

Benali,I.,Boutiba,Z.,Grandjean,D.,de Alencastro,L.F,Rouane-Hacene,O.,and Chevre,N(2017)spartial disteibution and biological effects of trace metals (Cu,Zn,Pb,Cd) and organic micropollutants (PCBs,PAHs)in mussels Mytillus galloprovincialis along the Algerian west coast.*Mar.poll.bull*.115,539-550.doi:10.101016/j.marpolbul.2016.12.028.

Bollmann,M.,Bosch,T.,ColijnF.,Ebinghaus,R.,Froese,R.,Gussow,k..Wein-berger,F(2010).World ocean review:Living with the Oceans.Hamburg Maribus.oceanrep.geomar.de/22086/1/WOR1.1-236

Byeong-Kyu Lee and Van Tuan Vu 2010. Source , Distribution and Toxicity of Polycyclic Aromatic Hydrocarbons (PAHs) in particulate matte*r.Department of Civil and Environmental Engineering University of Ulsan Korea.*Air pollution doi:10.5772/10045.99-122

Chaerum S.K.,Tazaki K.,Asada R.,Kogure K(2004). Bioremediation of coastal areas 5 years after the Najhodka oil spill in the Sea of Japan: Isolation and Characterization of hydrocarbondegrading bacteria. *Environ.Int.*30 911-922.10.1016/j.envint.2004.02.007

Chen M.,Xu P.,Zeng G., yang c.,Hung D.,Zhang 2015. Bioremediation of soil contanminated with polycycyclic aromatic Hydrocarbons, Petroleum, pesticides,chlorophenols and heavy metals by composting : applications microbes and future reseach needs.*biotechnol.Adv.*33 745755.10.1016/j.biotechachdv.2015.05.003

Christopher Bassett,Andone C Lavery, and Ted Maksym(2016).Broadband acoustic backscatter form crude oil under laboratory-grown sea ice.J.Acoust.Soc.Am.140(4),22742287.doi.10.1121/1.4963876

Firouz Abbasian,Robin Lockigton,Megharaj Mallavarapu,Ravi Naidu.A comprehensive Review of Aliphatic Hydrocarbon Biodegradation by Bacteria.*Applied Biochemistry and biotechnology2015.*doi:10.1007/s12010-015-1603-5

Gleaeser,J.L. and Vance GP:A Study of the behaviour of oil spills in the arctic. Offshore Technology Conference,1-3 May1972,Houston,TX.doi:10.4043/1551-MS

H.R.Melton, J. P. Smith,C.R.Martin, T.J. Nedwed,H.L .Mairs, D. L.Raught.2000. Offshore Discharge Fluids And Cuttings-A Scientific Perspective On Public Policy.Rio de Janeiro:IBP ;IBP44900;16-19

Heemken, O.P. ,Stachel,B.,Theobald,N.,and Wenclawiak,B.W(2000). Temporal variability of organic micropollutant in suspended paritculate matter of the River Elbe at Hamburg and the River Mulde at Dessau,Germany.*Arch environ. Contaminat . Toxicol.*38,11-31.doi:10.1007/s002449910003 S. Haque, N. Srivastava, D.B. Pal, M.F.Alkhanani,A.H. Almalki, M. Y. Areeshi, R. Naidu, V.K. Gupta(2022) Functional microbiome strategies for the bioremediation of petroleumhydrocarbon and heavy metal contaminated Soil: a review. Sci.Total Environ., Vol833,Article 155222. doi:10.1016/j.scitotenv.2022.155222

Jasbir Bagga,Mithilesh Pandey and Vibha Pandey.2015. Isolation, Characterization and Identification of Three petroleum Tolerant and Degrading Bacteria*(Micrococcus, Staphylococcus* and *Pseudomonas* Spp.)From Petroleum Oil Contaminated Soil.Vol.4(10):9993-10005doi:10.15680/IJIRSET.2015.0410098

Karanth,N.G.K.,P.G Deo and N.K Veenanadig,1999.Microbial Production of biosurfactants and their important.Curr.Sci.,77:116-126.

