جمهـــــــــورية العـــــــــراق
وزارة التعليم والعالي والبحث العلمي
جامعـــــة بابـــــل / كلية العلوم
قســـــم علـــوم الحيـــــاة

# دراسة الظروف المثلى والجزيئية للمجاميع البكتيرية المفككة للهايدروكاربونات وانتاج المستحلبات الحياتية

اطروحة مقدمة إلى
مجلس كلية العلوم/ جامعة بابل
وهي جزء من متطلبات نيل شهادة الدكتوراه فلسفة في العلوم /علوم الحياة

من قبل
لقاء يحيى محسن عبد الرضا
بكالوريوس علوم في الاحياء المجهرية / جامعة بابل (٢٠٠٧)
ماجستير علوم في الاحياء المجهرية / جامعة بابل (٢٠١٥)
بإشراف

أ. د. ايمان محمد جار الله
أ.د.اياد محمد جبر المعموري

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**Republic of Iraq**

**Ministry of Higher Education and Scientific Research**

**University of Babylon - College of Science**

**Department of Biology**

# Optimized Conditions and Molecular Study of Bacterial Consortium for Biodegradation of Hydrocarbons and Biosurfactant Production

**A Dissertation**

**Submitted to the Council of College of Science at University of Babylon in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy of Science in Biology**

**By**

**Liqaa Yehya Mohsen Abd Al-Redha**

**B.Sc. Microbiology/ University of Babylon (2007)**

**M.SC. Microbiology/University of Babylon (2015)**

**Supervised by**

**Prof. Dr.Eman Mohammad Jarallah** **Prof.Dr. Ayad M.J. Al-mamoori**

**2023 A.D** **1444 A.H**

الَّذِي جَعَلَ لَكُمُ الْأَرْضَ فِرَاشًا وَالسَّمَاءَ بِنَاءً وَأَنْزَلَ مِنَ

السَّمَاءِ مَاءً فَأَخْرَجَ بِهِ مِنَ الثَّمَرَاتِ رِزْقًا لَكُمْ ۖ فَلَا

تَجْعَلُوا لِلَّهِ أَندَادًا وَأَنتُمْ تَعْلَمُونَ

# سورة البقرة الآية (٢٢)

# *Acknowledgements*

*This Ph.D. journery started with a dream when I was first employed by the research. Dreams alone are not enough and it took hard work, perseverance, encouragement and faith to fulfill this dream. First of all, I would like to thank God Almighty for giving me the opportunity and guidance to achieve my goal and to be successful in this part my life's journey so far. Then I pray all my respects and Praises to the greatest man of the universe, the Holly Prophet* ***Mohammad*** *(Peace Be Upon Him) whose life and wordings are ultimate source of guidance.*

*I express my deep gratitude and special thanks to the Deanship of the College of Science and the presidency of Biology Department for their continued support. A great debt of gratitude and sincere thanks are extended to the Head of Biology Dept. Assist. Prof.Dr.* ***Adi Jassim Abd Al-Rezzaq*** *for his great help.*

*I would like to express my heartiest and sincerest sense of gratitude to my worthy Supervisor* ***Prof. Dr.Eman Mohammad Jarallah*** *,She is one of the most intriguing person I have encountered in my life who had more insight about me than myself. I thank her whole heartedly for providing me this opportunity. I remember being lost and confused. It was her who saw the potential in me and helped me to perceive or at least have a glimpse of my true potential., and Co-Supervisor* ***Prof. Dr. Ayad M.J.Al-***

***mamoori*** *, Who has created the invaluable space for me to do this research and develop myself as a researcher in the best possible way.*

*I express my deep gratitude and special thanks to* ***Dr.Basheer Abd-Alhamza*** *and* ***Dr. Shaker Hammad Mohammad*** *for the valuable guidance enduring support and inspired a kindness.*

*My profound thanks to* ***Wameedh Adil Khadim*** *for the support, and he has been a constant and strong pillar of support during my Ph.D. I thank him for everything.*

*My personal and very sincerest acknowledgment to my friend* ***Rafal Ahmed Lilo*** *for supporting and encouraging me to pursue this dissertation.*

*Finally, special thanks to my family especially my mother, brother(Sadiq), whom I do appreciate their long patience, support and help to everyone who gave hand to complete this work.*

***Liqaa***

# Summary

The spills of hydrocarbon due to the petrochemical industry are major contaminants in the environment. Bioremediation is an effective, economical and environmentally sound treatment. This work was designed to study the isolation, identification of bacterial species from polluted soil . Cultural, morphological, and biochemical investigations identified growth on blood agar, MacConkey agar, and chromogenic agar, which was validated by the VITEK 2 system. Also these isolates were identified by molecular identification by using 16rRNA. Seven Gram negative bacteria *Acinetobacter baumanni, Pseudomonas putida, Delftia acidovorans, Enterobacter cloacae, Pseudomonas aeruginosa, Pseudomonas stutzeri, and Pandoraea spp* and six Gram positive bacteria *Micrococcus luteus, Staphylococcus haemolyticus, Staphylococcus warneri, Staphylococcus lentus, Staphylococcus lugdunebsis and Kocuria kristinae*. In addition, this study was examined the best growth of collected bacteria by optical density, pH, and plate count. *K.kristinae ,S.warneri, S.haemolyticus, M.luteus, S.lugdunebsis, and S.lentus* were given the best growth and increased after 18 days at optical density 600 nm, respectively. The various pH curves revealed that the pH in the control flasks stayed nearly constant (7). Regardless of the treatment, the pH of the flasks containing crude oil decreased after 3 days and continued to decline after the last day of incubation (18 days) for *K.kristinae ,S.warneri, M.luteus, and S. lentus* respectively, while other isolates *S.haemolyticus, and S. lugdunebsis* were showed less growth after 18 days of incubation . While plate count ,After 3 days of incubation, the growth of the bacterial community increased, whereass after 18 days of incubation, it reached a peak, then began to decline at 21 days to evaluate plate count of gram-positive bacteria, *K.kristinae* had the highest count and followed by *S.warneri, M.luteus, S.lugdunebsis, and S. lentus* respectively.

Gram negative bacteria *D.acidovorans, Ps.stutzeri, Ps.aeruginosa, E.cloacae, Ps.putida, Pa.spp, and Ac.baumanni* gave the best growth after 18 days at optical density 600nm respectively. pH decreased after 3 days and continued to decrease after last day of incubation. These isolates were given the best growth after 3 days and continued to increase which reached maximum growth after 18 days to evaluate plate count .

This study was demonstrated biosurfactant production by using bacterial isolates (G+ve and G-ve) depending on the hemolysis test, CTAB agar plate method ,oil spreading, drop collapse test, emulsification index (Ei24%) test, BATH test, and foaming activity using crude oil as a carbon source. eight isolates *(K.kristinae, S.warneri, S.haemolyticus, M.luteus ,D.acidovorans, Ps.putida, Ps. aeruginosa, and, Ps.stutzeri,*) showed positive results for hemolytic activity by forming a clear zone. Only Six isolates (*D.acidovorans, Ps.putida, Ps.aeruginosa ,Ps.stutzeri, K.kristinae ,and S.warneri,*) generated dark blue halos on CTAB agar, indicating biosurfactant production. The ability of the cell free supernatant to displace the oil and form a higher clear zone in the oil spreading assay is also an indication of biosurfactant production, eight isolates :*K.kristinae, S.warneri, S.haemolyticus, M.luteus, D.acidovorans, Ps.putida , Ps.aeruginosa, and Ps.stutzeri* were gave positive result respectively. Also, the other parameters were given positive results for eight isolates to produce biosurfactant, so(*K.kristinae and D.acidovorans*) were the most efficient.

The optimum conditions for biosurfactant production by *K.kristinae* and *D.acidovorans* were determined. The results indicated that these bacteria are growing in mineral salt medium (pH7) ,containing 1% crude oil as a sole carbon source ,0.4% $(NH_4)_2SO_4$, at 35 ºC in shaker incubator (150 rpm) for 7 days. Biosurfactant was extracted using three methods, the results showed that the extraction with Ethyl acetate for *D.acidovorans* gave a better biosurfactant

activity, which showed (Ei24% 95.6%) ,while *K.kristinae* was gave the best biosurfactant activity (Ei24% 96.3%) with HCl. The biosurfactant was purified using preparative HPLC column chromatography, two peaks was obtained by *K.kristinae* and one peak by *D.acidovorans* . In order to investigate the purity of biosurfactant which is purified from *K.kristinae* and *D.acidovorans*, polyacrylamide gel electrophoresis under denaturing conditions was done, these results were showed *D.acidovorans* has molecular weight 70KD, while *K.kristinae* was given two protein bands seemed with different molecular weight (72 and 65 KD) while one band appeared in the other sample, with molecular weight 71KD. The partial purified biosurfactant of *K.kristinae* and *D.acidovorans* was characterized by fourier transform infrared spectrophotometer (FTIR) to complete the chemical characterization. The result of FTIR revealed that the biosurfactant contains protein and amount of lipid (lip glycoprotein).

In an attempt to detect of target genes responsible for contaminant Biodegradation all bacterial isolates (7 G+ve and 6 G-ve), genomic DNA of these bacterium were extracted and amplified using specific primers for *P34O, C230, NidA, C12O, C23O, Cat1,nah Ac, and sMO* gene coded for biodegradation produced by isolates was used. gene C23O that positive in 4 isolates that have molecular weight 900 bp. Gene C230 that gave positive results in 6 isolates with molecular size 450 bp. The third gene C12O that have molecular size 350 pb which showed positive result in 5 isolates . Gene Cat1 that have molecular size 650 pb, this gene give positive result in 3 isolates .*Gene 5* nahAc with molecular 487 bp was showed negative results for all isolates. The another gene NidA with molecular size 1400 pb that showed positive result in 3 isolates. P34 gene that have molecular size 800 pb, isolates were positive in this gene 6 .Also, last gene sMO with molecular size 374, all isolates are negative.

The current study was designed, which deals with a bioreactor, the use of bacteria (*K. kristinae* and *D. acidovorans*) to monitor for soil decontamination. These bacteria were immobilized after cultivation and purification for the purpose of conducting laboratory tests on it with varying times and to preserve it for the longest possible period, possibly months or even years, which paves the way for the using in different methods. Control concentration for soil contaminated oil was estimated before treatment by using HPLC to compare with immobilized isolates that used for treatment of soil contaminated oil which gave concentration (4598.6 ppm), while the soil concentrations after treatment with these immobilized (*K.kristinae*) and (*D.acidovorans*) were (479.0,and 658.5) ppm respectively. Finally, these results were conducted for development of Consortia of the Selected Bacteria, eight isolates were grown in culture media act as consortia to compare with single isolate used in this study. These results were given Consortium was more efficient in degradation compared with single strain cultures.

# الخلاصة

يعتبر انسكاب الهيدروكربون بسبب صناعة البتروكيماويات من الملوثات الرئيسية في البيئة. المعالجة الحيوية هي معالجة فعالة واقتصادية وسليمة بيئيًا. صمم هذا العمل لدراسة عزل تشخيص الأنواع البكتيرية من التربة الملوثة.حددت الصفات الزرعية والمظهرية والكيميائية الحيوية النمو في أجار الدم وأجار MacConkey وأجار الكروموجينيك ، والذي تم التحقق منه بواسطة نظام VITEK 2. كما تم التعرف على هذه العزلات عن طريق التعريف الجزيئي باستخدام rRNA١٦. سبعة من البكتيريا سالبة لصبغة غرام (*Acinetobacter baumanni* و *Pseudomonas putida* و *Delftia acidovorans* و *Enterobacter cloacae* و *Pseudomonas aeruginosa* و *Pseudomonas stutzeri* و *Pandoraea* spp) وستة انواع بكتيرية موجبة لصبغة غرام ( *Micrococcus luteus, Staphylococcus haemolyticus, Staphylococcus warneri, Staphylococcus lentus, Staphylococcus lugdunebsis and Kocuria kristinae*).

بالإضافة إلى ذلك ، اختبرت هذه الدراسة أفضل نمو للبكتيريا المجموعة من خلال الطول الموجي، ودرجة الحموضة ،والعد البكتيري. *Kristinae*، *S.warneri*، *S.haemolyticus*، *M.luteus*، *S.lugdunebsis*، *S.lentus* أعطيت أفضل نمو وزادت بعد ١٨ يومًا بطول موجي ٦٠٠ نانومتر على التوالي. وضحت منحنيات الأس الهيدروجيني المختلفة أن الأس الهيدروجيني في دوارق السيطرة ظل ثابتًا تقريبًا (٧). بغض النظر عن المعاملة ، انخفض الرقم الهيدروجيني للدوارق المحتوية على الزيت الخام بعد ٣ أيام واستمر في الانخفاض بعد آخر يوم من الحضانة (اليوم الثامن عشر) لكل من *K.kristinae* و *S.warneri* و *M.luteus* و *S. lentus* على التوالي ، بينما أظهرت العزلات الأخرى *S.haemolyticus* و *S. lugdunebsis* نموًا أقل بعد ١٨ يومًا من الحضانة. بينما العد البكتيري ، بعد ٣ أيام من الحضانة ، زاد نمو المجتمع البكتيري ، في حين أنه بعد ١٨ يومًا من الحضانة ، وصل إلى ذروته ، ثم بدأ في الانخفاض في اليوم الحادي والعشرين لتقييم العد البكتيري للبكتيريا الموجبة لصبغة غرام ،. معنوياً ، سجل *K.kristinae* أعلى عدد يليه *S.warneri* و *M.luteus* و *S.lugdunebsis* و *S. lentus* على التوالي.

أعطت البكتريا السالبة لصبغة غرام *D.acidovorans* و *Ps.stutzeri* و *Ps. aeruginosa* و *E.cloacae* و *Ps. putida* و *Pa.spp* و *Ac.baumanni* أفضل نمو بعد ١٨ يومًا عند

طول موجي ٦٠٠ نانومتر على التوالي. انخفض pH بعد ٣ أيام واستمر في الانخفاض بعد آخر يوم من الحضانة. أعطيت هذه العزلات أفضل نمو بعد ٣ أيام واستمرت في الزيادة التي وصلت إلى أقصى نمو بعد ١٨ يومًا لتقييم العد الكلي للأطباق .

أظهرت هذه الدراسة إنتاج المستحلب الحيوي باستخدام العزلات البكتيرية (G + ve و G-ve) اعتمادًا على اختبار انحلال الدم وطريقة CTAB ونشر الزيت واختبار تبادل القطرة واختبار مؤشر الاستحلاب (Ei24٪) واختبار BATH ونشاط الرغوة باستخدام الزيت الخام كمصدر للكربون. اظهرت ثمانية عزلات (*K.kristinae*، *S.warneri*، *S.haemolyticus*، *M.luteus*، *D.acidovorans*، *Ps.putida*، *Ps. aeruginosa*، and، *Ps.stutzeri*) نتائج إيجابية لتحلل الدم من خلال تشكيل منطقة شفافة واضحة. ستة عزلات فقط ( *D. acidovorans* و *Ps.putida* و *Ps.aeruginosa* و *Ps.stutzeri* و *K. kristinae* و *S. warneri* ) أنتجت هالات زرقاء داكنة على اكار CTAB مما يشير الى انتاج المستحلب الحيوي. إن قدرة المادة الطافية الخالية من الخلايا على إزاحة الزيت وتشكيل منطقة أعلى واضحة في مقياس انتشار الزيت هي أيضًا مؤشر على نشاط المستحلب الحيوي ، ثماني عزلات: *K.kristinae*، *S. warneri*، *S.haemolyticus*، *M. luteus*، *D. acidovorans*، *Ps.putida*، *Ps. aeruginosa*، و *Ps. Stutzeri*) اعطت نتيجة موجبة على التوالي. كما أعطت المتغيرات الأخرى نتائج إيجابية لثمانية عزلات لإنتاج المستحلب الحيوي ، لذلك كانت (*K.kristinae* و *D.acidovorans*) الأكثر كفاءة.

تم تحديد الظروف المثلى لإنتاج العامل الحيوي بواسطة *K.kristinae* و *D.acidovorans*. أشارت النتائج إلى أن هذه البكتيريا تنمو في وسط ملح معدني (pH7) يحتوي على ١٪ زيت خام كمصدر وحيد للكربون ، ٠.٤٪ $(NH4)_2SO_4$ ، عند ٣٥ درجة مئوية في حاضنة هزازة (١٥٠ دورة في الدقيقة) لمدة ٧ أيام. تم استخلاص المادة المستحلبة الحيوية باستخدام ثلاث طرق ، وأظهرت النتائج أن الاستخلاص باستخدام أسيتات الأثيل لبكتريا *D.acidovorans* أعطى فعالية أفضل للتفاعل الحيوي ، والتي أظهرت (Ei24٪95.6 ٪) ، بينما أعطى *K.kristinae* أفضل نشاط للتفاعل الحيوي. (Ei24٪96.3 ٪) مع HCl.

تمت تنقية المادة الحيوية باستخدام كروماتوغرافيا العمود HPLC التحضيري ، وتم الحصول على قمتين بواسطة *K.kristinae* وقمة واحدة بواسطة *D.acidovorans*.

# الخلاصة


لتنقية المادة المستحلبة من *K.kristinae*. و *D.acidovorans* ، تم إجراء الترحيل الكهربائي للهلام متعدد الأكريلاميد في ظل ظروف ماسخة ، وأظهرت هذه النتائج أن *D.acidovorans* كانت بوزن جزيئي ٧٠كيلو دالتون. بينما *K.kristinae* اعطت شريطين بوزن جزيئي مختلف (٧٢و٦٥ كيلو دالتون بينما ظهر شريط واحد في العينة الاخرى بوزن جزيئي ٧١ كيلو دالتون. تم توصيف المادة المنقاة من *D.acidovorans* و *K.kristinae*باستخدام FTIR لإكمال التوصيف الكيميائي. اثبتت النتائج ان المادة المنقاة تحتوي على البروتين وكمية من الدهون(لايبو كلايكو بروتين).

في محاولة لاكتشاف الجينات المحددة المسؤولة عن التحلل الحيوي الملوث لجميع العزلات البكتيرية (G + ve 6 و ve-G 7) ، تم استخلاص الحمض النووي الجيني لهذه البكتيريا وتضخيمه باستخدام بادئات محددة لـ *P34O، C230، NidA، C12O، C23O، Cat1 وsMO* ، المشفر للتحلل الحيوي الناتج عن العزلات. الجين C23O موجب النتيجة في ٤ عزلات التي لها وزن جزيئي ٩٠٠زوج قاعدي. الجين C230 الذي أعطى نتائج إيجابية في ٦ عزلات بحجم جزيئي ٤٥٠زوج قاعدي. الجين الثالث C12O ذو الحجم الجزيئي ٣٥٠ زوج قاعدي والذي أظهر نتيجة إيجابية في ٥ عزلات .الجين Cat1 ذو الحجم الجزيئي ٦٥٠ زوج قاعدي ، يعطي هذا الجين نتيجة إيجابية في ٣ عزلات .أظهر الجين nahAc ذو الوزن الجزيئي ٤٨٧ زوج قاعدي نتائج سلبية لجميع العزلات. الجين الآخر NidA ذو الحجم الجزيئي ١٤٠٠زوج قاعدي الذي أظهر نتيجة إيجابية في ٣ عزلات .الجين P34 ذو الحجم الجزيئي ٨٠٠ زوج قاعدي ، كانت العزلات موجبة في هذا الجين ٦. أيضا ، آخر جين sMO بحجم جزيئي ٣٧٤زوج قاعدي ، جميع العزلات سلبية.

كما تم تصميم الدراسة الحالية التي تتناول مفاعل حيوي واستخدام بكتيريا ( *K. kristinae* و *D. acidovorans*و إزالة التلوث من التربة. تم تجميد هذه البكتيريا بعد زراعتها وتنقيتها لغرض إجراء الفحوصات المختبرية عليها بأوقات متفاوتة وللحفاظ عليها لأطول فترة ممكنة ، ربما أشهر أو حتى سنوات ، مما يمهد الطريق لاستخدامها في الطرق المختلفة. تم تقدير تركيز السيطرة في الزيوت الملوثة بالتربة قبل المعالجة باستخدام HPLC للمقارنة مع العزلات الثابتة المستخدمة في معالجة الزيوت الملوثة بالتربة والتي أعطت تركيز (٤٥٩٨.٦ جزء في المليون) ، بينما تم تقدير تركيزات التربة بعد المعالجة بالبكتريا المعزولة ( *K kristinae. D.acidovorans*) كانت (٤٧٩.٠ و ٦٥٨.٥) جزء في المليون على التوالي.

أخيرًا ، أجريت هذه النتائج لتطوير التحالفات البكتيرية المختارة ، ونميت ثماني عزلات في وسط زرعي يعمل كاتحادات للمقارنة مع العزلة المفردة المستخدمة في هذه الدراسة. أعطيت هذه النتائج ان البكتريا مجتمعة كان أكثر كفاءة في التحلل مقارنة مع مزارع مستعمرة واحدة.