Ketil Hylland. 2006. Polycyclic Aromatic Hydrocarbon(PAH)Ecotoxiocology in Marine Ecosysystems.journal of toxicology and Environmental Health,part A,69:109123.doi:10.1080/15287390500259327

Kvenolden, K.A and cooper, C.K(2003)Natural seepage of crude oil into the Marine Environment.Geo-Maeine Letter,23,140-146.doi 10.1007/s00367-003-0135-0

Lodungi JF, Alfred DB, Khirulthzam AFM, Adnan FFRB, Tellichandran S (2016) A Review in Oil Exploration and Production Waste Discharges According to Legislative and Waste Management Practices Perspective in Malaysia. Int J Waste Resour 7: 260. doi: 10.4172/22525211.1000260

Robert L.Bohon and W.F. Claussen..1951.The Solubity of Aromatic Hydrocarbons in Water. *J.Am.chem.Soc*.73,4,1571-1578 doi:10.1021/ja01148a047

Sara Beresford, Jessie Kastler, Rachel Mcdoonald , Dan Dini Cola , And Katie Fillingham..2019. Deepwater Horizon Oil Spill Impacts On Organisms and Habitats.*The journal of marine education.* Vol33.No1.winter 2019. 1-48

Sara Kleindienst and Samantha B.Joye.2019.Global Aerobic Degradation of Hydrocarbons in Aquatic symstems. In book: Aerobic Utilization of Hydrocarbons, Oils, and Lipid. pp.797-814.doi:10.1007/978-3-319-50418-6_46

Silva S, FariasC, RufinoR, Luna J, Sarubbo L.Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992.Col-loids surf,B 2010;79:174-83

Sunita j.Varjani.2017.Microbial degradation of petroleum hydrocarbons.Bioresource Technology.doi:10.1016/j.biortech.2016.10.037.Vol 223,277-286

Uchechukwu V Okere and Kirk T Semple.2012. Biodegradation of PAHs in Pristine from Different Climatic Regions.J.Bioremed Biodegrad .*Lancaster Environment Centre , Lancaster University, Lancaster,LA14YQ,UK* S:1doi10/4172/2155-6199.S1-006 . 1-11

Wang C., Liu.,Guo J.,Lv Y(2018). Biodegradation of marine oil spill residues using absoriginal bacteria consortium based on penglai 19-3 oil spill accident. China. *Ecotoxicol. Environ.Saf.*159 20-27. 10.1016/j.ecoenv.2018.04.059.

X. Wang ,C. Wang, W. Jiang, Y. Pan, F. Li, H. Tian. The occurrence and partition of total petroleum hydrocarbons in sediment, seawater, and biota of the eastern sea area of Shandong Peninsula,China.Environ.Sci,Pollut.Res.,(2022), 29(54) 82186-82198.doi:10.1007/s11356-02221376-7

Zheng J,Chen B,ThanyamantaW,Hawboldt K,Zhang B et al(2016)offshore produced water management:A review of current practice and challenges in harsh/Arctic environments.Mar Pollut Bull 104:7-19.

Zuloaga, O., Prieto, A., Usobiaga, A., Sarkar, S. K., Chatterjee, M., Bhattacharya, B. D., et al. (2009). Polycyclic aromatic hydrocarbons in intertidal marine Bivalves of Sunderban mangrove wetland, India: An approach to biomarker species. *Water, Air, Soil Pollution, 201*, 305–318.