# *Dedication*

***To:***

*The Light of my eyes, the soul of my heart, endless support, and the source of compassion , the one who was in my soul whenever I went away*

***My brother who did not complete the road with me (Sami) may Allah rest his soul in peace***

*The source of my strength, patience and all love…*

***My mother***

*The best Who supported and encouraged me to reach my dreams…*

***My brother (Sadiq)***

*The source of love, inspiration and help to me in everything…*

***My brother's wife (Shrooq)***

*The moons that light up my life, the source of my laughs*

*My brothers' children (**Ali, Montazer. and Tho-alfiqar**)*

*My beloved family, I present the fruit of my effort to you…..*

***Liqaa***

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# *Abbreviations*

| Abbreviated Form | Meaning |
|---|---|
| **µl** | **Microliters** |
| **APS** | **Ammonium persulfate** |
| **BSA** | **Bovin serum albumin** |
| **CTAB** | **cetyl trimethylammonium Bromide** |
| **D.W** | **Distil water** |
| **E.C** | **Electrical conductivity** |

| | |
|---|---|
| **GC** | **Gas chromatography** |
| **Mg** | **Milligram** |
| **MSM** | **Mineral salt medium** |
| **O.D** | **Optical density** |
| **PAG** | **Poly Acrylamide gel** |
| **PAHs** | **Polycyclic aromatic hydrocarbons** |
| **PCR** | **Polymerase chain reaction** |
| **SDS** | **Sodium dodycle sulphate** |
| **TBE** | **Tris borate ethylene diamine tetra acetic acid** |
| **TDS** | **Total dissolved soild** |
| **TEMED** | **(N.N.N.N tetramethylethylenediamine)** |
| **TOC** | **Total organic carbon** |

## *Appendices*

| No of appendix | Meaning |
|---|---|
| **Appen.1** | **Determination of PAH compound by using GC- mass** |

## Introduction:-

Polycyclic aromatic hydrocarbons (PAHs), which are among the most pervasive and persistent pollutants, have gotten more and more attention. A common class of organic pollutants, they are made up of two or more single or fused aromatic rings (Cabrerizo *et al.*,2014). They come from biological processes as well as by-products of incomplete combustion from either naturally occurring sources (volcanic eruptions , bush or forest fires) or man-made sources (coal, charbroiled meats, cigarettes, wood, rubbish, gasoline, oil, and other organic waste) (Primost *et al.*,2018).Although PAHs received various treatments, they are still regarded as environmental pollutants that persist and exhibit a variety of toxicological traits (Cristaldi *et al.*,2017). Because of their highly resistant molecular structure, high hydrophobicity, and low aqueous solubility, polycyclic aromatic and heavier aliphatic hydrocarbons are difficult to leach and volatilize out of soil ( Li *et al.,* 2020). Desorption is only possible in the aqueous phase due to the hydrophobicity of PAHs. Volatilization, Photoxidation, chemical oxidation, sorption, leaching, and biodegradation are the six basic methods of dissipation, or disappearance, that are known in the environment. Petroleum-derived compounds (PAHs) have toxic, mutagenic, and carcinogenic effects on organisms and are highly persistent in the environment. One technology for cleaning up the Antarctic environment is bioremediation using microorganisms such as; *Pseudomonas, Vibrio, Corynebacterium, Arthrobacter, Brevibacterium, Flavobacterium, Sporobolomyces, Achromobacter, Bacillus, Aeromonas, Thiobacillus, Lactobacter, Staphylococcus, Penicillium, and Articulosporium* are some of the different genera that have been reported to contain species that degrade hydrocarbons. These organisms have been identified in considerable quantities from numerous oil-

contaminated bodies of water and soil, but they are rare in uncontaminated environments (Agnello *et al.*, 2016). Bacteria often degrade PAH through the actions of certain enzymes like oxygenase and peroxidases. These enzymes include, Alkane monooxygenase, such as AlkB from *Pseudomonas* and Alkm from *Acinetobacter sp*. Strain, DP-1, AlkB1 and AlkB2 from *Rhodococcus* sp; XylE, catechol 2, 3 dioxygenase; NdoB, naphthalene monooxygenase from *Pseudomonas putida* and NidA, pyrene dioxygenase large subunit from *Mycobacterium sp.* strain PYR-1, as well as various dehydrogenases and protocatechuate dioxygenase in Stenotrophomonas spp. (Das and Chandran, 2011). Environmental biodegradation capacity can be evaluated with the aid of a better understanding of the diversity of the natural microbial population and particular metabolic genes (Fernández-Luqueo *et al.*, 2011). However, as the majority of microorganisms are challenging to cultivate due to a lack of suitable conditions and media, using purely culture-dependent methodologies may not be sufficient to estimate microbial diversity in the environment. Because of this, indigenous genera have been identified using molecular techniques based on phylogenetically relevant genes, such as denaturing gradient gel electrophoresis (DGGE) and 16S ribosomal RNA (rRNA) gene-based clone libraries (Das and Kazy,2014). In addition, culture-independent techniques that target certain metabolic genes can be used to determine the biodegradation capacity in settings. Key enzymes that catalyze the first stage of the PAH degradation pathways are ring-hydroxylating dioxygenase (Peng *et al.*, 2008). The target genes for the terminal dioxygenase -α subunit have been extensively used to describe the potential for PAH degradation in various conditions (Jurelevicius *et al.*, 2012). Bio-surfactant synthesis can also part of the bacterial breakdown of PAHs. In their chemical makeup, surface active agents (surfactants) have both hydrophilic and hydrophobic groups. Biosurfactants

are surfactants produced by microorganisms, typically during their stationary phase of life (Anaukwu *et al.*,2016). Their amphiphilic nature makes them good at reducing surface and interfacial tension. They are more environmentally friendly, non-recalcitrant, degradable, and non-toxic than their synthetic equivalents, and improved production yield through the use of recombinant strains (Hu *et al.*,2019). *Acinetobacter, Arthrobacter, Pseudomonas, Halomonas, Bacillus, Rhodococcus, Enterobacter*, *Serratia*, and several fungal species, including *Saccharomyces cerevisiae, Fusarium fujikuroi, Candida tropicalis, Pseudozyma, and Xylaria regalis*, have all been documented to create biosurfactants (Adnan *et al.*,2018).

The objective of study is considered as initial attempt is to investigate the Molecular aspects of some bacterial species in bioremediation of Polluted soil samples with PAHS . To achieve such goals the following steps were performed:

1- Isolation and identification of bacterial species responsible of biodegradation from polluted soils.

2- Determination some environmental properties polluted soils.

3- Detection of target genes responsible for biodegradation in bacteria.

4- DNA sequencing of the genes responsible for biodegradation.

5- Design lab experiment for bioremediation of petroleum oil from soils by using efficient bacterial species.

## 2.1Hydrocarbons:

Crude oil found in underground geologic formations contains combustible organic chemicals called hydrocarbons that are present in nature. Alkanes, naphthenes, aromatics, and alkenes are the four categories of hydrocarbons that are found in crude oil based on their molecular makeup. Alkanes have the chemical formula CnH(2n+2) and are also referred to as paraffin's or saturated hydrocarbons. Alkanes are substances that contain only single carbon-carbon bonds and no double or triple bonds between carbon atoms. They can be either branched or unbranched chains of carbon atoms with bound hydrogen atoms. The hydrocarbons known as aromatics are composed of alternating double and single bonds between carbon atoms. They are recognized as the part of crude oil that is most immediately harmful and are linked to both long-term and cancer-causing effects. Since many low weight aromatics are water soluble, the possibility of exposure to aquatic resources is increased. Because many of the chemicals have a sweet scent, the term "aromatic" was first used before the physical process regulating aromaticity. They are commonly known based on the quantity of rings they have, which can be one to six. Polycyclic aromatic hydrocarbons are aromatic hydrocarbons that have two or more rings (PAH). Alkenes, which have the chemical formula CnH, are also known as olefins or Isoparaffins (2n-2). Similar to alkanes with the exception of the presence of double-bonded carbon atoms, alkenes are distinguished by branched or unbranched chains of carbon atoms. Although aromatic compounds are frequently represented as cyclic alkenes, they are not regarded to be alkenes because of differences in their structure and characteristics. Alkenes are typically absent from crude oils but are frequently present in refined goods like gasoline (Harayama, 2004). PAHs are present in a variety of habitats,

including soils, sediments, ground waters, and the atmosphere, as a result of their ubiquity . According to estimates, terrestrial ecosystems, and more specifically the top 20 cm of the soil horizon, contain 90% of the entire ambient PAH load (Maliszewska- Kordybach, 1999). The ambient air contains more than 2800 substances, such as metals, nitrated and halogenated organic compounds, sulfur derivatives, and polycyclic aromatic hydrocarbons (PAHs) .According to Liu *et al.*, (2003) the main sources of PAHs from coal burning are floating dusts and flues. The creation of species with two and three benzene rings, like naphthalene, acenaphthene, and acenaphthene, causes the incomplete combustion to manifestly increase PAH emission as compared to other combustion processes. The toxicity equivalent value (TEQ) is significantly influenced by benzo[a] pyrene, dibenz[a,h]anthracene, and benzo[a]anthracene (Liu *et al.*, 2012).

## 2.2 Polycyclic aromatic hydrocarbons (PAH)

The important environmental contaminants known as polycyclic aromatic hydrocarbons (PAHs) are widely dispersed and have fused aromatic rings (Luke *et al.*,2013). The incomplete burning of wood, coal, oil, and gasoline results in the formation of PAHs. It has been reported that asphalt, coal tar, and crude oil all contain PAHs. The unintentional or intentional release of hydrocarbons is the primary source of soil and water contamination. Due to their toxicity, carcinogenicity, and mutagenicity, PAHs have a negative impact on human health and their presence in the environment is of major concern. The US EPA has 16 PAHs on its list of priority pollutants, and they are continuously monitored in industrial effluents (Balaji *et al.*,2013). As environmental concerns have grown, potential solutions have been created and put into practice. Bioremediation is a technique that turns various contaminants into carbon dioxide, water, inorganic

salts, and other byproducts by using microbes (bacteria, fungi, and algae). Polycyclic aromatic hydrocarbon (PAH) breakdown has been accomplished by bacteria, fungus, or algae (Dangi *et al.*, 2019).

## 2.3 Sources of PAHs

Over the past century, a lot of dangerous chemicals have been produced due to the growth of industrial, agricultural, and medical activities, which has increased environmental contamination (Figure 2.1). Numerous resources are interested in the mechanisms of PAH degradation and their potential fate in nature as a result of their widespread distribution and harmful impact on human health (Samanta *et al.*,2001). Polynuclear aromatic hydrocarbons, or PAHs, are byproducts of incomplete combustion of organic molecules. Organic pollutants called PAHs are pervasive, harmful, and persistent in the environment (Wang *et al.*,2009). Petroleum-based products are the main source of energy for daily life and industry in the industrial age. Therefore, there is always a potential that something will spill or leak while petroleum products are being explored, produced, refined, transported, or stored. In general, PAHs are non-polar, neutral, organic compounds that can be found in both naturally occurring and artificially created environments. Volcanoes and forest fires are examples of natural sources. Among the anthropogenic sources are ;

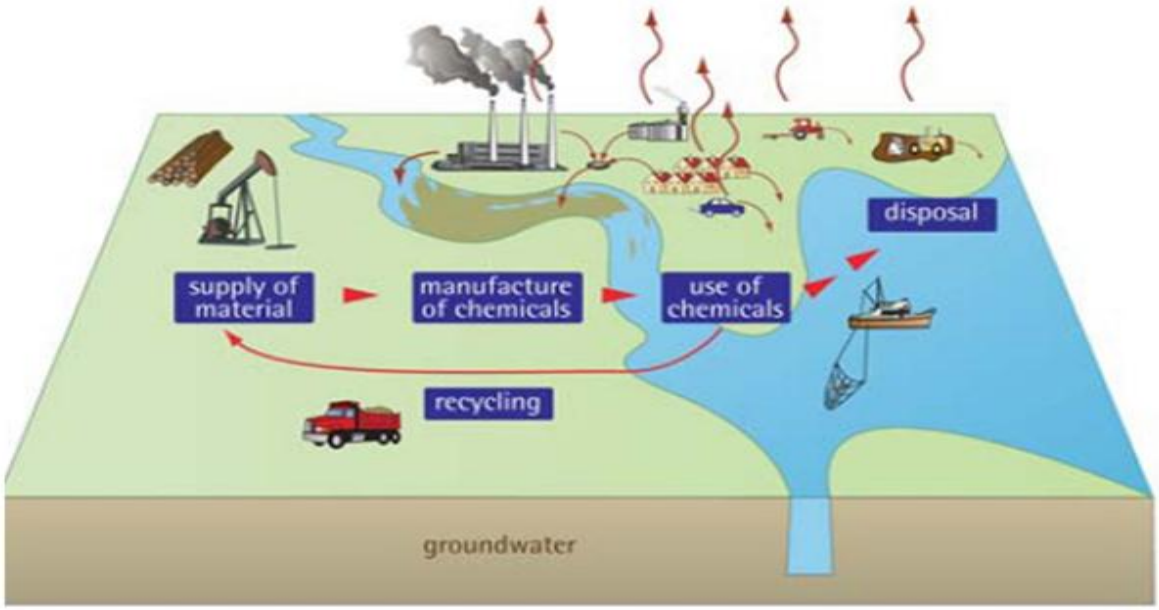



**Figure 2-1 :Transfer of PAHs in the environment (Wick *et al.*,2011).**

- Combustion of fossil fuel- including motor vehicle emission and power generation.
- Wood burning.
- Municipal and industrial waste inceration.
- Coal tar, coke, asphalt, crude oil, creosote, asphalt roads, roofing tar.
- Discharges from industrial plants and waste water treatment plants.
- Hazardous wastes sites, coal gasification sites, smoke houses, aluminum production plants.
- Atmospheric contamination of leafy plants.
- Cigarette smoke and Charbroiled meat ((Kastner,2008).

## 2.4 Structure of PAHs

Carbon and hydrogen atoms with two or more fused benzene rings arranged in linear, angular, or cluster fashion make up the structure of PAHs (Hesham *et al.*,2014). In addition to having carbon and hydrogen, heterocyclic (heteroatom) compounds, such as those with N, S, and O atoms, are also referred to as PAHs. Typically, molecular weight is between 166 and 328. Molecular weight of the chemical generally depends on its number as well as position of fused rings and other compounds (Hussein and Mona,2016) . Different organic compounds in the environment undergo thermolysis, resulting in the formation of PAHs. The incomplete combustion of organic compounds at high temperatures (500–800°C) to low temperatures (100–300°C) for extended periods of time results in the production of PAHs. Due to the dense concentration of electrons on both sides of the ring structure, the PAH molecule displays biological persistence. Consequently, the compounds made of PAHs are more resistant to nucleophilic attack (Haritash and Kaushik,2009). Although PAHs have a high melting and boiling point, they have a low vapor pressure and are not water soluble. PAHs can be colorless, white, or pale yellow-green in appearance. Naphthalene, Fluroene, and Phenanthrene are examples of Low Molecular Weight (LMW) PAHs, which have two or three fused benzene rings, while Pyrene is an example of a High Molecular Weight (HMW) PAH, which has four or more fused benzene rings. The chemical becomes more refractory as its molecular mass grows along with its hydrophobicity and lipophilicity, water solubility, and vapor pressure. Therefore, compared to LMW, HMW are more teratogenic, carcinogenic, and mutagenic, in addition to being poorly bioavailable and resistant to microbial breakdown (Okere and Semple,2012). The ring structure of PAHs is another factor used to categorize

them: (a) Alternant (b) Non-Alternant. Anthracene, Phenanthrene, and Chrysene are three alternative PAHs that are produced from benzene by the fusing of extra 6-membered benzoid rings and have fewer rings than benzoid rings (Harvey,1998). Fluoranthene is a non-alternant PAH that also has a 6-membered ring and rings with fewer than 6 carbon atoms. Four fused, six-carbon benzene rings make up chrysene. On the other hand, fluoranthene is a product of lower temperature and less effective combustion because it contains naphthalene and a benzene unit joined by a five-membered ring (in the middle of the structure). A "bay-region," a "K-region," and a "L-region" make up the majority of PAHs. For instance, the Bay-region, or internal open inner corner, of the phenanthrene structure. The L-region symbolizes the pair of opposed anthracene point atoms, and the K-region is an external closed corner. These bay- and K-region epoxides are chemically reactive. Consequently, they grow biologically and metabolically (Ramesh *et al.*,2004).

## 2.5 Pathway for PAHs degradation

Pollutant biodegradation includes several processes and the use of various enzymes (Abbasian *et al.*,2015). Individual microorganisms or groups of microorganisms from the same or different genera can selectively metabolize hydrocarbons; however, studies have shown that group cultures are more effective than individual cultures at metabolizing or degrading pollutants (Déziel *et al.*, 1996; Abo-State and Mostafa, 2005; Saleh *et al.*, 2013; Varjani *et al.*, 2015). The fundamental phase in the degradation of aromatics and polycyclic aromatic hydrocarbons (PAHs), which often involves diol production followed by ring cleavage and creation of dicarboxylic acid, is an initial oxidative attack followed by benzene ring cleavage (Hendrickx *et al.*,2006). The first step in the microbial

breakdown of PAHs is oxidation, which is performed by monooxygenase or dioxygenase and results in the synthesis of cis-dihydrodiol at the two carbon atoms of the benzene ring. Succinate, Acetyl COA, Pyruvate, and Gluconeogenesis were the primary precursor metabolites used in the formation of cell biomass, which led to the synthesis of sugars and growth. The most frequent method of early oxidation is the development of a diol, which is subsequently followed by ring cleavage, the creation of dicarboxylic acid, the incorporation of both oxygen atoms from an oxygen molecule to create Cis-dihydrodiol, and finally the creation of catecols (Zhang *et al.*,2011). A large number of peripheral degradation routes are involved in the aerobic catabolic pathway, which converts PAHs into a limited number of intermediates that enter the tricarboxylic acid (TCA) cycle (Peng *et al.*,2008). Ortho- or meta-cleavage pathways produce core intermediates, such as protocatechuate and caticols, which are then transformed into intermediates for the TCA cycle (Abbasian *et al.*,2015). Compared to aerobic degradation, anaerobic degradation is more recent. This is because there is less knowledge about the genes and enzymes that are involved in these pathways (Wilkes *et al.*,2016). Natural isolates with active ortho-pathway genes and inactive meta cleavage of catechol oxidation had lower levels of Naph. biodegradation than isogenous bacteria with active meta-pathway genes. The activity of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, two of the enzymes involved in the ortho- and meta-pathways of catechol oxidation, were demonstrated in all strains under study that had functional genes for the meta-pathway (Filonov *et al.*, 1999 and 2000). Naphthalene to 4-hydroxy-1-tetralone was bio transformed in an excellent yield by Streptomyces griseus. Additionally, 2-methyl-1-4-naphoquinone and 2-methyl-4-hydroxy-1-tetralone were produced as intermediates (Gopishetty *et al.*, 2007). NahAc and C23O were proven to be present in the system, and the aerobic tank had 2–3 orders

more copies of each gene than the influent water sample did. The distinct behavior of C23O showed that the aerobic unit may be where mineralization of PAHs takes place primarily (Wang *et al.*, 2007). The metabolism of phenanthrene by bacteria, particularly Pseudomonas, has been extensively investigated. The majority of bacteria use the dioxygenase enzymatic system as their primary mode of attack before converting it to dihydroxy phenanthrene (Sutherland *et al.*, 1990).

A novel mechanism developed by Staphylococcus sp. PN/Y to break down phenanthrene involved 2-hydroxy-1-naphthoic acid (2H1NA), which was then further broken down by a special meta-cleavage dioxygenase to produce TCA cycle intermediates (Mallick and Dutta, 2008). Finding the gene that breaks down phenanthrene in bacteria that break down PAHs typically shows the same degradation mechanism. Phenanthrene is first converted to cis-dihydrodiol by PAH dioxygenase (a multicomponent dioxygenase enzyme system); dihydrodiol dehydrogenase then transforms catechol from catechol into aldehydes or acids; and catechol 2,3-dioxgenase subsequently degrades catechol into these aldehydes or acids (Haritash and Kaushik, 2009). There are several places on the molecule where anthracene can degrade, including the 1, 2, or 3, 4 carbon positions, an angular Kata-type initial deoxygenating, the 9, 10, or 11, carbon positions, a linear Kata-type initial deoxygenating, or the K-region at the 5, 6 carbon position. Only six species *Shingobium yanoikuyae* mutant strain B8/36 have had their metabolites from the biotransformation of Benzo[a]anthracene (B[a]A] by identified bacteria. The first step in the synthesis of B[a] anthracene was oxidation, which resulted in Benzo[a]anthracene 7, 12 dione. Following additional oxidation and ring fission, indo-5-aldhyde, benzene ethanol, and a variety of acids, alcohols, and esters were produced (Kunihiro *et al.*,2013). The results of the pyrene degradation process

showed that the breakdown of PAH would result in the production of several acidic intermediate compounds, including succinic acid, acetic acid, fumaric acid, and pyruvic acid.

The buildup of the acid intermediate products led to the pH drop and this could be the cause of why the pH change matched the change in PAH content (Lin and Cai, 2008). *Pseudomonas stutzeri CECT930* uses the pyrene degradation pathway to create 1-hydroxy-2-naphthoic acid, phthalic acid, and cinnamic acid. While also revealing the synthesis of 1-[(hexadeulerio)phenyl] naphthalene, trans-4, 4-di methoxy -beta methyl chalcone, phthalic acid monocyclohexyl ester, phthalic acid monobutyl ester, dimethoxybenzyl-ide neacetone, and phathalic anhydride via the degradative pyrene suggested pathway by Bacill (Moscoso *et al.,*2015). To produce 2-hydroxymethyl benzoic acid or monomethyl and dimethyl esters of phthalic acid, benz(a) anthracene-7, 12-dione was degraded to 1,2-naphthalene dicarboxylic acid and phthalic acid. Another degradation product of BaAQ was identified as 1-tetralone. Its transformation via 1,4-naphthalenedione, 1,4-naphthalenediol and 1,2,3,4-tetrahydro-1-hydroxynaphthalene resulted again in phthalic acid. None of the intermediates were identified as dead-end metabolites. Metabolites produced by ring cleavage of benz[a] anthracene using the ligninolytic fungus are firstly presented in this work (Cajthmal *et al.*, 2006).

## 2.6 Low Molecular weight PAHs (LM W-PAHs) degrading bacteria

*Pseudomonas panipatensis, Pseudomonas putida, Pseudomonas vesicularis, Pseudomonas paucimobilis, Bacillus cereus, Mycobacterium sp., Alcaligenes dentrificans, Rhodococcus sp., Corynebacterium venale, Vibrio species, Streptomyces species Bordetella avium, and Cyclotrophicus sp.* are just a few of the numerous bacteria that may break down PAHs (Samanta *et al.,* 2001; Abo-

State *et al.*, 2018a). In the case of bacteria that break down naphthalene, various bacteria, such as *Arthrobacter polychromogenes, Aeromonas sp., Beijerincka sp., Micrococci species ,Alcaligenes faecalis* and a variety of *Mycobacterium ,Nocardia sp, Bordetella sp, Flavobacterium sp. Vibrio sp., Bacillus sp. And Rhodococcus sp* have been identified (Abo-State *et al.*,2018b). The taxonomically diverse degrading bacterial strains that have been identified primarily come from the genera *Mycobacterium, Pseudomonas, Bacillus, Sphingomonas,* and *Alcaligenes* (Aitken *et al.*,1998; Abo-State *et al.*,2017). Several studies were demonstrated that after five days of incubation, *Bacillus subtilis* had the highest catechol, 1, 2 dioxygenase activity in MSM supplemented with anthracene, that have 99% of degradation (Abdelhaleem *et al.*,2019).

## 2.7 High Molecular weight PAHs (HM W-PAHs) degrading bacteria

High molecular weight (HMW) PAHs are described as substances that contain the structure of a PAH and consist of more than three aromatic benzene rings. PAHs could be broken down by a wide variety of microorganisms. Phenanthrene, anthracene, and pyrene may all be degraded by *Mycobacterium RJG* II-135 at rates that are 10 to 20 times higher than those of Benzo[a]anthracene and Benzo[a]pyrene (Mclellan *et al.*,2002). *Mycobacterium vanbaalenii* PYR-1 can also break down a variety of PAHs with low and high molecular weights (Kim *et al.*,2006). *Bacillus subtilis* was found in soil that had PAH contamination. Pyrene and Benzo[a]pyrene can both be transformed by *Bacillus subtilis,* although Benzo[a]pyrene degrades more quickly than Pyrene (Hunter *et al.*,2005). Two microbes that may degrade PAHs, *Bacillus SPO2* and *Mucor SFO6,* were immobilized on vermiculite to test their capacity to degrade benzo[a]pyrene. In another study, pyrene degradation was carried out using *Bacillus subtilisDM-04*

and *Pseudomonas aeruginosa* mucoid (M) and Non-Mucoid (NM) strains isolated from petroleum-contaminated soil samples of North East India. Removal rate in case of immobilized bacterial-fungal mixed consortium was higher than that of freely mobile mixed consortium (Dan *et al.*, 2006).*Bacillus subtilis* showed higher utilization of pyrene than *Pseudomonas.* Syakti *et.al.,* (2013) isolated 6 viable and cultural bacterial strains from contaminated mangroves. *Bacillus aquimaris, Bacillus megterium, and Bacillus pumilus* were identified as the bacterial strains by 16S rRNA, whereas *Flexibacteraceae, Halobacilus trueperi,* and *Rhodobacteraceae* were connected to the other three strains. These strains could thrive on PAHs (Phenothiazine, fluorine, fluoranthene, dibenzothiophene, phenanthrene and pyrene). Both alone and in combination, two bacteria, *Bacillus* PY-1 and *Sphingomonas* PY-2, and a fungus, *Fusarium* Py-3, isolated from polluted soils, were able to breakdown pyrene and volatize arsenic. Pyrene removal at a high rate was observed to have taken place after 9 days in liquid media and 63 days in soil (Liu *et al.*,2013). Five bacterial strains were isolated by Abo-State *et al.* in (2013) and Abo-State *et al.,* (2014) from petroleum oil-contaminated soil and water in Cairo, Egypt. The two strains that were the most effective were *Bacillus amyloliquifaciens MAM-62* with accession number 038054 and *Achromobacter xylosoxidans MAM-29* with accession number 038055 according to 16S rRNA analysis, as the only carbon and energy source, pyrene was effectively degraded by both of the two bacterial strains. Within 60 hours, the *Pseudomonas* isolate PAHs As-1 eliminated both the entire 60 mgl-1phenanthrene and half of the 20 mgl-1pyrene (Feng *et al.*,2014). Pyrene was initially adsorbed by *Brevibacillus brevis* cells, after which it was transported and intracellularly degraded. After 168 hours, 0.75 mg of the pyrene had been removed. The 8 bacteria that were modified to use a mixture of 4 PAHs and included *Bacillus, Acinetobacter, Stenotrophomonas,*

*Alcaligenes, Lysinibacillus, Brevibacterium, Serratia,* and *Streptomyces* were the most effective (anthracene, fluorine, phenanthrene and pyrene),within 7 days, a group of the four most potent isolates was able to degrade PAHs more effectively (Chaudhary *et al.*,2015). *Achromobacter xylosoxidans*, a marine halo-tolerant bacteria, has been found to degrade LMW-PAHs. Triton X-100 and b-cyclodextrine along with glucose caused the degradation of LMW-PAHs to increase by 2.8 and 1.4 fold, and the degradation of HMW-PAHs to increase by 7.59 and 2.23 fold, respectively (Dave *et al.*,2014). *Mycobacterium gilvum strain PYR-GCK* isolated from an estuary polluted with PAHs and was able to breakdown pyrene efficiently. *Staphylococcus nepalensis,* which can break down pyrene at PH 8 and 30 °C within 5 days of incubation, was isolated from a diesel-contaminated soil sample and identified by 16S rRNA. When co-substrates (glucose 4% and sucrose 2%) were introduced, the best bacterial growth and effective pyrene breakdown were observed (Valsala *et al.*,2014).

It was shown that mono culture of Pseudomonas monteilii P26 and *Pseudomonas sp.* The *Actinobacteria, Rhodococcus p18, Gardonia H 19* and *Rhodococcus F27* were able to degrade HMW-PAHs efficiently but were unable to remove LMW-PAHs from the culture medium. Number 3 was able to degrade LMW-PAHs efficiently but did not demonstrate any noteworthy HMW-PAHs removal skills. Naphthalene and phenanthrene were completely removed by the combination of four of these five strains (known as C15 mixed culture), which also displayed the highest pyrene biodegradation activity with removal values close to half, nearly 6 times higher than those values recorded with strains in pure culture (Isaac *et al.*,2015). From soil on the long-term manufacturing gas plant site, four strains that could break down both LMW-PAHs and HMW-PAHs were found. These isolates included *Stenotrophomonas (MTS-2), Citrobacter (MTS-3),* and

*Pseudomonas (MTS-1)*—the most effective strain for the breakdown of HMW-PAHs (Kuppusamy *et al.*,2016). Isolate IT B II was identified by 16SrRNA as *Mesoflavibacteer zeaxanthinifaciens*. This strain uses Benzo[a]Pyrene as its exclusive source of carbon and energy (Okai *et al.*,2015). Over the course of 33 days, microorganisms with the potential to specialize in adhesion sorbed more than half of the pyrene on hydrophobic filters than the five-ring Benzo[a]pyrene and Benzo[a]fluoranthene. Most bacteria enhanced by HMW-PAHs were *Bacillus*, *Mycobacterium and Pseudomonas* (Folwell *et al.*,2016).

## 2.8 Fate of PAHs in the environment

There are many different ways to degrade PAHs in the environment, such as chemo-oxidation and photo oxidation, although microbial degradation is thought to be the main method in soils (Juhasz and Naidu, 2000). Depending on how susceptible their structural makeup is to chemical, physical, or biological breakdown, PAHs have varying half lifetimes in various environmental compartments (such as soil, air, and water). Changes in the toxicity, mobility, or other chemical properties of the compound are frequently the result of changes in the structure of the PAH brought on by environmental modification. This change may cause the PAH to attach to soil components or cause the molecule to be destroyed by biotransformation (Mueller *et al.*, 1996). The term "biotransformation" refers to the chemical modification of a substance's molecular structure carried out by living things (such as bacteria, fungus, and algae), which results in a change in the substance's complexity or loss of a distinguishing attribute. Environmental systems' biotransformation rates are influenced by a variety of physiochemical processes that regulate bioavailability as well as bio kinetic processes including electron acceptors, nutrient availability, toxicity,

inhibition, and competitive substrate usage .PAHs in the environment are subject to a number of processes that affect their fate. The following briefly details some of the environmental fate of PAHs prior to a more detailed analysis of microbial degradation (Ramaswami and Luthy, 1997).

## 2.8.1 Volatilization

Although this is only possible with molecules with higher vapor pressures, volatilization can happen in the atmosphere, at or near the surface of water, soil, and plants, allowing the destruction or substantial change of organic substances (Alexander, 1999). Few PAHs are lost from soils as a result of volatilization due to their predominantly low vapour pressures (particularly naphthalene and fluorene). Most PAHs are in their solid form at environmental temperatures (0 - 40 °C), which prevents them from sublimating into the vapour phase. PAHs (e.g. naphthalene, fluorene, fluoranthene, pyrene) are often found in ambient air in homes, although this is more often a result of temperature increases associated with activities such as the use of heating appliances or cigarette smoking (van Winkle and Scheff, 2001).

## 2.8.2Photochemical Oxidation

The PAHs can absorb solar light and undergo change. By absorbing infrared electromagnetic radiation from ultraviolet and visible light, certain molecular groupings in the PAH molecule increase their rotation or vibration, which may cause fragmentation, oxidation, or polymerization (Connell *et al.,* 1997). Solar radiation can partially oxidize PAHs in the presence of gaseous or aqueous oxygen, but this usually only affects PAHs that aren't angular or clustered.There is a paucity of research on photochemical oxidation of PAHs in environmentally relevant conditions, particularly soil, with most studies being undertaken with

artificial light or in the presence of catalysts such as titanium oxide (TiO2) (Kirso *et al.,* 1991)

## 2.8.3Chemical Oxidation

Although non-enzymatic or non-photochemical reactions are common in soil, they rarely lead to a compound's total alteration. Usually, minor change happens that resulting in a product that is chemically identical to the original substance (Alexander, 1999). In soils, PAHs can be altered by any abiotic oxidants present, including metal ions (Mn and Fe) and clay minerals (including oxides and oxyhydroxides of Al, Fe, Mn and Si) (Gramss *et al.*, 1999).

## 2.8.4 Sedimentation

due to variables like the sorption of PAHs to sediment organic material (adsorption to sediment minerals is inhibited by the presence of water) and limited diffusion of O2 into organic rich sediments, act as a sink for deposited PAHs from industrial discharges or other pollutants. A portion of the PAHs in the sediment become highly refractory after sorption to organic matter, preventing their dissolution into the water column and microbial degradation, leading to accumulation (Chiou *et al.,* 1998). The PAH's octanol-water coefficient (Kow) and sediment characteristics like organic matter content and sediment aromaticity can be used to predict the likelihood of deposition and accumulation in sediments. Either through gas flux or through the deposition of particles with sorbed PAHs, PAHs can build up in sediments in water. Due to their low solubility, PAHs that enter water by gas flux often interact with organic matter particles (such as plankton) and are then excreted in feces or consumed by other animals. The PAH may cycle through the biota throughout this process before being deposited, remain

floating in the water column, or build up in a higher organism (Arzayus *et al.*, 2001).

### 2.8.5Bioaccumulation

The accumulation of PAHs in organisms is highly variable, depending on: the PAH; the environment of exposure; the organism's potential to metabolically transform the PAH and the lipid content of the organism (in which the PAH may collect) .Molluscs and other species with low or no metabolic ability can collect substantial levels of PAHs, whereas fish and other organisms with some metabolic capacity can acquire very little or no PAHs. The capacity to digest PAHs can be harmful since it can produce reactive metabolites that increase toxicity(Schuler *et al.*, 2004). Similar to trophic transfer, bio magnification depends on the ability to metabolize the absorbed PAH (Hofelt *et al.*, 2001). Due to their low water solubility and low reactivity as a result of their large size and angularity, high molecular weight PAHs have a propensity to bio-amplify through trophic transfers in the environment (Kanaly and Harayama, 2000).Phytoplankton are thought to play an important role in the fate and transport of persistent organic pollutants like PAHs and their consumption an important initial phase in bioaccumulation (Fan and Reinfelder, 2003).

## 2.9 Biodegradation of hydrocarbons by microorganisms

Organisms that break down PAHs are frequently found in the soil's microbial population. Fungi and bacteria that can degrade PAHs receive the carbon and energy needed for growth in non-contaminated soils by breaking down organic molecules (Liu *et al.*,2020). Microbial degradation processes often exhibit broad substrate specificity and can happen both aerobically and anaerobically. Since lignin is the most prevalent type of aromatic carbon in nature and serves to shield

plants' polysaccharides from enzymatic attack, many of these organisms are lignin-degraders. Lignin degraders are consequently widely dispersed in nature and have been isolated from a variety of sources, including soil and the feces of animals that consume wood. The degradation of lignin is achieved via the production of enzymes that are relatively non-specific and can be exploited for the degradation of some organic pollutants (Juhasz and Naidu, 2000). Petroleum hydrocarbon biodegradation is a complicated process that is influenced by the kind and quantity of hydrocarbons present. There are four different categories of petroleum hydrocarbons: saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and resins (pyridines, quinolones, carbazoles, sulfoxides, and amides). Cooney *et al.*, (1985) revealed various parameters affecting hydrocarbon decomposition. Because microorganisms may only access a limited amount of oil pollutants, biodegradation of these pollutants in the environment is restricted. Petroleum hydrocarbon compounds stick to soil elements and are challenging to remove or break down (Barathi and Vasudevan,2001).

Different hydrocarbons are more or less vulnerable to microbial assault. A general ranking of hydrocarbons' vulnerability to microbial breakdown is as follows: linear alkanes > branched alkanes > tiny aromatics > cyclic alkanes (Ulrici,2000). Some substances, such the high molecular weight polycyclic aromatic hydrocarbons (PAHs), might not even decompose (Atlas and Bragg,2009). The term "bioremediation" refers to the employment of biological systems or agents to eliminate or lower hazardous waste concentrations at contaminated sites. Bottom line: According to evidence coming in from all directions, it's not difficult to locate microbes that have the ability to break down petroleum molecules. The findings of several studies have been published on the response of the bacterial community to bioremediation treatment of soils polluted

with crude oil or specific hydrocarbon classes. Sites with petroleum contamination are rife with native microorganisms that can be inoculated during bioremediation (Odokuma and Dickson, 2003). The primary and most effective natural method for removing petroleum hydrocarbon contaminants from the environment is microbial degradation.

According to Tian *et al.*, (2002), microbial degradation of PAHs is thought to be the primary decomposition pathway for these toxins in nature and may be a viable remedy for the environmental issues they cause. Since they are the primary agents in the biodegradation and mineralization of organic molecules, microorganisms play a significant role in the biogeochemical cycling of both organic and inorganic elements (Madigan *et al.*,2000). According to Pan *et al.*, (2004), many different types of microbial resources have been discovered to be useful for PAH breakdown. Many isolated bacterial and fungal species have been found to be capable of biodegrading petroleum hydrocarbons, even polynuclear aromatic hydrocarbons, according to Márquez-Rocha *et al.*, (2005). Numerous bacteria, fungi, and algae that are found in soil and aquatic environments have the enzymatic ability to completely mineralize hydrocarbons (Jahangeer and Kumar,2013).

The goal of the bioremediation process can be one of three things: a) mineralization, which is the complete oxidation of organic pollutants; b) biotransformation of organic contaminants into small, less toxic intermediates; or c) reduction of compounds with highly electronic nitro and halo-groups into less toxic forms by transfer of electrons from an electron donor, typically a sugar or fatty acid, to the contaminant (Rockne and Reddy,2003). Carbon dioxide and water, which are byproducts of the breakdown of petroleum hydrocarbons and

indicators of soil microbial respiration and activity (Obire and Nwaubeta, 2001). The Figure 2.2, which details the mineralization process, is shown below.

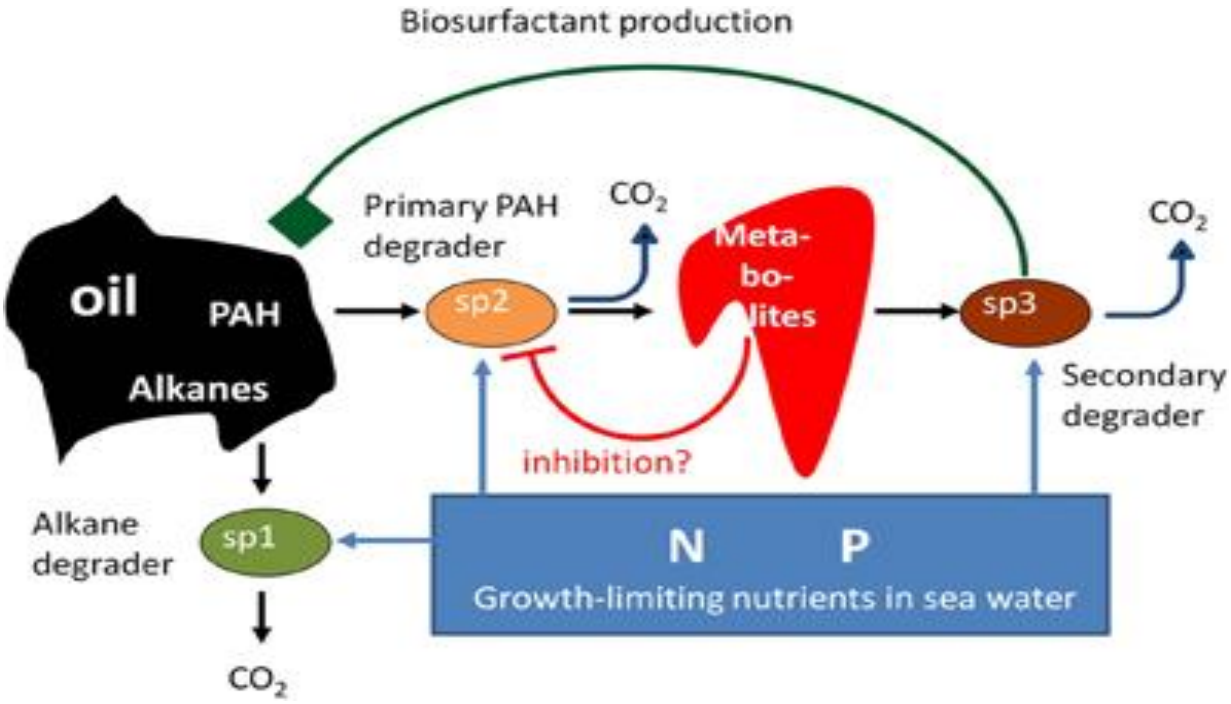



**Figure 2-2:A diagram showing mineralization of organic contaminant (Kanaly *et al.*,2002).**

Oil cleanup by microbes is significantly influenced by environmental factors; Salinity, ambient nutrients, oxygen availability, and temperature all influence the choice and activity of the microbial community (Figure 2-3).