APPENDIX

APPENDIX

A.1 Gram staining

- Take clean slide using a nichrome loop add one drop of saline ,make an even smear of the culture suspension on a clear grease free slide

- Allow the smear to dry and heat fix it.
- Discard the stain and gently wash with tap water
- Flood the smear with gram Iodine for 2min than discard
- Treat with alcohol for 3 min till decolourizer
- Wash the slide with water and counter stain with saffranin.
- Discard the stain gently wash with Tap water air dry and observe under oil

A.2 Biochemical reactions

- Indole test:The medium used is tryptone water which is rich in the amino acid tryptophan. Some organisms produce an enzyme called trytophanase which breaks drown tryptophan into in-dole and pynvic acid .Indole is concentrated from the medium in an organic solvent like. Composition:typtophan broth g/l peptone10g;sodium chloride5g;D/L Tryptophane1g. Xylene/xylol 2to3 drops of Kovac's reagent are added to this which gives a pink colour in the xylol layer.

Composition:Amyl or isomyl alcohol 150ml p-dimethyl amino benzaldehyde 10gm concentration Hcl 50ml and slowly add the acid.

- Methyl Red Test: the medium used is glucose phosphate peptone water. This test is employed to delect the production of sufficient acid during the fermentation of glucose.K_2HPO_4 added to the medium maintains the pH at around 4.5(which is obtained due to acid production) Methyl Red indicator gives red colour at this pH value .The Test is most useful in differentiating between *Escherichia and Aerobacter*
- Voges Proskaur test:The medium used is again glucose phosphate peptone water.Many organism which ferment glucose produce acetyl Methyl carbinol or its reduction production product 2,3 butane diol.These 2 products on oxidation under alkaline conditions react with the guanidine group of creatine to give cosin pink colour in the solution. After the growth of organisms in the glucose-PO_4 medium, to one part of the

medium(I.e about 2 to 3ml) 0.5ml of O'mearas reagent is added.The tude is incubated at 37c for 2-4hours and the result is noted O'meara's reagent contains 0.3% creatine in 40%KOH

- Citrate Test: This Test to detect the ability of an organism to utilize citrate as the sole carbon and energy source for growth and on NH_4 salt as the sole source for growth and on NH_4 salt as the sole source of nitrogen.Two types of media can be used koser's citrate-a liquid medium and simmon's citrate-a solid medium. simmon's citrate medium is a modified koser's medium used in the form of a slant.The organisms are inoculated on the slant surface. After 24hours incubation, a positive is shown by the appearance of growth and blue colouration due to alkaline pH of the slant surface. pH range 6&7.6 colour change from yellow to blue

- Triple surgar iron agar:This medium is used for the identification of Gram negative enteric pathogens in the routine examination of stools. The medium indicates the ability of an organism to ferment lactose,sucrose and glucose with the formation of acid and gas and also its ability to produce H_2S form sodium Thiosulpate and ferric/ferrous ions phenol red is the pH indicator.Organisms the ferment glucose produce a variety of acid, turning the colour of the medium from red to yellow more amount of acid are liberated in butt(fermentation) than

  in the slant (respiration) Growing bacteria also from alkaline products neutralize the large amounts of acid present in the butt/slant.Thus the appearance of an alkaline (red) slant and an acid(yellow)butt after incubation indicates that the organism is a glucose - fermention but is unable to ferment lactose or sucrose . bacteria the ferment lactose or sucrose (or both) in addition to glucose,produce large amounts of acid that prevent reversion of pH in that region and thus bacteria exhibit an acid slant and acid butt.Gas production(co_2)is delected by the presence of cracks or bubbles in the medium,when the accumulate gas

escapes.Thiosulphate is reduced to H_2S by several speceis of bacteria and combines with ferric ions of ferric salts to produce the insoluble black precipilate of ferrous sulphide

- Urease test :The medium used is chistensen's urea medium the oraganism which produce the enzyme urease,break down urea to NH_3 and CO_2 . Due to release of NH_3 ,the pH of the medium becomes alkaline and goes above 6.4 .This change of pH is detected in the urea medium by the indicator phenol red ,which has a pH range of 6.8-8.4 and shows yellow to pink colour change.
- Catalase test: catalase test determine the ability of the test organism to produce enzyme catalase that can degrade hydrogen peroxide. Catalase production was determined by the addition of H_2O_2 to the organism. Production of effervescence was seen which is positive test .