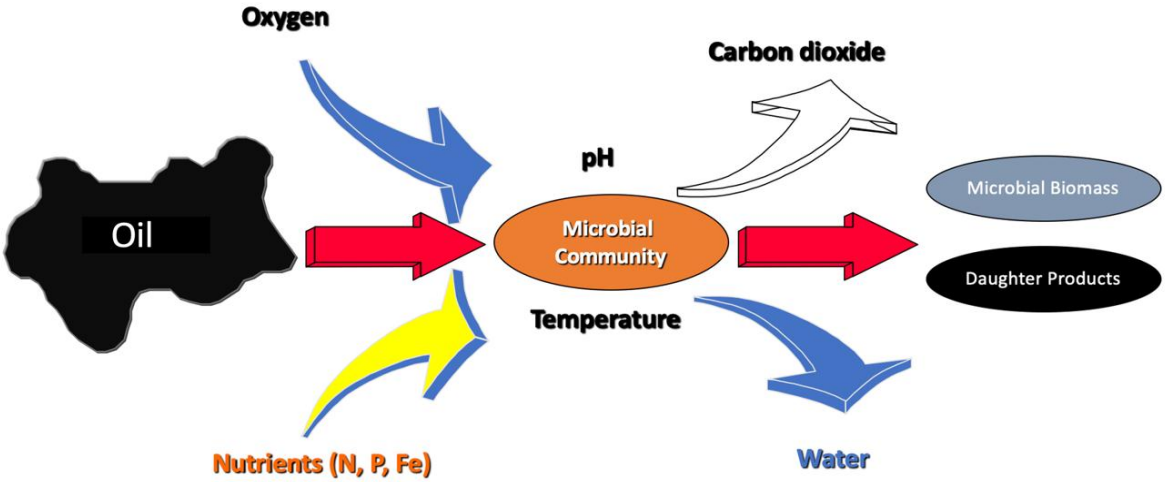



**Figure 2-3: A conceptual model of oil biodegradation (Hazen *et al*,. 2016).**

The most abundant microorganisms are bacteria, which can have up to 1026 cells in the phyllosphere and 108 to 1012 cells in a 1 g sample of rhizosphere soil (Morris and Kinkel,2015). Bacteria play a significant role in the microbial breakdown of organic pollutants as an integral component of the global ecosystem (Xu and Zhou, 2017). Since Davies and Evans discovered for the first time that Pseudomonad bacteria can digest naphthalene, in the past few decades, tremendous progress in the study of biological species, degradation processes, and catabolic genes has been accomplished by researchers. Of all the crude oil's constituents, polycyclic aromatic hydrocarbons (PAHs) are one of the most difficult to remove (Kanaly and Harayama, 2000). *Micrococcus, Corynebacterium, Bacillus, Enterobacter, Pseudomonas, Alcaligenes, Flavobacterium, Moraxella, Aeromonas, Acinetobacter, and Vibrio* are the genera that comprise the isolated crude oil degraders. The native genera appear to be crude oil consumers, and the flora reflects the typical heterotrophic bacteria found in soil. The mentioned genera were demonstrated by a number of other researchers as microbes that degrade hydrocarbons (Banat *et al.*, 2000).

*Rhodococcus, Pseudomonas, Sphingomonas, Bacillus, Flavobacterium, Mycobacterium, Nocardia, Vibrio, Micrococcus,* and *Acinetobacter* are the principal bacteria with a high capacity to breakdown PAHs (Czarny *et al.*,2020).*Rhodococcus, Mycobacterium, Nocadiodes, and Terrabacteres* are capable of degrading PAHs with high molecular weight having more than three rings (Picariello *et al.*,2020). Metabolic pathways including the enzymes and involved catabolic genes for several Gram-negative bacteria such as *Pseudomonas, Sphingomonas, Burkholderia, and Comamonas* are proposed. Despite the fact that several of these bacteria have been found to digest both low and high molecular weight PAHs, knowledge of the genetic and biochemical mechanism is still lacking. After 20 days of incubation, a bacterial consortia typically exhibits the highest percentage (78%) of crude oil degradation. According to Kuppusamy *et al.*, (2017) semi-continuous crude oil-fed reactor operated by a four-member consortium degraded crude oil by about 60%.

## 2.10 Biosurfactant

One of the harmful contaminants that have accumulated in the environment as a result of both anthropogenic and natural activity is petroleum (Banat, 1995). Since the contamination is hazardous to plants, animals, and people, it has a negative impact on the environment and human health. Petroleum is difficult for the environment to break down. However, it is asserted that the use of biosurfactants can accelerate the breakdown of hydrocarbons by making insoluble substrates more accessible to microbes (Haba *et al.,* 2000). Due to its advantages over synthetic products, such as biodegradability, low toxicity, manufacturing from renewable sources, and usefulness under extreme pH and temperature settings, biosurfactants have attracted increasing interest in recent years ( Shekhar *et al.,*

2015). Biosurfactant, however, is less competitive than synthetic alternatives due to high production costs. The carbon requirements for the generation of biosurfactant were typically supplied by agro-industrial substrates with high carbohydrate and lipid contents (Sarubbo *et al.,* 2007; Coimbra *et al.,* 2009; Gusmo *et al.,* 2010). Amphiphilic substances known as biosurfactants are formed on living surfaces, mostly on the surfaces of microbes. They can also be secreted extracellularly, and they include both hydrophilic and hydrophobic moieties, which lower surface and interfacial tension, respectively. Even though emulsifiers may not lower surface tension, biosurfactant and bio emulsifiers both display emulsification capabilities, hence they are commonly studied together. Glycolipids, mycolic acid, polysaccharide-lipid composites, lipoprotein/lipopeptides, phospholipids, or the microbial cell surface itself can all be structural components of a biosurfactant (Banat *et al.,* 2010). Although the majority of biosurfactants are thought to be secondary metabolites, some may be crucial for the survival of microorganisms that produce biosurfactants by facilitating nutrient uptake, microbial host interactions, acting as biocide agents, or by encouraging the swarming motility of microorganisms and taking part in physiological signaling and differentiation processes in cells (Gaur *et al.*,2021). For instance, rhamnolipids are crucial for preserving the structure of biofilms and are regarded as one of *Pseudomonas sp. virulence* factors (Van - Hamme *et al.,* 2006; Arutchelvi *et al.,* 2009). According to Eduardo *et al.,* (2011), who focused on the production of biosurfactant from *lactobacilli*, the composition of mineral salt medium has a significant impact on biosurfactant production in addition to the type of microorganism used, it was discovered that *lactobacilli* produce less biosurfactant than other microorganisms like *Bacillus subtilis* or *Pseudomonas aeruginosa* and also consume a variety of nutrients. Considering that these microorganisms are

typically regarded as GRAS and are already used in many food manufacturing and industrial processes, *lactobacilli* represent a promising source of biosurfactant. It was also discovered that improving the culture conditions could boost the yield of biosurfactant production. *B. subtilis, P. aeruginosa, and R. erythropolis*, three bacteria that produce biosurfactants, were isolated from reservoir formation water by Wen *et al.*, (2011). Using these three bacteria, three biosurfactants were recovered and examined using crude oil as a carbon source. *P. aeruginosa* was found to have significantly higher biosurfactant production rates, resistance, and stability than the other two bacteria. Additionally, this achieved an emulsion index for crude oil of 80% and decreased the medium's surface tension from 71.2 to 22.6 mN/m. According to the results of a biosurfactant flooding experiment, *P. aeruginosa* recovered 14.3% of its oil after being submerged in water. Heavy metals, organic solvents, and hydrocarbons are all complicated mixtures that make up petroleum. Bacteria produce exopolysaccharides/bioemulsifiers, which are amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers that stabilize oil-in-water emulsions, to overcome the harsh effects of organic solvents and heavy metals in contaminated soil (Robinson *et al.,* 1996). It can improve the dispersion, emulsification, and degradation of hydrocarbon pollutants in the polluted site by decreasing the surface tension, interfacial tension, and cell surface hydrophobicity of bacteria (Al_ Tahhan *et al.*, 2000). Numerous bacteria create bioemulsifiers. The oil industry has found tremendous use for the extracellular polymeric bioemulsifier emulsan, which is produced by Acinetobacter caicoaceticus RAG-1 (Fiechter, 1992). While *Candida lipolytica* produces the bioemulsifier liposan, which is mostly made of carbohydrates (Amaral *et al.*,2006). Enhancing oil recovery, creating stable oil-in-water emulsions for the food and cosmetic industries, and bioremediation of oil-

polluted soil and water are some of the possible commercial uses of bioemulsifiers (Klekner and Kosaric, 1993,Banat *et al.*, 2000; Christofi and Ivshina, 2002).

## 2.11 Classification of biosurfactants

Biosurfactants can be separated into high molecular mass polymers, which are better emulsion stabilizers, and low molecular mass molecules, which effectively lower surface and interfacial tension. Lipopeptide, glycolipid, and phospholipid are the three primary types of low-mass surfactants, whereas polymeric and particulate surfactants are the two main classes of large-mass surfactants. The hydrophilic moiety of biosurfactants can be a carbohydrate, phosphate, amino acid, or cyclic peptide, while the hydrophobic moiety is based on long-chain fatty acids or fatty acid derivatives (Erum *et al.*,2013)

Following is a brief explanation of each category of biosurfactant (Table 2.1):

**Table 2-1: Major biosurfactant classes and microorganisms involved**

| Surfactant class Microorganism | Surfactant class Microorganism |
|---|---|
| **Glycolipids** | ***Pseudomonas aeruginosa*** |
| **Rhamnolipids** | ***Rhodococcus erithropolis*** |
| **Trehalose lipids** | ***Arthobacter* sp.** |
| **Sophorolipids** | ***Candida bombicola, C. apicola*** |
| **Mannosylerythritol lipids** | ***C. antartica*** |
| **Lipopeptides** | ***Bacillus subtilis*** |
| **Surfactin/iturin/fengycin** | ***P. fluorescens*** |
| **Viscosin** | ***B. licheniformis*** |
| **Lichenysin** | ***Serratia marcescens*** |
| **Serrawettin** | |
| **Phospholipids** | ***Acinetobacter* sp.** |
| | ***Corynebacterium lepus*** |
| **Surface-active antibiotics** | ***Brevibacterium brevis*** |
| **Gramicidin** | ***B. polymyxa*** |

| | |
|---|---|
| **Polymixin**<br>**Antibiotic TA** | ***Myxococcus Xanthus*** |
| **Fatty acids or neutral lipids Corynomicolic acids** | ***Corynebacterium insidibasseosum*** |
| **Polymeric surfactants**<br>**Emulsan**<br>**Alasan**<br>**Liposan**<br>**Lipomanan** | ***Acinetobacter calcoaceticus***<br>***A. radioresistens***<br>***C. lipolytica***<br>***C. tropicalis*** |
| **Particulate biosurfactants Vesicles Whole microbial cells** | ***A. calcoaceticus***<br>***Cyanobacteria*** |

## 2.12 Methods used to detect biosurfactant producing microorganisms:

There are many methods used to screen and detect potential biosurfactant producing microorganisms. These methods were as follows:

### 2.12.1Drop collapse test

The biosurfactant synthesis was screened using the drop collapse test. It is a qualitative technique. For a 24-hour equilibration period, crude oil was administered to the well regions marked on the covers of 96-well microplates. The oil-coated well areas received the supernatant containing biosurfactant, and a drop size was seen using a microscope after 1 minute. When the drop's diameter grew by 1mm from the diameter produced by the distilled water used as the negative control, the outcome was deemed positive (Salamat *et al.*, 2018).

### 2.12.2 Blood hemolysis test

Blood hemolysis testing can identify the isolate that produces biosurfactant by looking for new single colonies of the isolated cultures that were obtained and streaked on blood agar plates. These plates were incubated at 37 °C for 48 to 72 hours. After looking at the plates, it was discovered that the presence of a clear

zone around the colonies indicated the presence of organisms that produce biosurfactant (Anandaraj and Thivakaran, 2010).

## 2.12.3 Emulsification index

Petroleum was used to test the emulsification index (Jazeh *et al.,* 2012). Five ml of hydrocarbon, or gasoline, was placed in a test tube. Five ml of cell-free supernatant, obtained after centrifuging the culture, was then added. The test tube was vortexed for two minutes to ensure a homogenous mixture of both liquids. After 24 hours, the emulsification index was measured and calculated using the following formula:

**Ei24% = total high of the emulsified layer / total high of the liquid layer ×100.**

The calculations were done for all the cultures individually and their emulsification activities were compared with each other

## 2.12.4Emulsification activity

By assessing the emulsification activity, the biosurfactant activity was assessed. To do this, 0.5 ml of cell free supernatant was added to 7.5 ml of Tris-Mg buffer and 0.1 ml of dodecanese, and the mixture was vortexed for two minutes. After one hour, the tubes were checked for absorbency at 540 nm. Emulsification activity was determined by measuring optical density; the blank was a culture-free solution of Tris-Mg, dodecanese, and mineral salt (Bodour *et al.,* 2004).

## 2.12.5 Surface tension

One of the most basic methods is the employment of a du Nouy ring-type tensiometer to measure surface tension. By monitoring changes in surface and interfacial tensions, biosurfactant activities can be identified. A tensiometer can be used to quickly measure the surface tension at the air/water and oil/water interfaces. Distilled water has a surface tension of 72 mN/m, and adding a

surfactant of some kind reduces this value. Surface tension is reduced when a surfactant is applied to air/water or oil/water systems at increasing concentrations, but only until a critical point (Satpute *et al.*, 2010).

## 2.13 Purification of biosurfactant

A combination of conventional methods can easily extract the majority of the biosurfactant from the culture media (Sen and Swaminathan, 2005). In general, numerous structural kinds of the target biosurfactant, which are produced in different amounts, are frequently co-extracted during the extraction together with a number of contaminants. These might require separation and impurity removal in order to be evaluated. The type of charge, solubility characteristics, whether the product is intracellular or extracellular, as well as the economics of recovery and downstream processing, as well as the physicochemical characteristics of the desired biosurfactant, all play a role in the method for purification and recovery of surfactants (Shaligram and Singhal, 2010). Precipitation, adsorption-desorption, ion exchange chromatography, centrifugation, crystallization, filtration and precipitation, foam fractionation, isoelectric focusing, solvent extraction, ultrafiltration, and dialysis are some of the different techniques used to purify biosurfactants and bioemulsifiers (Satpute *et al.*, 2010). According to Phetrong *et al.*, (2008), the most effective method for precipitating the emulsifier from A. calcoaceticus subsp. Anitratus SM7 was ethanol. The acid precipitation method is also simple, affordable, and easily accessible for recovering crude biosurfactants like surfactin, lipopeptides, and glycolipids. By employing concentrated HCl to lower the pH for acid hydrolysis, biosurfactant becomes insoluble at lower pH levels (Mukherjee *et al.*, 2006). The extraction of the culture medium of the microorganisms responsible for creating biosurfactants using an organic solvent is

the approach utilized the most frequently in the first step of biosurfactant purification. Chloroform-methanol, dichloromethane-methanol, ethyl acetate, hexane, hexane-methanol, butanol, pentane, acetic acid, or ether are among the common solvents used. In addition, certain biosurfactants were isolated using acid precipitation and ultrafiltration. Following extraction, column chromatography is the recommended technique; examples include silica-gel, sephadex, ODS, and HPLC for purification, depending on the type, characteristics, and goal of the biosurfactant application. Thin layer chromatography with the various specific sprays was used for detection of the purity of the product (Desai and Banat,1997).

## 2.14Techniques used for biosurfactant characterization

### 2.14.1 Fourier transform infrared spectrophotometer (FTIR)

The activity of biosurfactant depends on their structural components, the types of hydrophilic and hydrophobic groups and their spatial orientation .Surfactin, lichenysin and rhamnolipids have been characterized by the FTIR technique . Also the Alkyl, carbonyl, ester compounds of biosurfactant are detected clearly . FT-IR spectra of the purified bioemulsifier, which exhibited the typical feature characteristics of hetero polysaccharide, in which abroad band was observed around 3428 cm and a weak C–H stretching band at 2923 cm which attributed to the characteristic for O–H content, typical of polysaccharide (Sood *et al.,* 2020).

### 2.14.2 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a type of liquid chromatography used to separate and quantify compounds that have been dissolved in solution. HPLC is used to determine the amount of a specific compound in a solution. In HPLC and liquid chromatography, where the sample solution is in

contact with a second solid or liquid phase, the different solutes in the sample solution will interact with the stationary phase. The differences in interaction with the column can help separate different sample components from each other (Kupiec, 2004). Since the compounds have different motilities, they exit the column at different times, they have different retention times, Rt. The retention time is the time between injection and detection. There are numerous detectors which can be used in liquid chromatography. It is a device that senses the presence of components different from the liquid mobile phase and converts that information to an electrical signal. For a qualitative identification one must rely on matching retention times of known compounds with the retention times of components in the unknown mixture (Brown *et al*., 1997; Holler and Saunders, 1998). High performance liquid chromatography (HPLC) is not only appropriate for the complete separation of different biosurfactant, but can also be coupled with various detection devices (UV, MS, evaporative light scattering detection, ELSD) for identification and quantification of biosurfactant (Heyd *et al*., 2008). *S. marcescens* can cause biodegradation for palmarosa oil (green oil); the compounds have been identified by HPLC, the HPLC of palmarosa oil shows three peaks, it indicates that oil contains three compounds (Mohanan *et al*., 2007).

## 2.15 Applications, environmental impact and economics

Biosurfactants are attracting interest in applications where their environmental compatibility and stability under extreme conditions can be exploited. For instance, biosurfactants can be used in the biomedical field as drug delivery systems and antimicrobial agents, as specialty chemicals in flavor compound production, in bioremediation and microbial enhanced oil recovery (MEOR) (Desai and Banat 1997; Makkar and Cameotra 2002; Banat *et al.*, 2010;

Reis *et al.*, 2013). Bioremediation, dispersion of oil spills, enhanced oil recovery and transfer of crude oil are some examples of environmental applications of biosurfactant (Shafiei *et al.*, 2014). Heavy crude oil recovery, facilitated by microorganisms, was suggested in the 1920s and received growing interest in the 1980s as microbial enhanced oil recovery (MEOR), although there were not many reports on productive microbial enhanced oil recovery project using biosurfactant and microbial biopolymers. Emulsification properties of biosurfactant make them potentially useful tools for oil spill pollution-control by enhancing hydrocarbons degradation in the environment (Bertrand *et al*., 1994). The biosurfactant are involved in bioremediation in two ways: by increasing the surface area of hydrophobic water insoluble substrate and by increasing the bioavailability of hydrophobic water insoluble substances. The bioremediation of some contaminated sites are limited due to the low water-solubility of many hydrocarbons, which reduce their availability to micro-organisms. It has been assumed that surfactants can be used to enhance the bioavailability of hydrophobic compounds (Atlas and Cerniglia, 1995). In addition to other application of biosurfactant, food line have been given many enhancements by decrease surface and interfacial tension, thus facilitating the formation and stabilization of emulsions. In addition to control the aggregation of fat globules, stabilization of aerated systems, improvement of texture and shelf-life of products containing starch, modification of rheological properties of wheat dough and improvement of constancy and texture of fat-based products (Shoeb *et al*., 2013). They are also utilized as fat stabilizers and anti-spattering agent during cooking of oil and fats (Kosaric, 2001).Also, Biosurfactant have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection sites, making them useful for treating many diseases as therapeutic and probiotic agents. Pre-coating vinyl urethral catheters by running the surfactin

solution through them before inoculation with media resulted in a decrease in the amount of biofilm formed by *S. typhimurium*, *S. enterica*, *E. coli* and *P. mirabilis* (Rodrigues *et al*., 2004).

## 2.16 Molecular approaches of PAH degradation

There are microorganisms that exhibit physiological and metabolic versatility and have been established as proficient PAH degraders. There are some others, which have no proven capacity to degrade PAHs in contaminated environmental matrices, but possess other desirable characteristics such as ability to withstand extreme conditions and produce certain desirable compounds like enzymes, surfactants and emulsifiers, which might enhance desorption (Pazos *et al.*, 2010). Also, many times, microorganisms exhibit optimal proficiency in PAH degradation at the laboratory scale, but show less efficiency at the field scale. This might be due to factors such as adverse environmental conditions or other unfavorable potential or survival of an otherwise efficient PAH degrader (Bustamante *et al.*, 2011). It is possible to improve the bioremediation potential of such microorganisms prior to their field scale use by manipulating their genetic properties through genetic engineering. There are available molecular tools and techniques that can efficiently modify remediation genes (Fernández-Luqueño *et al.*, 2011). There is a proven relationship between the relative abundance of the genes involved in bioremediation and the potential for contaminant degradation. However, sometimes, a microbial strain might possess the required gene for PAH remediation but the genes might not be fully expressed . There are two options available for genetically stimulating microorganisms to achieve optimal bioremediation results: gene introduction or gene manipulation (Gentry *et al.*, 2004). Genome sequencing has been used to explain detailed characteristics of

many bacteria including their metabolic behavior ( Oyedara *et al.*, 2018). Genome sequencing of some bacteria with the potentials to degrade hydrocarbons and PAHs has given profound insight into the genes involved in the degradation and mineralization of PAHs ( Das and Chandran, 2011; Pal *et al.*, 2017). This genome sequence analysis has also provided information on the peripheral pathways associated with the PAH degradation process by bacteria including *Stenotrophomonas* species (Elufisan *et al.*, 2019). Several bacteria with good potentials for hydrocarbon degradation have been sequenced (Das *et al.*, 2015; Pal *et al.*, 2017). Identification and detection of indigenous PAH degraders are therefore necessary to better understand natural biodegradation processes and for the successful application of bioremediation technologies. Culture-independent methods targeting the 16S rRNA gene can effectively reveal the microbial diversity of many PAH-polluted systems (Singleton *et al.*,2013).

## 2.17 Enzymes and genes involved in PAHs-degradation

Enzymes are crucial for the microbial breakdown of many different substances, including oil, gasoline, and PAHs (Gupta and Pathak, 2020). Enzymes called Oxidoreductase break down chemical bonds and move electrons from a reduced organic substrate (donner) to another chemical molecule (acceptor). Contaminants are oxidized to harmless compounds during these oxidation reduction reactions. Oxygenase classified under the Oxidoreductase group of enzymes (Karigar and Rao ,2011). The primary enzymatic process in aerobic biodegradation, oxidation reaction, is catalyzed by oxygenase. Organic compounds are broken down by Oxygenases, which also increase their reactivity, water solubility, and cleave the organic ring. Oxygenase can be further subdivided into two types based on the quantity of oxygen atoms used for oxidation: I)

Monooxygenase and II) Dioxygenase. On a variety of substrates, monooxygenase are highly region and stero selective, adding one atom of molecular oxygen to the organic molecule (Arora *et al.*,2009). *Pseudomonas stutzeri* produces catechol 2, 3 dioxygenase, which is responsible for Meta cleavage of catechol. Members of the genus *Pseudomonas* are known to have varied metabolic pathways and thrive using a variety of substrates as sources of carbon (Lalucat *et al.*,2006). *Mycobacterium gilvum* PYR-GCK contains the four aromatic ring cleavage dioxygenase genes Phd F, Phd I, Pea G, and Pca H that are essential for the biodegradation of pyrene (Badejo *et al.*,2013). several species of *Mycobacterium* possessing many dioxygenase (Lee *et al.*,2007). The genes designated mid A3B3 that encode the subunits of the terminal dioxygenase detected enzyme of Mycobacterium vanbaalenii PYR-1 shared many similarities with PAH-ring hydroxylating dioxygenases from Mycobacterium and Rhodococcus species, but they were most similar to the -subunit of NocardioidesKP7 fumarase (Kim *et al.*,2006). The NahAc gene was found in 13 isolates of Gram-negative bacteria, and the sequences of Nah Ac-like genes from strains of *Pseudomonas brenneri*, *Enterobacter*, *Pseudomonas entomophila*, *Pseudomonas koreensis*, and *Stenotrophomonas* were also obtained (Buckova *et al.*,2013). Ring-hydroxylating dioxygenase (RHD) genes were linked to I), nid A3 of *Mycobacterium* Py146 to II) Pdo A of *Terrabacterium* HH4 to III) Mid A of *Diaphorobacter* KOTLB, to IV) Pdo A2 of *Mycobacterium* CH-2 in clone libraries of Gram +ve bacteria. *Burkholderia* satisoli's Phn Ac, *Burkholderia* glathei's Naphthalene dioxygenase, and an uncultured bacterium's RHD - component were connected to the RHD genes of Gram-Ve (Muangechinda *et al.*,2015). The 1-hydroxy-2-naphthoate dioxygenase 1H2Dase genes, which code for 1H2Dase enzymes, and the ring-hydroxylating dioxygenase RHDase genes, which code for RHDases, both play significant roles in the breakdown of

PAH intermediates that can be separated from Arthrobacter sp. SAO2 and are capable of degrading phenanthrene (Li *et al.*,2015). For the first time, gene-targeted metagenomics was employed by Liang *et al.*, (2019 a, b) to look into the variety of PAH-degrading bacterial communities in oil field soils and mangrove sediments. Instead of Pah Ac, which served as a functional marker gene for PAH-degrading bacteria and encoded the alfa-subunit of PAH ring-hydroxylating dioxygenase, Pah E was a superior biomarker for PAH-degrading bacteria.

## 3.1. Instruments analysis and equipment

The general laboratory instrument and equipment are listed in (Table 3-1)

**Table (3-1) :List of general laboratory instrument and equipment**

| Equipment | Supplied company |
|---|---|
| Autoclave | Labtech/Korea |
| Balance | Sartorius /U.K. |
| Cooling centrifuge | Hettich |
| Deep freezer | Almateen/ China |
| Different size Micropipettes (µl) | Dragon/ German |
| Distiller | H- Jurgens- CO |
| Electrical conductivity | Hanna |
| Gas chromatography | Shemadzo/Japan |
| Gel electrophoresis | CE cleaver/ Germany |
| Horizontal gel electrophoresis | ATTA/ Japan |
| Incubator | Binder/ Germany |
| Laminar flow capinate | Bio Tech/Korea |
| Light compound microscope | Zeiss /Germany |
| Magnetic stirrer | Scientific industries/ U.S.A |
| Micro-Centrifuge | Hettich/ Germany |
| Oven | Memmert/ Germany |
| PCR | Ependroff/ USA |

| Equipment | Supplied company |
|---|---|
| PCR apparatus | BIOMETRA/ Germany |
| pH meter | Hanna |
| Power supply | Shndon |
| Racks for Eppendorf tubes | Meheco/ China |
| Refrigerator | Vestal |
| Sensitive electronic balance | Sartorius /U.K. |
| Shaker incubators | Binder/ Germany |
| UV Transilluminator | Quantum/ France |
| UV visible Spectrophotometer,6300PC | Cambridge/ UK |
| Vortex | Griffen and George Ltd/ UK |
| Water bath | Memmert/ Germany |

## 3.2 Chemicals

All the chemicals used in this study are listed in Table (3-2)

**Table (3-2): List of chemicals used in the work**

| **Chemicals and Media** | **Supplied company** |
|---|---|
| Acrylamide | Himedid/India |
| Agarose | Pronadisa/Spain |
| APS | Sigma/German |
| Biosafety dye | Intron/ England |
| Bis acrylamide | Scr/China |

| | |
|---|---|
| Bovin serum albumin | Merk |
| Bromophenol blue | Analar |
| $CaCl_2$ | Fluka |
| Coomassie brilliant blue G-250 | BDH/England |
| CTAB | Himedia/India |
| DNA loading buffer | Promega/USA |
| EDTA | Scr/ Chain |
| Ethanol (70% ) | Fluka chemika/ Switzerland |
| Ethyl acetate | BDH/England |
| $FeSO_4.7H_2O$ | Fluka |
| Glucose | Himedia/India |
| Glycerol | Analar |
| Glycine | BDH/England |
| HCl | BDH/ England |
| $K_2HPO_4$ | Fluka |
| KCL | Fluka |
| $KH_2PO_4$ | Fluka |
| Ladder DNA | Cyntol/Russain |
| Master Mix Kit | Cyntol/Russain |
| methylene blue | Analar |
| $MgSO_4.7H_2O$ | Fluka |
| $MnSo_4.6H_2O$ | Fluka |

| | |
|---|---|
| $Na_2HPO_4$ | BDH/England |
| $Na_2MoO4$ | Fluka |
| NaCl | Fluka |
| $NaNO_3$ | Merck |
| $(NH_4)_2SO_4$ | Fluka |
| NaOH | BDH/ England |
| Nuclease free water | Cyntol/Russain |
| Peptone | Himedia/India |
| Phosphoric acid | Analar |
| Proteinase K | Pioneer/India |
| SDS | Ghem supply |
| Silver nitrate | BDH/England |
| TBE | Thomas baker/India |
| TEMED | Sigma/german |
| Tris- base | Thomas baker/India |
| Tris- HCl | BDH |
| Yeast extract | Himedia/India |

## 3.3 Cultural media

A variety of media were used for bacterial isolation and bacterial growth , these cultural media are listed in Table (3-3):

**Table (3-3):Media for bacterial isolation and bacterial growth**

| No. | Name of cultural media | Supplied company |
|---|---|---|
| 1. | Blood agar base medium | Himedia |
| 2. | Brain heart infusion broth medium | Himedia |
| 3. | Chromogenic agar medium | Himedia |
| 4. | MacConkey agar medium | Himedia |
| 5. | Nutrient agar medium | Himedia |
| 6. | Nutrient broth medium | Himedia |

## 3.4 Mineral salt medium (MSM): (Bezza and Chirwa, 2016).

This medium was prepared by dissolving material shown below:-

| Compound | Quantity /gm |
|---|---|
| $(NH_4)_2SO_4$ | 1.1gm |
| $K_2HpO4$ | 2.2 gm. |
| $KH_2pO4$ | 0.9gm |
| $MgSO4.7H_2O$ | 0.1gm |
| $MnSO4.6H_2O$ | 0.025gm |
| $FeSO_4.7H_2O$ | 0.005 gm. |
| $CaCl_2$ | 0.1gm |

These components were dissolved in 1000 ml D.W and sterilized in autoclave at 121˚C for 15 minutes. After medium is cooled, added crude oil (1 %) for each flask.

## 3.5 Methods

### 3.5.1 Sample Collection

Fifteen soil sample taken from an oil-contaminated site in Babylon province during January 2021. Samples were collected from several sites using a soil auger to a depth of 2-5 cm and stored in sterile dry bottles before being transferred to the laboratory and stored at 4°C .

### 3.5.2 Environmental measurement

### 3.5.2.1 Physical and Chemical properties of soil

In this study used (10gm) soil and (50ml) distilled water for obtaining (1:5) soil-over-water mass ratios (soil : water) extracts for soil electrical conductivity measurements (EC , Tds,PH,Cl,$CaCO_3$ and TOC ).

#### 3.5.2.1.1 Salinity:

Salinity was measured by using apparatus for salinity and expressed with (%).

$$‰ \text{ Salinity} = \frac{EC(\mu s\backslash cm) * 0.64}{1000}$$

#### 3.5.2.1.2 Total dissolved soild (TDS)

Total dissolved solid (TDS) was measured and expressed the units with (mg/L).

#### 3.5.2.1.3 PH

The PH was measured of soil samples using pH meter.

#### 3.5.2.1.4 Electrical conductivity (E.C)

The EC was measured and expressed the units with (µs/cm).

## 3.5.2.1.5 Total organic carbon(TOC):

Organic matter the residue of soils in various phases of decomposition ,O.M has the main action on soils nutrient reserve , aggregation and it's availability , moisture reservation and biological activity , Organic Carbon (O.C) rang from being the controlling constituent of peat or rich sediments and soils in cold regions with soils naturally have more than (3-4) % O.M while soils of semi-arid rain –fed area have naturally less than (1% ) O.M .The most popular steps include the reduction of potassium dichromate ( $K_2Cr_2O_7$) by organic carbon compound and subsequent determination of the unreduced dichromate by ( Oxidation –reduction titration with ferrous –ammonium sulfate ( Walkley , 1947 ).

1- One gram of air -dry soils was taken and put in beaker (volume 500ml) .
2- Ten ml of 1.0 N-potassium dichromate solution was Added by pipette, then added 20ml concentrated sulfuric acid by dispenser rotate the beaker for mix the suspension . leave it to settle down for 30 min.
3- Two hundred ml of deionized water was Added, then added 10ml concentrated phosphoric acid ,leave the mixer to cool .
4- Ten- Fifteen drop diphenylamine sulphate was Add as an indicator .
5- -Titration with ferrous sulfate solution until the color change from (blue - violet to bright green) . The percentage organic matter in soils

X=V sample \V blank

Y=1-X

%O.M (w\w) = (Y*0.67*10.5)\Wt.

Where :

V blank=Volume of (ferrous –ammonium sulfate solution) needful to titration the blank (ml).

V sample=Volume of (ferrous –ammonium sulfate solution) needful to titration the sample (ml).

Wt.= Weight of (air-dry) soils (gm)

### 3.5.2.1.6 $CaCO_3$

Calcium carbonate was measured as: According to (Chaney *et al.*,1982)

1- Ten gram m of dry soil was grind.
2- One to five gram of dry soil was weighed and put in beaker (volume 250 ml).
3- Ten ml of HCl was Added and mixed well.
4- The beaker was left for the next day or heat at (50-60°C) and left to cool.
5- Fifty ml of d.w was Added.
6- Soil solution was filtered into flask volume 250 ml with D.W. Then wash soil by calcium chloride (1 N) to remove hydrogen as a result of adding acid.
7- Added 2-4 drops of phenolphthalein, and equilibrate by NaOH until appear pink color for 15 seconds.

$$CaCO_3\% = [(V_{HCL} * N_{HCL}) - (V_{NaOH} * N_{NaOH})] * \frac{50}{1000} * \frac{100}{W}$$

### 3.5.2.1.7 Chloride (Christoph,2019)

Chloride was measured by taken 10 ml of sample and added 3 drops of potassium chromate. After that, sample was mixed with silver nitrate. Blank was made from D.W with the same quantity and sample size, then added 3drops of chromate and mixed with silver nitrate.

The percentage of chlorides is measured according to the formula:

$$\text{Cl mg/L} = \frac{(A-B) * N*35450}{\text{Ml of sample}}$$

A: Sample descending volume

B:Blank descending volume

N:Silver nitrate molarity (0.0141)

Ml: sample size

### 3.5.2.1.8 Determination of PAH compound by using GC-MS

Three grams of dry, homogenized sediment sample was placed in a clean centrifuge tube along with 20 mL of a 5:5 (v/v) acetone/n-hexane solution. Blanks were made using the same method but without the addition of sediment sample. For the purpose of extracting PAH, all samples were vortexed for 1 min. after which the mixture underwent a 15-min. ultrasonic treatment. After that, the sample tubes were centrifuged for 10 min at 4,000 rpm. After centrifuging, the extracted compounds' organic layer was removed using a pipette, and the sediment was twice more extracted using a 2:2 (v/v) acetone/n-hexane solution (10 mL ) (Cheng *et al.*,2012).

### 3.5.2.1.9 GC Conditions

In the laboratories of the Ministry of Science and Technology, Shimadzu (2010, Japan) gas chromatography was used to detect PAHs. Column separation was done on a 30 m 0.25 mm i.d. DB-5 column (J and W Scientific, Folsom, CA) coated with a 0.25-mthick sheet of 5%diphenyl-polydimethylsiloxane. At a temperature of 280 °C, the samples were injected in the split mode. The column temperature was first maintained at 40 °C for one minute, then increased to 120 °C at a rate of 25 °C/min, 160 °C at a rate of 10 °C/min, and ultimately 300 °C at a rate of 5 °C/min, which was then

maintained for 15 minutes. The detector (FID) was maintained at 330 °C. As a carrier gas, helium was employed at a constant flow rate of 5 mL/min (Cheng *et al.*,2012).

## 3.6 Enrichment, Isolation, and identification of PAHs-Degrading bacteria Samples

Ten grams of oil-contaminated soil was placed in 100 mL of mineral salt medium , pH 7.0,prepared in 3.4 .The flasks were shaken for seven days at 150 rpm at 30°C on an orbital shaker. To isolate bacterial species, MSM agar plates coated with the same PAHs (crude oil) as the only carbon source were utilized. Individual colonies with different morphologies were selected. The ability of each isolate to degrade PAHs were examined. Using sterile distilled water as diluents, all of the homogenized mixture was serially diluted. For the serial dilution, six test tubes holding 9ml of distilled water were used (Juhasz and Naidu, 2000).

### 3.6.1 Identification of Bacterial species

Using the pour plate technique, 100 µL aliquots from $10^{-5}$ and $10^{-6}$ dilutions were plated in duplicate on sterile Nutrient Agar plates prepared in 3.2.1. The bacteria were cultivated for 24 hours at 35°C. All of the isolates were inoculated on MacConkey agar, blood agar, and chromogenic agar plates . The streaked plates were incubated at 35°C for 24 hours. Colony morphology and Gram staining were utilized to identify isolates, and the Vitek-2 system was used to identify them using ID-GN and ID-GP cards, as per the manufacturer's instructions (Cheesbrough ,1991).

## 3.6.2Growth and activation of bacterial species

Sterilized nutrient broth medium was prepared as mentioned in 3.2.2, put in sterilized test tubes .The media was inoculated with fresh bacterial isolates .Tubes were incubated at 35˚C for 18-24 hours.

## 3.6.3 Determining of PAHs compounds biodegradation activity

The 56 isolates were cultured on MSM broth medium supplemented with 1% of crude oil. Three replicates were used for each isolate which cultured at 35°C in an orbital shaker incubator at 150 rpm for 18 days. Optical density (O.D.) at 600 nm was measured using a spectrophotometer at 3, 6, 9, 12, 15, and 18 days, as well as pH and plate count.

## 3.7 Screening the bacteria for biosurfactant production:

## 3.7.1 Mineral salt medium for biosurfactant compounds

This medium was prepared as following:

| Compound | Quantity /gm |
|---|---|
| $CaCl_2.2H_2O$ | 0.1 g |
| $FeSO_4.7H_2O$ | 0.05 g |
| $K_2HpO_4$ | 1 g |
| KCl | 3g |
| $MgSO_4.7H_2O$ | 0.2g |
| $Na_2MoO_4.2H_2O$ | 0.001g |
| NaCl | 30g |
| $NaNO_3$ | 0.1g |

All components were dissolved in 1 Liter of D.W, pH was adjusted to 7. After that, this medium was sterilized in autoclave at 121C° for 15 minutes, after cooled at 40-45 C°, crude oil(1%) was added and distributed in flasks.

## 3.7.2 Methods

### 3.7.2.1 Screening the bacteria for biosurfactant production:

The ability of bacterial isolates to produce biosurfactant was tested using 250 ml Erlenmeyer flasks and 50 ml of MSM . Following sterilization, 1% of crude oil was added to the flasks as a sole carbon source. The flasks inoculated by 1% fresh bacterial growth (24 hrs.)and incubated under shaking (150 rpm) at 35 $^{o}$C for 7 days. Then, the cultures were centrifuged at 4 $^{o}$C, 10000 rpm, for 15 min. Production of biosurfactant was investigated in cell –free supernatant (Roy,2017).

### 3.7.2.2 Determination of biosurfactant compounds:

#### 3.7.2.2.1 Hemolysis test

By plate assay using blood agar medium, all bacterial isolates were screened to select more active isolates for biosurfactant production .To test for hemolytic activity, each isolate was streaked on blood agar medium and incubated at 35 °C for 24–48 hrs. Visual inspection of the plates revealed zones of clearing surrounding the colonies, indicating biosurfactant production(Siddiqui *et al*., 2015).

#### 3.7.2.2.2 CTAB agar plate method

On CTAB (Cetyl trimethylammonium bromide) (/methylene blue agar medium with (g/l)), all bacterial isolates were grown on this media which composed of (Agar-Agar 15g, CTAB 0.2g, Glucose 5g, Methylene blue 0.005g, Peptone10g, and Yeast extract 0.5g, pH was adjusted to 7.3). Using a cork borer, 100 microliters of previously activated bacterial culture in

MSM were grown on CTAB agar by well method. The plates were then incubated at 35°C for 24-48 hrs. A dark blue halo zone that surrounded the culture suggested the release of biosurfactants The radius of the inhibitory zone was calculated in mm (Satpute *et al.*,2008).

### 3.7.2.2.3Oil spreading test

The oil spreading test was determined by using twenty ml of D.W that put into a plastic Petri dish, and 20 µl of crude oil covered the top of the water. After that, 10 µl of cell-free culture broth(supernatant) was added onto the oil surface. The oil will be replaced by an oil-free clearing zone, whose diameter shows surfactant activity, also known as oil displacement activity. Water was used as a negative control (Morikawa *et al.,* 2000).

### 3.7.2.2.4 Drop collapse test:

This test was used to screen biosurfactant production by determined the interfacial tension between the drop containing the surfactant and the parafilm surface is used in this experiment, resulting in the drop spreading. were pipetted as a droplet on the parafilm. As a negative control, distilled water was used. It was noticed that the droplet flattens and spreads across the parafilm surface. The parafilm was covered with 2 microliters of crude oil, and 13 isolates were transferred to the oil-coated parafilm sections, where they were allowed to equilibrate for 24 hours. The result was considered positive for biosurfactant production when the drop was flat, but negative when the drop was rounded indicating a lack of biosurfactant production (Nanjundan *et al.*,2019).

### 3.7.2.2.5 Emulsification index test

The ability of isolates to emulsify crude oil was measured using an emulsification index (E24). 1.5 mL crude oil was combined with 1.5 mL cell-free broth for 2 minutes in a test tube before being vortexed at high

speed for 2 minutes and set aside for 24 hours. The following equation was used to calculate the percentage of the emulsification index: (Datta *et al.*,2018)

**(Ei24 = Height of emulsion formed /total height of solution) ×100.**

### 3.7.2.2.6 Bacterial adhesion to hydrocarbons (BATH):

The BATH assay can be used to determine the cells hydrophobicity by determined the suspension absorbance at 600 nm (A0), then 100 µl of crude oil was mixed with 2 ml of cell culture in test tubes and mixing by vortex for 3 minutes. The crude oil and aqueous phase were allowed to separate for 1 hour after mixing. The aqueous phase was removed with care. The O.D of the aqueous phase was then estimated in a spectrophotometer at 600nm (A1). The following formula was used to compute hydrophobicity, which is given as a % of cell adhesion to crude oil: **(**Van der Vegt *et al.*,1991):

$$H\% = (1-A1/A0) *100$$

**H= Hydrophobicity**

**A1= OD of aqueous phase**

**A0= OD of initial cell suspension**

### 3.7.2.2.7 Foaming activity

Different bacterial isolates were grown in 250 mL flasks that each contained 100 mL of nutrient broth medium. In a shaker incubator, the flasks were incubated for 72 hours at 37 °C and 150 rpm. The duration of foam stability, foam height, and foam form are used to evaluate foam activity in the graduated cylinder.(Abouseoud *et al.*,2008).

*Kocuria kristinae* and *Delftia acidovorans* was selected to complete other methods due to high activity for all parameters.

## 3.7.3 Optimization condition for biosurfactant production:

The flasks were cultured in a shaker incubator (150 rpm) at 35 °C for 7 days during the optimization experiments. The flasks included 50 ml of MSM with 1% of crude oil inoculated with 1 ml of fresh bacterial culture (G+ve *Kocuria kristinae*, G-Ve *Delftia acidovorans*). The emulsification index was calculated after the incubation period.**(** Jameel and Haider,2021)

### 3.7.3.1 Effect of pH

To find the optimum pH, (MSM)was modified with several pH values (3, 4, 5, 6, 7 and 8). After being infused with the bacterial isolate, the flasks performed a 7 day of incubation period in a shaker incubator at 150 rpm and 35 °C. And in a subsequent experiment, the optimum pH value was used.

### 3.7.3.2Effect of temperature

MSM (pH 7), after being inoculated with bacteria, was incubated in shaker incubators (150 rpm) for seven days at various temperatures (20, 25, 30, 35, 40, and 45 °C) in order to determine the optimum temperature for the production of biosurfactants, emulsification index was calculated.

### 3.7.3.3 Effect of carbon source:

To find the best source of carbon for the synthesis of biosurfactants, various carbon sources (fructose, glucose, manitol, sucrose, date extract, and crude oil) were employed. Each of these sources was added to the medium at a concentration of 1% separately. After adjusting the pH to 7.0, bacteria were added, and the mixture was shaken (at 150 rpm) in an incubator for 7 days at 35 °C. The optimum carbon source was later used after measuring the emulsification index.

### 3.7.3.4Effect of carbon source concentration:

The bacterium was grown MSM with various concentrations of crude oil(0.5%, 1%, 1.5%, 2%, 2.5%, and 3%), in an attempt to identify the optimum concentration for the production of biosurfactant. Flasks were incubated in a shaker incubator (150 rpm) at 35 °C for 7 days after the pH adjustment to 7.0. emulsification index was determined.

### 3.7.3.5 Effect of different nitrogen sources:

The ideal conditions for the bacterial isolate to produce the biosurfactants were determined by using various nitrogen sources ($(NH_4)_2SO_4$, $NH_4Cl$, yeast extract, peptone, $NH_4NO_3$, and urea). The pH was adjusted to 7.0 before these nitrogen sources were added to the MSM at a concentration of 0.4%. Following inoculation, the flasks were incubated for 7 days at 35 °C and 150 rpm in a shaker incubator. The optimum nitrogen source was chosen and used subsequently. The emulsification index was calculated.

### 3.7.3.6 Effect of nitrogen source concentration:

The optimum nitrogen source ($(NH_4)_2SO_4$) was progressively introduced to the MSM at concentrations of 0.1%, 0.2%, 0.3%, 0.4%, and 0.6%. After adjusting the pH to 7.0, the bacterium isolate was inoculated, and the mixture was shaken at 150 rpm for seven days at 35 °C. The best concentration was then used. emulsification index was calculated.

### 3.7.3.7 Effect of incubation period:

Bacterial isolates were incubated at different incubation period (1–8) days in MSM pH 7, at 35°C ,150 rpm .Ei24% was calculated to determine the optimum incubation period for these isolates.

### 3.7.4 Extraction of biosurfactant compound:

One litter of a mineral salt medium (pH 7) containing crude oil (1%) as a sole carbon source and ammonium sulphate (0.4%) as a nitrogen source was incubated in a shaker incubator (150 rpm) at 35 °C for seven days after being inoculated with 1% fresh cultures of *K. kristinae* and *D. acidovorans*. After incubation, supernatant of bacterial culture was obtained by centrifugation at 10000 rpm for 15 min at 4 °C. The supernatant subjected to extraction with different methods as follows:

1- To treat crude biosurfactant, pH 2.0 was achieved using 2 N HCL, and the acidified supernatant was then left overnight at 4°C to allow the biosurfactant to completely precipitate. Samples that precipitated in acid were centrifuged, and the pellets that were produced serve as the unpurified biosurfactant for these samples.

2- A second extraction technique involved extracting a crude biosurfactant sample three times in equal amounts of ethyl acetate and chloroform/methanol (2:1). A rotary evaporator was used to evaporate the organic solvent, and the resulting residue served as the unpurified biosurfactant (Saravanan and Vijayakumar,2015).

### 3.7.5 Purification of biosurfactant compound:

Purification and analysis of biosurfactant material was carried out by using high-performance liquid chromatography (HPLC) system (Knauer Germany system component) equipped with a Binary high pressure gradient pump, sample loop , and Diode array detector Table (3-4). The separation was achieved on C18 protein column and peptide (pore size 300A°,length 250mm ,diameter 10 mm, particle size 5 µm ) ( vydac corporation, USA) was used for biosurfactant purification as described earlier with

modification. Preparative -HPLC was run by injecting , 300 µl of extracted sample of biosurfactant injected into column, purification of biosurfactant attempted by using mobile phase (gradient of mobile A (0.05% TFA in HPLC grade water) and mobile B (Acetonitrile) the gradient profile is listed in Table (3-5) and Figure (3-1).The flow rate was maintained at 3 ml/min,the fraction collector parameters are set to Peak recognition : level 10 mAU , slope 0.2 Au/min

Fraction size : 5 ml. Biosurfactant peak was detected by diode array detector at 214 nm at semi-preparative scale. each peaks produced by HPLC was collected, concentrated by evaporation at 40 °C and dried,, and used for further analysis.

**Table 3-4 : Component Model or version Company and origin for HPLC**

| No. | **Component** | **Model or version** | **Company and origin** |
|---|---|---|---|
| 5 | **Analyses and system control software** | **Claritychrom , V 7.4.2.107** | **Dataapex, Czech Republic** |
| 1 | **Binary high pressure gradient pump** | **P6.1L** | **Knuaer , Germany** |
| 2 | **Diode array detector** | **DAD 2.1L** | **Knuaer , Germany** |
| 4 | **Fraction collector** | **Foxy R1** | **Teledyne Isco, USA** |
| 3 | **Sample loop (200 µl) and injector** | **D1357** | **Knuaer , Germany** |

**Table 3-5 : The mobile phase gradient profile of preparative HPLC**

| Time | Mobile A Concentration % | Mobile B Concentration % | Flow rate ml/ min |
|---|---|---|---|
| 0 | **90** | **10** | **3** |
| 5 | **90** | **10** | **3** |
| 15 | **50** | **50** | **3** |
| 16 | **0** | **100** | **3** |
| 25 | **0** | **100** | **3** |

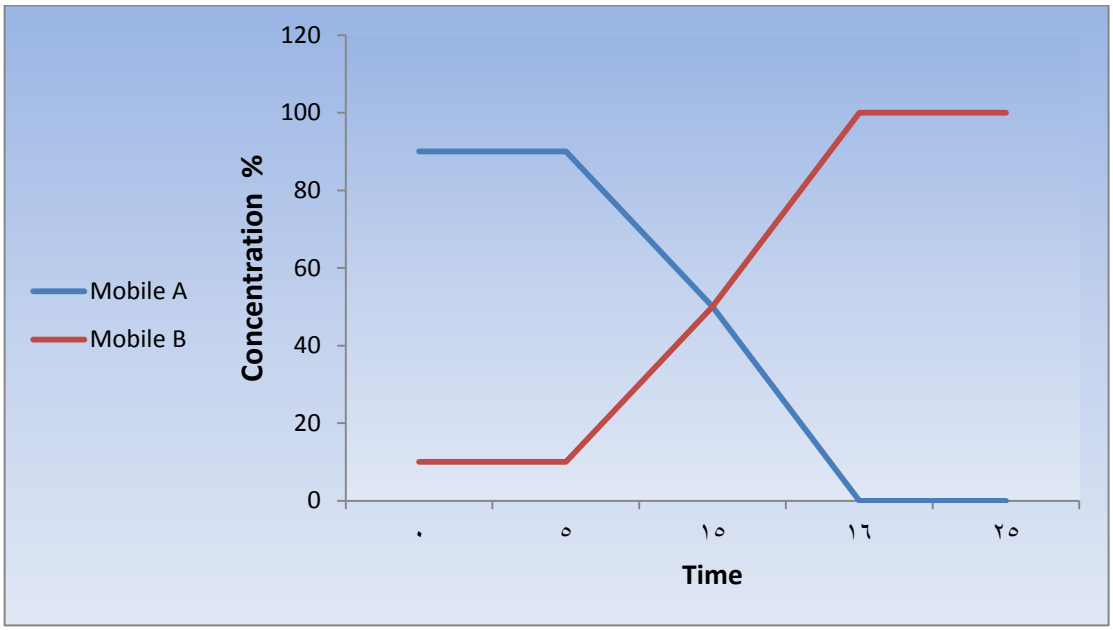



**Figure 3-1 : The mobile phase gradient profile of preparative HPLC**

## 3.7.6 Chemical composition of biosurfactant compounds

## 3.7.6.1Determination of protein concentration by using the following solutions and buffers

### 3.7.6.1.1 Bovine serum albumin (mg /ml) (stock solution)

It was prepare by combining 10 mg of BSA with 100 ml of distilled water, and it was kept in the refrigerator until it was needed.

### 3.7.6.1.2 Coomassie Brilliant blue G-250 (Protein dye reagent)

One liter of distilled water was added after 100 ml of 85% phosphoric acid and 100 mg of Coomassie Brilliant blue G-250 ($C_{47}H_{48}N_3NaO_7S_2$) had been dissolved in 50 ml of 95% ethanol.

## 3.7.7 Determination of lipid concentration by using the following solutions and buffers:

The following solutions were used for estimation the total lipid (Kaufmann and Brown, 2008):

1- Sulfuric acid (98 %).

2- Vanillin – phosphoric acid reagent :

It was prepared by dissolving 600 mg vanillin in 100 ml deionized hot water, then 400 ml of 85 % phosphoric acid was added and stored in dark.

## 3.7.8 Determination of carbohydrates by using the following solutions and buffers:

The following solutions were used for estimation of total carbohydrate by phenol – sulfuric acid method (Dubois *et al.*, 1956):

1- Stock solution of glucose:

This solution was prepared by dissolving 100 mg of glucose in 100 ml of sterile D.W., to obtain a final concentration of 1000 μg/ml. Different

concentrations of glucose (0-1000 µg/ml) were prepared to obtain the standard curve of glucose. Table (3-6).

2- Phenol solution (5 %):

A weight of (5) gm of phenol crystal was dissolved in 10 ml of D.W., the volume was completed to 100 ml.

- Sulfuric acid (98 %).

## 3.7.9Estimation of the protein concentration:

Based on the bovine serum albumin (BSA) standard curve, the Bradford method was employed to estimate the protein concentration of a partially purified biosurfactant (Bradford, 1976).

### 3.7.9.1Preparation of standard curve

The standard protein (BSA) was prepared as in (3.7.6.1.1),and numerous concentrations of protein (BSA) were prepared in duplicate as per the Table (3-6). Next, 100 µl of the protein concentration was mixed with 2.5 ml of coomassie brilliant blue G-250, which was prepared as in (3.7.6.1.2). The combination was left at room temperature for 5 minutes, after which the absorbance at 595 nm was measured. The blank was made by combining 2.5 ml of Coomassie G-250. The standard curve was plotted between the protein amount and the corresponding absorbance of the standard protein as show in Figure (3-2).

**Table 3-6: Volume used in preparing standard curve of (BSA)**

| Tube number | Volume of stock solution (BSA)(µl) | Volume of DW.(µl) | Protein concentration (mg) |
|---|---|---|---|
| 1 | **0** | **100** | **0** |
| 2 | **20** | **80** | **5** |
| 3 | **40** | **60** | **10** |
| 4 | **60** | **40** | **15** |
| 5 | **80** | **20** | **20** |
| 6 | **100** | **0** | **25** |

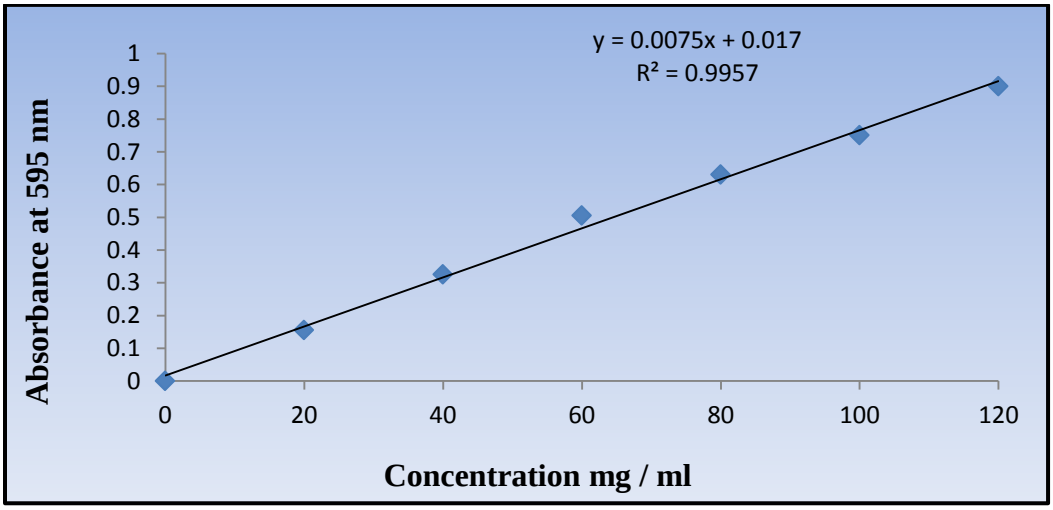



**Figure (3-2): Standard curve of BSA concentration (Bradford method, 1976).**

## 3.7.10Estimation of the lipid concentration

The lipid concentration of partial purified biosurfactant was determined according to Kaufmann and Brown (2008) as follows:

1- One mg of commercial vegetable oil was dissolved in 1 ml of chloroform (1000µg/ml).
2- The standard curve was prepared by preparing a serial amount of lipid (50-600µg).
3- The previous solution was added as the following 50, 100, 200, 400 and 600 µl separately to each glass tube in triplicate.
4- These glass tubes were placed in a heating block at 90-110 ºC to evaporate the solvent.
5- Two hundred microliter of sulfuric acid (98%) was added to each tube separately and heated for 10 min at 90-110 ºC.
6- Vanillin reagent was added to 5 ml level and mixed.
7- The tubes were removed from heating block and allowed to cool.
8- The absorbance at 625 nm was measured.
9- The lipid amount of unknown sample was treated in the same manner by dissolving 1 mg of biosurfactant in 1 ml of chloroform with mixing and the lipid was determined according to the standard curve (Figure 3-3).

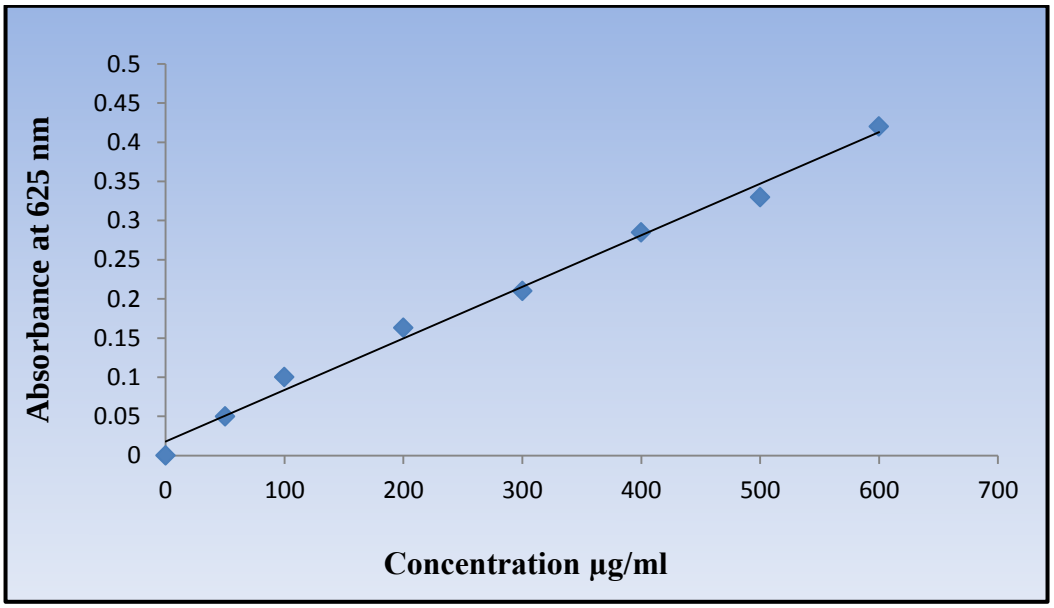



**Figure 3-3: Standard curve of lipid determining by Kaufmann and Brown (2008) method.**

## 3.7.11Estimation of carbohydrate concentration

The carbohydrate content of the partially purified biosurfactant sample was determined by the preparation of glucose standard curve (Dubois *et al*., 1956) as follow:

1- Different concentrations ranged from (0-1000 µg /ml) were prepared from glucose stock solution . The final volume was 1 ml, as shown in Table (3-7).

2- One ml of phenol solution was added to each tube with shaking.

3- Five ml of concentrated sulphuric acid (98%) was added to the mixture, mixed well and left to cool at room temperature.

**Tube (1*) represents the blank solution.**

- The absorbency (O.D) was measured for each tube at 490 nm.
- The curve of glucose was demonstrated the relationship between absorbency (O.D) and glucose concentration as shown in the Figure (3-4).

- The unknown samples used for carbohydrate estimation were prepared by dissolving 1mg of biosurfactant powder in 1ml of Tris – HCl with mixing with a magnetic stirrer.

**Table (3- 7): Preparation of different glucose concentrations**

| Tube No. | volume of Sugar solution(ml) | Volume of D.W (ml) | Final concentrations ( µg /ml ) |
|---|---|---|---|
| 1* | 0 | 1 | 0 |
| 2 | 0.1 | 0.9 | 100 |
| 3 | 0.2 | 0.8 | 200 |
| 4 | 0.3 | 0.7 | 300 |
| 5 | 0.4 | 0.6 | 400 |
| 6 | 0.5 | 0.5 | 500 |
| 7 | 0.6 | 0.4 | 600 |
| 8 | 0.7 | 0.3 | 700 |
| 9 | 0.8 | 0.2 | 800 |
| 10 | 0.9 | 0.1 | 900 |
| 11 | 1 | 0 | 1000 |

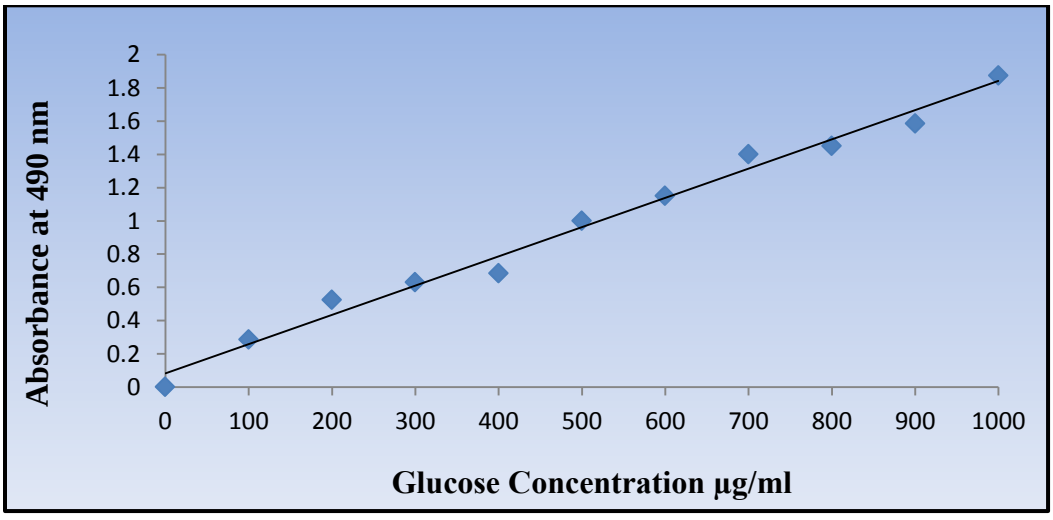



**Figure (3-4): Standard curve of glucose by Dubois *et al*. method for glucose concentration.**

## 3.8 Polyacrylamide gel electrophoresis of purified biosurfactant

According to their electrophoretic mobility, biological macromolecules—typically proteins or nucleic acids—are separated by electrophoresis. It was demonstrated that a gel created by cross-linking two organic monomers, acrylamide and N, N, N, N- methylene bis acrylamide, is an appropriate medium for electrophoresis .When separating proteins using gel electrophoresis, solubility denaturing agents like sodium dodecyl sulphate (SDS) are frequently utilized (Laemmli, 1970). SDS causes protein denaturation and has a strong affinity for proteins**.** Regardless of charge, the proteins separated by SDS page move in accordance with their molecular weight. By varying the cross-linking agent concentration, polyacrylamide gel (PAGE) can have varied pore sizes. Electrophoresis by (PAG) is dependent on both electrophoretic mobility and molecular weight (Magdeldin, 2012).

### 3.8.1Preparation of solutions

### 3.8.1.1 Acrylamide – bisacrylamide

It was made by continuously dissolving 29.2 g of acrylamide and 0.8 g of bis-acrylamide in 80 ml of D.W. The final volume was then adjusted to 100 ml by D.W. and stored at 4 °C in a dark bottle.

### 3.8.1.2 Stacking gel buffer (pH= 6.8)

It was produced by dissolving 12.1 g of Tris-HCl(1M) in 80 ml of deionized water, bringing the pH down to 6.8 using HCL, and then adding deionized water to get the volume up to 100 ml.

### 3.8.1.3 Resolving gel buffer (pH= 8.8)

It was created by dissolving 18.15 g of Tris-base in 80 ml of D.W., and then increasing the amount to 100 ml of D.W. after adjusting the pH to 8.8 using HCL.

### 3. 8.1.4 Laemmli buffer (2X)

This solution was created by dissolving 1.52 g of Tris-base in 50 ml of D.W, adding 20 ml of glycerol, and adjusting the pH to 6.8 before adding another 10 ml of D.W to make a total volume of 100 ml.

### 3.8.1.5 Running buffer

In 500 ml of D.W., SDS(10%) 5ml, 1.5 g of Tris and 9.4 g of glycine were dissolved to make it.

### 3.8.1.6 Ammonium persulphate solution (10%)

It was freshly made by combining 1 ml of D.W. with 0.1 g of ammonium persulphate, and put to use that same day.

### 3.8.1.7 Bromophenol Blue (0.2%)

A final volume of 10 ml of sample buffer was added to which 0.2 g of bromophenol blue was dissolved.

## 3.9Procedure

### 3.9.1 Preparation of the gel

The stacking gel (5%) and resolving gel (12%) were prepared according (Sambrook and Russell,2006)**.**

### 3.9.1.1 Preparation of Resolving gel (12%, Lower layer, Volume 10 ml):

This gel was prepared by taken 3.3 ml of $H_2O$, 4 ml Acrylamide-Bisacrylamide, 2.5 ml of Tris (1.5M ,pH 8.8), 0.055 ml of ammonium persulphate, and added 0,002 ml of TEMED. Swirl the mixture rapidly. After that, pour the solution into the gap between the glass plates and leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1cm),added $H_2O$ and leave the gel to solidify for 30 minutes. After this time the gel was dried by using paper towel to remove any remaining $H_2O$.

### 3.9.1.2 Preparation of stacking gel (5%, upper layer, Volume 2ml):

This gel was prepared by taken 1.4 ml of $H_2O$, 0.330 ml Acrylamide-Bisacrylamide, 0.25 ml of Tris-Cl (1M ,pH 6.8), 0.020 ml of ammonium persulphate, and added 0,002 ml of TEMED. Swirl the mixture rapidly. After that, Pour the solution directly onto the surface of the polymerized resolving gel. Avoid trapping air bubbles. Put the comb and leave the gel to solidify for two hours. After this time, the comb was raised and wash the well by running buffer.

### 3.9.1.3 Preparation of Sample

This sample was prepared by taken 20µl from sample with equal volume from (Laemmli buffer), added 2 µl of mercaptoethanol, and 10 µl of Bromophenol blue. This mixture was heated to 100°C for 5 minutes to denature the proteins. Attach the electrophoresis apparatus (60 volt, 50mA) to an electric power supply. After the dye front has moved into the resolving gel, increase the voltage until the dye (Bromophenol blue) reaches the

bottom of the resolving gel, then turn off the power supply. Finally remove the glass plates and place them on paper towel

### 3.9.1.4 Gel staining

**Step A: preparation of solution**

This step was made by taken 25 ml of methanol, 5 ml of acetic acid and volume was completed to 50 ml of DD.W and put onto the gel for 30 minutes**.** After this time, this solution was removed and complete other step.

**Step B:** This step was made by taken 2.5 ml of methanol for and complete volume to 50 ml and put on gel, shaking for 15 minutes.This solution was washed by DD.w 3 times, each time for 5 minutes. This solution was removed and complete other step.

**Step C:** This step was prepared by taken 0.1 gm of thiosulphate sodium ($Na_2S_2O_3$) must be fresh and cold, volume was completed to 50 ml with DD.W, and incubated for 40 minutes.

This solution was washed 3 times by DD.W, each time for 1 minutes. After that, this solution was removed.

**Step D:** In this step, solution was prepared by taken 0.1 gm of silver nitrate (cold and fresh), and volume was completed to 50 ml of DD.W. Incubated for 40 minutes, and this solution was washed 3 times, each time for 1 minutes.

**Step E:**This step was made by taken sodium carbonate ($Na_2CO_3$) 1.5 gm and added formaldehyde (37%) 25µl with sodium thiosulphate 1ml and complete volume to 50 ml, this solution was added to gel until appear bands.

**Step F:** 7 gm of $Na_2EDTA$ was taken and added to 50 ml of D.W, after that washed by DD.W.

Finally; The gel was put on the white lid light for take photo.

## 3.10Characterization of biosurfactant compound

### 3.10.1Analysis with Fourier Transform Infra-Red (FTIR)

Fourier Transform Infrared (FTIR) was used to qualitatively examine the basic functional groups of the partially purified biosurfactant from *K.kristinae and D.acidovorans* .Using a (BRUKER) spectrophotometer, the biosurfactant sample's Fourier transform infrared spectroscopy was acquired after the sample's 2 mg of partial purification was combined with 150 mg of KBr and compressed into a tablet form in a dry environment. With a resolution of 2 cm and 50 scans, the FT-IR spectra were measured in the 400–4000 cm' frequency range (Özer *et al.*,2016).

## 3.11 Molecular study

### 3.11.1 Detection of target genes responsible for contaminant Biodegradation.

### 3.11.1.1DNA extraction and purification

Bacterial isolates were extracted according to Cheng and Jiang, 2006 ,this method was described as follow:

1- One ml of cultured cell was taken.
2- Centrifuge was made of 10000rpm for 2 minutes.
3- The pellet of bacteria was put in 200µl of TE buffer.
4- A 100 µl of phenol that saturated with Tris PH=8 was added.
5- Vortex was made for 60 second.
6- Centrifugation was made for 10000rpm for 5 minute to remove residues.

7- A 160 µl of upper layer was taken and transferred to new tube.

8- Amount of 40 µl of TE buffer and 100 µl of chloroform were added to tube and mix well.

9- Centrifugation at 10000rpm for 5 minute and supernatant was taken.

10- Step 8 and 9 was repeated until disappear of white color (chloroform in tube was found in lower layer, water and DNA was found in upper layer. 160 µl of supernatant was transferred to new tube.

11- Amount of 40 µl of TE buffer was added, in addition, 5 µl of RNase was added and mixture was incubated at 37°C for 15 minutes.

12- A 100 µl of chloroform was added, mix well and centrifuge 10000 for 5 minutes.

13- A150 µl was taken and transferred to new tube (represent purified DNA).

### 3.11.1.2 Determination of purity and concentration of DNA.

The DNA amount and quality were determined using nano-drops and a scanning diode array from 200 to 320 nm wave length. The absorbance profile was then processed and evaluated to calculate the 260/280 and 260/230 ratios to estimate the DNA amount and quality. The sample is re-extracted if the 260/280 ratio is less than 1.8.

### 3.11.1.3 Molecular Weight and Integrity Estimation of DNA

The M.wt of DNA extracted and the integrity of extracting DNA was determined-by 0.8% agarose gel-electrophoresis, the setting device at 100 volts for 60 minutes. The electrophoresis was carried out as described below in abbreviated. Table (3-8).

### 3.11.1.4 Material and solutions used in gel electrophoresis

**Table 3-8:Solutions used in gel electrophoresis**

| No. | Name of material | Supplied Company |
|---|---|---|
| 1- | **TBE (Tris – borate EDTA)** | **Thomas baker Company, stored at 25˚C until used.** |
| 2- | **Biosafety eco dye** | **Intron Company** |
| 3- | **DNA loading buffer** | **Promega Company.** |
| 4- | **DNA ladder** | **Cyntol Company.** |
| 5- | **Agarose** | **Pronadisa Company.** |

### 3.11.1.5 Agarose gel electrophoresis for extracted DNA

1. The gel was prepared at a concentration of 1% by dissolving 1gm of agarose in 100 ml a buffer solution TBE 1X and then heated the mixture for 45 seconds in the microwave.
2. The homogeneous solution of agarose left until its temperature reaches 55°C, then add 2 µl of its Safety dye added to it and mix with the mixture by turning the beaker.
3. The homogeneous mixture of agarose is poured into the gel tray and left to polymerize for 30 minutes.
4. After hardening, the agarose is transferred to an electrophoresis device and immersed in a TBE running buffer at a concentration of 0.5X.
5. Five µl of DNA extraction was combined with 2 µl of loading-dye and carefully loaded by a mechanical pipette into the wells of the gel.
6. The electrophoresis was set to 100 volts for one hour and switched on.

7. After completing the electrophoresis, the gel was imaged and the image was analyzed in order to determine the molecular weights of the DNA segments.

## 3.11.2PCR technique

### 3.11.2.1 Specific primer used in this study are illustrated in Table (3-9):

**Table 3-9: Primers used in this study**

| Name of gene | | Sequence of Primers (F and R) | Size | References |
|---|---|---|---|---|
| Protocatechuate 3,4-dioxygenase (P340) | P34OF<br>P34OR | 5-CTCACGCAGCACGACATCGACCT-3<br>5-CCGGGCGCGACTGTCGATCGTGGT-3 | 800 | Spring *et al.*,(2014) |
| Catechol 2,3-dioxygenase (C23O) | C230F<br>C230R | 5-AAGAGGCATGGGGGCGCACCGGTTCGATCA-3<br>5-CCAGCAAACACCTCGTTGCGGTTGCC-3 | 450 | Spring *et al.*,(2014) |
| Ring-hydroxylating dioxygenase (Nid A) | NidAF<br>NidAR | 5-ATGACCACCGAAACAACCGGAACAGC-3<br>5-TCAAGCACGCCCGCCGAATGCGGGAG-3 | 1400 | Barbara *et al.*,(2003) |
| nahAc F<br>nahAc R | | 5-TGGCGATGAAGAACTTTTCC-3<br>5-AACGTACGCTGAACCGAGTC-3 | 487 | Laurie and Jones,(2000) |
| Catechol dioxygenase | C12OF<br>C12OR | 5-GCCAACGTCGACGTCTGGCAGCA-3<br>5-CGCCTTCAAAGTTGATCTGCGTGGTTGGT-3 | 350 | Sei *et al.*,(1999) |
| Catechol dioxygenase | C23OF<br>C23OR | 5-AAGAGGCATGGGGGCGCACCGGTTCGA-3<br>5-TCACCAGCAAACACCTCGTTGCGGTTGCC-3 | 900 | Sei *et al.*,(1999) |
| Cat1-2F<br>Cat1-2R | | 5-AAACCCGCGCTTCAAGCAGAT-3<br>5-AAGTGGATCTGCGTGGTCAGG-3 | 650 | Marta *et al.*, (2006) |
| sMO_F<br>sMO_R | | 5-GTCCGTTGCAGATCGGACTGCGTA-3<br>5—CAGCACTGCATTGATCCGGTG-3 | 374 | Tanghe *et al.*, (1999) |

### 3.11.2.2 Polymerase chain reaction method

Using the exact primer shown in the table, this technique amplifies DNA . These techniques rely on the volumes found in the Master Mix provided by the Cyntol Company; the volumes are listed in the Table (3-10).

**Table 3-10: Volumes of chemical material that used in PCR reaction**

| Chemical material | Concentration | Volumes |
|---|---|---|
| Master Mix | 2.5X | 8µl |
| Primer forward | 10Pmol/ml | 1µl |
| Primer reverse | 10Pmol | 1µl |
| DNA | 10-20ng/ µl | 2µl |
| Nuclease- free water | | 7.5µl |
| $Mgcl_2$ | 25mM | 0.5 µl |
| Total volume | | 20 µl |

After that, transfer the tubes to PCR apparatus, adjusted the temperature and time according to Table (3-11):-

**Table 3-11:Thermocycling Program of PCR technique**

| No.steps | Steps | Temperature | Time | No.of cycle |
|---|---|---|---|---|
| 1 | Initial denaturation | 95˚C | 5 minute | 1 |
| 2 | Denaturation | 95˚C | 30 second | 35 cycle |
| 3 | Annealing | 58 ˚C | 30 second | |
| 4 | Elongation | 72 ˚C | 30 seconds | |
| 5 | Final extension | 72 ˚C | 5 minute | 1 |
| 6 | Storage | 4 | Hold | |

### 3.11.2.3 Gel electrophoresis of PCR products

It applied the same technique as in (3.11.1.5), but it employed 1.5% concentration of agarose gel instead of 1%.

### 3.11.3DNA sequencing

Primers and volumes of chemical material were used in DNA sequencing illustrated in Table (3-12) and Table (3-13) respectively:

**Table 3-12: Primers used in DNA sequencing**

| Sequence of primers | size | References |
|---|---|---|
| 5′- CTACGGGGGGCAGCAG-3′<br>5′-GGACTACCGGGGTATCT-3′ | 465bp | (Mori *et al.*,2014) |
| 5′ -GTTTGATCCTGGCTCAG-3′<br>5′-AAGGAGGTGATCCAGCC-3′ | 1500bp | (Kane *et al.*,1993) |

**Table 3-13: Volumes of chemical material that used in reaction for DNA sequencing**

| Chemical material | Concentration | Volumes |
|---|---|---|
| Master Mix | 2.5X | 10µl |
| Primer forward | 10 Pmol/ml | 1µl |
| Primer reverse | 10 Pmol | 1µl |
| DNA | 10-20ng/ µl | 1µl |
| Nuclease- free water | | 11.5µl |
| $Mgcl_2$ | 25mM | 0.5 µl |
| Total volume | | 25 µl |

The PCR thermo cycling was optimized by using different annealing temperature (55-60°C), also different cycles to acquire the most specific and efficient PCR product. The Table below (3-14) represent the final optimized thermo cycling that employ to amplify ribosomal rRNA gene region(16S rRNA).

**Table 3-14: Program of PCR technique for DNA sequencing**

| No.steps | Steps | Temperature | Time | No.of cycle |
|---|---|---|---|---|
| 1 | Initial denaturation | 95˚C | 5 minute | 1 |
| 2 | Denaturation | 95˚C | 30 second | 30 cycle |
| 3 | Annealing | 60 ˚C | 30 second | |
| 4 | Elongation | 72 ˚C | 60 seconds | |
| 5 | Final extension | 72 ˚C | 5 minute | 1 |
| 6 | Storage | 4 | Hold | |

## 3.11.3.1 Methods for DNA Sequencing of PCR results:

In this work, two distinct PCR fragments with various lengths (465 bp and 1500 bp, respectively) were chosen, each of which partially covered two different regions of the 16S rRNA sequences. To evaluate the pattern of genetic polymorphism in the bacterial samples that were obtained, the amplified fragments were directly subjected to direct sequencing tests. In order to evaluate the accuracy of the observed variations' identification and their phylogenetic distribution, unique comprehensive trees were created. Following the sequencing company's instructions, the resolved PCR

amplicons were commercially sequenced in both (forward and reverse) directions (Macrogen Inc. Geumchen, Seoul, South Korea). To confirm that the annotation and variations are not the result of PCR or sequencing artifacts, additional analysis was only performed on clear chromatographs acquired from ABI (Applied Bio system) sequence files. The virtual locations and other information of the obtained PCR fragments were determined by comparing the observed nucleic acid sequences of local samples with the retrieved nucleic acid sequences.

### 3.11.3.2 Interpretation of sequencing data

Using Bio Edit Sequence Alignment Editor Software Version 7.1, the sequencing results of the PCR products of the targeted samples were edited, aligned, and assessed along with the corresponding sequences in the reference database (DNASTAR, Madison, WI, USA). Each sequenced sample's detected changes were given a number in both its matching position in the reference genome and its PCR amplicons. The detected nucleic acids were assigned numbers in both their respective places in the reference genome and in PCR amplicons. Snap Gene Viewer ver. 4.0.4 (https://www.snapgene.com) annotated each variant found in the bacterial sequences.

### 3.11.3.3 Comprehensive phylogenetic tree construction

In this work, a specific comprehensive tree was built using the neighbour-joining method outlined by Hashim *et al*., (2020). Using the NCBI-BLASTn server, the detected variations were matched to their neighbor homologous reference sequences (Zhang *et al.*, 2000). Then, using the iTOL suit, a full inclusive tree was constructed using the neighbour-joining approach, incorporating the observed variant (Letunic and Bork,

2019).The sequences of each incorporated species in the comprehensive tree were colored in an appropriate color to be differentiated from the other species. Whereas the sequences color of each species was taken as one unified color.

## 3.12 Immobilization of Microorganisms

The bacterial isolates *K.kristinae* and *D.acidovorans* which previously identified as the more active were used in this study. The isolates were cultured in BHI broth prepared by dissolving 37 g from media in 1 L of D.W, then sterilized in autoclaved at 121°C for 15 minutes. After that, The flasks were inoculated with bacterial isolates and incubated for 24 h at 35 °C on a rotary shaker operated at 150 rpm.

### 3.12.1 Beads Formation:

The concentrated organisms were combined with an equal volume of the 2% sodium alginate soluble solution and vigorously shaken to homogenize the ingredients before being placed in a medical syringe. 100 ml of the studied organisms culture in the log phase had been taken and concentrated by filtering through Millipore filter paper 0.45 µm. The calcium chloride solution (1.0M), which was already made in a different beaker at this point, is gradually filled with the contents of the syringe. After being left in the beaker for 5–10 minutes, a drop of the solution (organisms and sodium alginate) solidifies and becomes immobilized in the form of beads. The calcium chloride solution is then passed through a tea strainer to remove the beads, which are then gently washed with tap water and completely rinsed with distilled water (Adlercreutz and Mattiasson, 1982).

## 3.13 Soil treatment

Soil was used in this experiment sterilized by oven at 160-180°C, after that this soil was contaminated with naphthalene (10 ppm) to test the efficiency of immobilized organisms with sodium alginate in their treatment. This soil was used as control before treatment by immobilized organisms.

A bioreactor was constructed as shown in Figure ( 3-5). The soil sample was placed in the bioreactor and left for 24 hours for each bacteria. The sample was then collected and measured by HPLC and FTIR.

### 3.13.1Measurment By HPLC

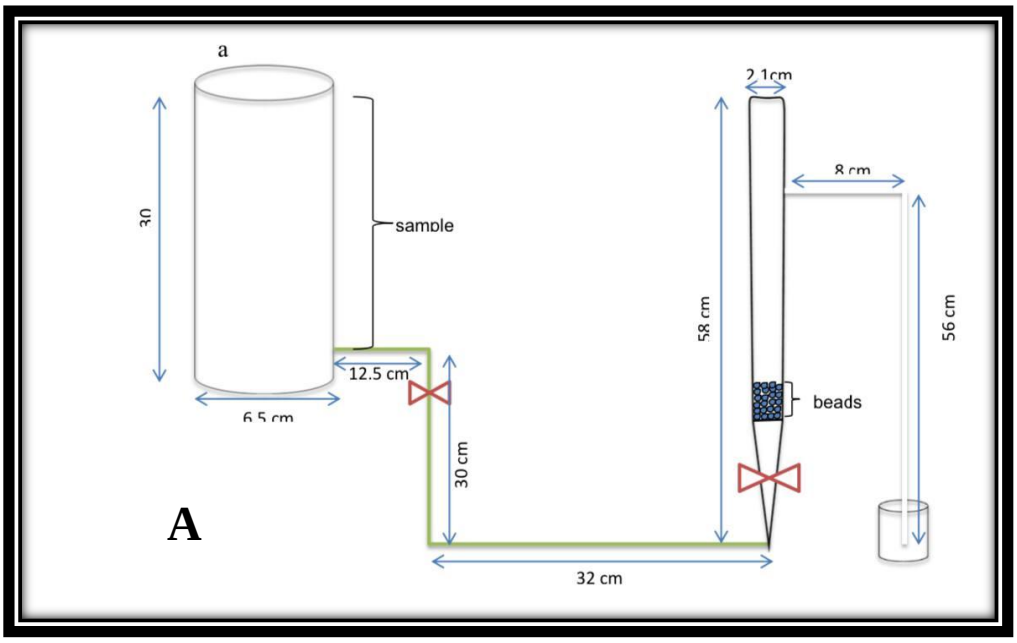
Measurement was carried out by using high-performance liquid chromatography (HPLC) system equipped with a Binary high pressure gradient pump, sample loop , and Diode array detector . The optimized conditions included the use of an HPLC system (SYKAM ). A Supercoil C18 - ODS (25 cm × 4.6 mm, 5 µm) column was used (Supelco). The mobile phase consisted of acetonitrile–water (70:30, v/v). The flow rate was 0.8 mL/min. The analysis was performed at room temperature (~ 30°C). was used as the detector. The wavelength was 220 nm.(Guiherme and Fernandoo,2006).

### 3.13.2 Detection of Beads Efficiency by FT-IR:

By using a mixture of dried samples (one milligram) and KBr (200 mg), FT-IR was used to determine efficiency according to the functional groups that shift, are absent, or emerge. The results were read by the X-axis and Y-axis of the spectrum. The "Wavenumber" axis of an IR spectrum has a number range of 400 on the far right to 4,000 on the far left. The absorption number can be found on the X-axis. The typical peaks in the IR spectrum are represented by the numbers 0 at the bottom and 100 at the top

of the Y-axis, which is designated as "Percent Transmittance." Every IR spectrum has numerous peaks. However, determine the large peaks on the spectrum because they will provide the data necessary to read the spectrum. (Murdock and Wetzel, 2009).

This work done in the laboratories of the Ministry of Science and Technology - Department of Environment and Water.



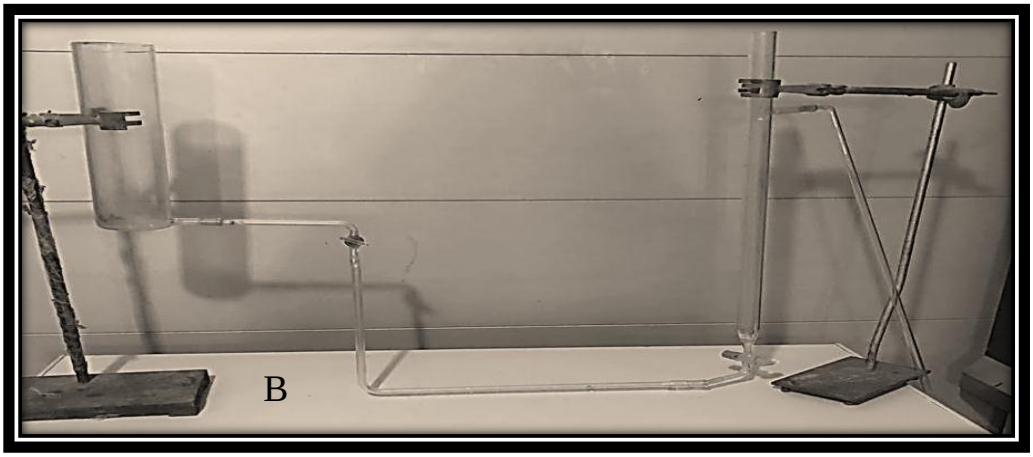




**Figure 3-5 Bioreactor used in this study, A:Scheme of bioreactor , B:Photo of bioreactor**

## 3.14 Development of Consortium of the Selected Bacteria

The 13 well-grown HDB isolates were cultured on MSM broth supplemented with 5% ml of crude oil and inoculated by 1 ml of each isolates. On each chemical, three replicates were used for bacterial consortia. For 18 days, the flasks were cultured at 30°C in an orbital shaker incubator at 150 rpm. Optical density (O.D.) at 600 nm was measured using a spectrophotometer at zero time (initial), 3, 6, 9, 12, 15, and 18 days, as well as pH and plate count.

## 3.15 Screening the consortium for biosurfactant production:

### 3.15.1Biosurfactant production

The 500 ml of MSM was used to test the capacity of bacterial consortiums to create biosurfactant. Following sterilization, 5% of crude oil was added to the flasks as a sole carbon source. The flasks were shaken at 150 rpm for 7 days at 35 °C after being injected with 1 ml of each fresh bacterial growth over the course of 24 hrs. The cultures were then centrifuged for 15 minutes at 4 °C and 10,000 rpm. Investigations on biosurfactant production in cell-free supernatant.

### 3.15.2Determination of biosurfactant compounds:

For determination of biosurfactant compound, the same previous methods were used to determine the biosurfactant production (Hemolysis test, CTAB agar plate method, oil spreading test, Drop collapse test,Emulisification index test, Bacterial adhesion to hydrocarbons, and foaming activity).

## 3.16 Statistical analysis:

In this work, assays were performed in triplicate, and results were described using mean and standard deviation by SPSS program.

## 4.1 Determination of soil properties

A variety of micro and macro creatures, minerals, organic matter, gases, and liquids make up soil, which can support plant life. Effect of bioremediation on chemical parameters such as compost pH has a great effect on the soil. These results were revealed several parameters for properties of soil such as; (Salinity, pH, TDS,TOC,$CaCo_3$, and chloride. The first parameters ,soil pH in this study was neutral, because it directly influence on the availability of nutrients to plants. Optimum range of pH of compost was from 6.5-7.3. The second parameters, the electrical conductivity is an important parameter used to estimate the level of dissolved salts in water and soil. Electrical Conductivity of soil was higher in top layer of soil, the values vary from 544-8350 μS cm-1. Salinity, the third parameters include mineral plant nutrients that occur naturally in soil and water, fertilizers applied and also other dissolved minerals that are not plant nutrients and might even be harmful to plants. While fourth parameters ,TDS is a measure of the dissolved combined content of all inorganic and organic substances present in a liquid in molecular, ionized, or micro-granular (colloidal sol) suspended form. TDS concentrations are often reported in parts per million (ppm). TOC, $CaCo_3$, and chloride were quick, easy, and affordable way to assess the health of soils is to use these parameters. It is a gauge for the ions in a solution, with an increase in ion concentration, a soil solution's E.C rises. These results were showed in Table (4-1). As a broad phrase, "soil" refers to the thin, fluctuating layer of unconsolidated minerals and organic material that covers the majority of the earth's land surface and is typically biologically active. Indicators can be utilized with soil characteristics that are susceptible to management changes. Environmental impact from engine oil contaminating soil was severe (Solly *et al.*, 2015). The fractional composition of oil and petroleum products that fall into the soil determines how the

pH of the soil changes. The results of the trials showed that following re-cultivation, the soil quality improves. The chemical make of the soil affects the pollutant's concentration. Only within a limited pH range can the majority of microbial species live. Additionally, soil pH might impact the nutrients that are available (Agamuthu *et al.,* 2013). The pH of the soil can be raised to neutral (6.6–7.5) depending on the contaminants. Aggregates in soil have been impacted by oil and oil derivatives, such as gasoline, engine oil, and kerosene. However, as the PAH is associated with the carbonaceous particle materials, the PAH contamination in the various granulometric fractions is exactly proportional to the respective total organic carbon concentration (Trellu *et al.,* 2017). Another crucial characteristic of soil is E.C, which is used to assess the soil's quality. It measures the number of ions in a solution. As ion concentration rises, a soil solution's electrical conductivity(Smita and Sangita,2015). Gas chromatography coupled to mass spectrometry (GC-MS) was used to identify and quantify PAHs in sediment samples with the use PAH internal standards and surrogate standards. The same GC-MS settings as the standards were used to separate and quantify PAHs in the sediment samples. On the basis of a five-point calibration curve for each chemical, sixteen PAHs were measured using the response factors associated with the corresponding internal standards. Appendix 1 contained these findings.

**Table 4-1:Estimation of soil parameters**

| Sample number | TOC % | $CaCO_3$ % | Cl mg/L | E.C Conductivity µs/m | Salinity ppm | PH | TDS mg/L |
|---|---|---|---|---|---|---|---|
| 1 | 3.56 | 36 | 980 | 921 | 750 | 6.93 | 642 |
| 2 | 1.36 | 22 | 735 | 955 | 761 | 7.06 | 645 |
| 3 | 2.98 | 11.5 | 490 | 544 | 446 | 6.8 | 371 |
| 4 | 0.68 | 35 | 3920 | 2190 | 1920 | 6.98 | 1200 |
| 5 | 3.88 | 23 | 6860 | 2900 | 2620 | 6.77 | 2070 |
| 6 | 2.53 | 18 | 4410 | 575 | 465 | 6.53 | 379 |
| 7 | 3.79 | 17.5 | 4361 | 1953 | 1700 | 7.07 | 1380 |
| 8 | 3.29 | 30 | 1078 | 853 | 670 | 7.08 | 982.1 |
| 9 | 2.93 | 29.5 | 588 | 829 | 693 | 7.3 | 679.7 |
| 10 | 3.76 | 20.5 | 1029 | 8350 | 834 | 7.3 | 711 |
| 11 | 3.72 | 28.5 | 3038 | 164 | 1450 | 6.94 | 1190 |
| 12 | 3.67 | 23 | 2597 | 1543 | 938 | 7.2 | 796 |
| 13 | 3.54 | 23.5 | 6223 | 2680 | 2400 | 7.35 | 1910 |
| 14 | 0.72 | 24.5 | 1470 | 2110 | 1980 | 7.21 | 1780 |
| 15 | 3.79 | 29.5 | 1225 | 1070 | 890 | 7.05 | 753 |

## 4.2 Isolation and Identification of Bacterial isolates

A total of 13bacterial isolates were selected from 56 oil-contaminated soils based on their ability to use crude oil as their a sole carbon source. The identification of these isolates were accomplished on several culture media (Nutrient agar, Blood agar, MacConkey agar, and, Chromogenic agar).The results revealed that there were 7 Gram negative bacteria (*Acinetobacter baumanni, Pseudomonas putida, Delftia acidovorans, Enterobacter cloacae, Pseudomonas aeruginosa, Pseudomonas stutzeri, and Pandoraea spp*.) and 6 Gram positive bacteria (*Micrococcus luteus, Staphylococcus haemolyticus, Staphylococcus warneri, Staphylococcus lentus, Staphylococcus lugdunebsis and Kocuria kristinae)*. Organic substances that have contaminated the environment can enter the cytoplasm membrane and disrupt physiological membrane functioning. To

withstand the harm caused by toxic chemicals pollutants and to prevent toxic compounds from accumulating in the cell, bacteria had to adopt specific mechanisms. Cell adaptation regulates membrane fluidity, the ratio of non-bilayer to bilayer phospholipids, as well as the efflux of toxic substances, protein repair mechanisms, and the breakdown of harmful pollutants. Other physiological processes demand less energy than cells that have evolved to adapt. Bacteria that can thrive in a hazardous environment could be used to clean up contaminated regions as a bioremediation agent (Murinova and Dercova, 2014). Based on the ability of bacterial isolates exhibited in degrading hydrocarbons, it reveals that petroleum microbial degradation is a natural process involving microorganisms that may transform and degrade petroleum hydrocarbons into other simpler components (Xenia and Refugio, 2016). Bacteria's transformation and degradation technique demonstrates the breakdown of n-alkane compounds. To break down the n-alkane hydrocarbon molecule, microorganisms use a multi-complex enzyme monooxygenase (-hydroxylase) system. This system can oxidize alkanes into primary alcohols. The main alcohols are subsequently converted to aldehyde molecules, which are then converted to fatty acids. Through the a-oxidation process, the released fatty acids can be directly broken down into CO2 or used as nutrients (carbon supply and energy) for cell growth through the -oxidation process (Holst *et al.*, 2007; Das and Chandran, 2011). In order to metabolize and multiply, bacteria need carbon molecules as a source of nutrients and energy. Hydrocarbonoclastic microorganisms, in particular, are microorganisms that can use carbon sources produced from hydrocarbons (Harayama *et al.*, 1999).

## 4.3 Determining of PAHs compounds biodegradation activity

In order to analyze and compare alternative PAH degradation pathways, the current study aims to look out at the possible degrading capacity of G+ve and G-ve isolates in the presence of PAH. Three parameters were studied to determine the activity of isolates for degrading of PAHs. The first portion of this study focuses on an experiment employing optical density and PH, while the third section evaluates plate count for 13 isolates. *K.kristinae ,S.warneri, S. haemolyticus, M. luteus, S. lugdunebsis, and S.lentus* gave the best growth and increased after 18 days at optical density 600 nm, which showed different significant (0.99±0.01, 0.97±0.03, 0.90±0.02, 0.89±0.01, 0.66±0.01, and 0.66±0.01) respectively for mentioned isolates. The various pH values revealed that the pH in the control flasks stayed nearly constant (7). The pH of the flasks containing oil decreased after 3 days and continued to decreased after the last day of incubation (18 days) that given different significant (3.0±0.1, 3.61±0.02, 4.40±0.24.17±0.06) for *K.kristinae ,S.warneri, M.luteus, and S.lentus*. Other isolates *S.haemolyticus, and S.lugdunebsis* were showed not significant after 18 days of incubation (3.80±0.10,*and* 4.13±0.06*),*respectively. After 3 days of incubation, the growth of the bacterial community increased, and after 18 days of incubation, it reached a peak, then began to decrease at 21 days to evaluate plate count of Gram-positive bacteria, which revealed *K.kristinae* had the highest count and , followed by *S.warneri, M.luteus, S.lugdunebsis, and S.lentus* respectively (222.0±1.4, 200.7±0.5, 167.00±1.0, 128.0±1.0, and 118.7±1.2), respectively, these results were conducted in (Figure 4-1) Table (4-2).

Gram negative bacteria ,*D.acidovorans, P.stutzeri, P.aeruginosa, E.cloacae, P.putida, Pa.spp, and Ac.baumanni* were given the best growth and the

growth increased with different significant after 18 days at optical density 600nm (0.89±0.01, 0.86±0.02, 0.80±0.02, 0.77±0.01, 0.76±0.02,0.71±0.04,and 0.61±0.01),respectively. The multiple pH values revealed that the pH in the control flasks stayed nearly constant (7). The pH of oil-containing flasks decreased after 3 days and continues to drop after the last day of incubation (18 days) which showed different significant for *D.acidovorans and E.cloacae* (4.10±0.02 and 4.20±0.01),respectively for these isolates. While other isolates *Ps.stutzeri*, *Ps.aeruginosa, Ps.putida, Pa. sp.* and *Ac.baumanni*, were given not significant. The growth of Gram-negative bacteria increased after 3 Incubation period days and continued to increase after 18 days of incubation, giving the best results on day 18 of incubation, Then, after 21 days, it started to go down. to evaluate plate count of Gram-negative bacteria that showed optimal growth and different significant of *D.acidovorans,* followed by *Ps.stutzeri, Ps.aeruginosa, E.cloacae, Ps.putida, Pa.spp, and Ac.baumanni,* respectively (221.0±4.2, 213.67±0.5, 201.0±1.2, 194.3±0.6, 156.33±2.6, 145.3±0.6, and 132.3±1.9), these results were showed in Figure (4-2) and Table (4-2).

In mangroves, (Brito *et al.*, 2006) found and confirmed a wide range of bacteria that destroy PAHs. According to the study, fluoranthene and pyrene are degraded by *Marinobacter* and *Pseudo* species, octane is decomposed by *Alcanivorax* species, fluoranthene and naphthalene are degraded by *Microbulbifer* species, and pristane is degraded by *Sphingomonas* species. Baker (1994) showed that biodegradation occurred more rapidly at neutral pH levels. According to numerous research, the digestion of some pollutants is hampered in groundwater and certain waters with an acidic pH. (pH 4.87). The acidic metabolites generated by PAH decomposition (such as phenanthrene dihydrodiol, dihydroxy

phenanthrene, phthalic acid, dihydroxy pyrene, lactone, and others) are most likely to be responsible for the pH decline. pH decreased due to production of organic acids according to activity of bacterial isolates to biodegradation of PAHs. The rate of hydrocarbon breakdown in contaminated mangrove ecosystems is influenced by environmental conditions.

Nutrients are required in addition to temperature, pH, and the presence of PAH degraders. Nutrients encourage bacterial growth, which has a direct impact on hydrocarbon breakdown. The release of particles and ions caused by PAH breakdown was most likely the source of this decline. The pH reduction is most likely due to acid metabolites produced by the breakdown of different PAHs in the environment. In this type of complex environment, the pH could drop due to the biodegradation of other molecules in the sediments, such as organic compounds. (Mori *et al.*,2011) used two biodegradation models to investigate the most effective hydrocarbon degradation pathway in mangrove sediments (assisted phytoremediation with Rhizophora mangle and simply bioremediation). Although the ability to break down PAHs varies among bacterial strains from different genera isolated from PAH-contaminated soils, the *Pseudomonas* and *Rhodococcus* genera are the most commonly employed bacteria in bioremediation.

Mangrove soils, as according (Ramsay *et al.,* 2000), have a very active micro flora capable of digesting PAHs. Microorganisms need time to adjust and acclimate to poisons in intentionally contaminated environments before they can break them down. Microorganisms such as *Rhodococcus spp.*, *Bacillus spp.,* and *Pseudomonas spp* were discovered in diverse hydrocarbon-contaminated soils. Anthracene, phenanthrene, pyrene, and fluoranthene were the only carbon and energy sources available to these microorganisms (Samanta *et al.,* 2002). These

microorganisms' strong degradation capacity, according to *Tian et al.*, (2008) is attributable to the presence of key enzymes like PAH dioxygenase and catechol oxygenase. These microorganisms destroy PAHs by metabolism or co-metabolism.

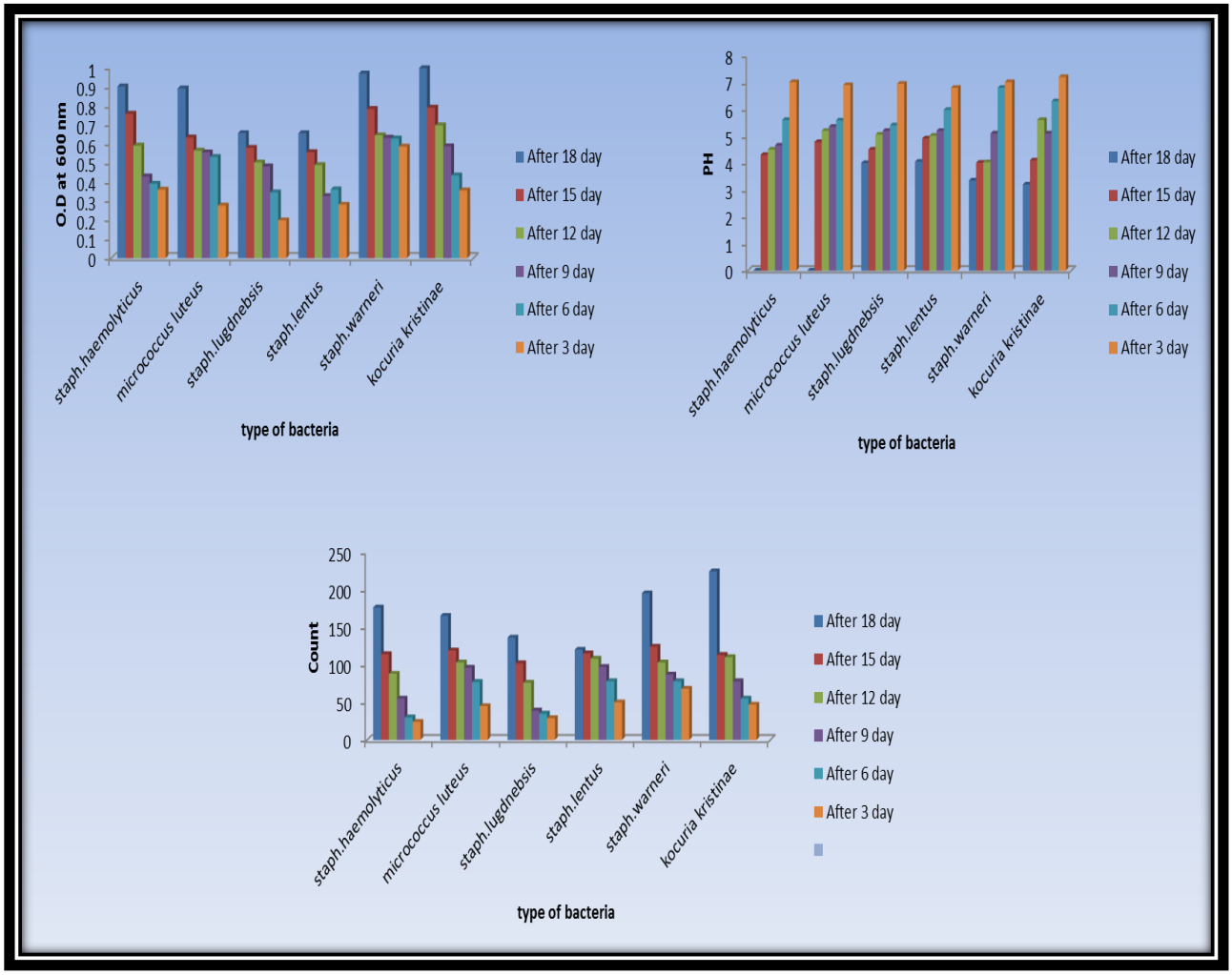



**Figure 4-1:Optical density, pH, and plate count for Gram positive bacteria(*Mi.luteus, S.haemolyticus, S.warneri, S.lentus, S. lugdunebsis, and K.kristinae)***

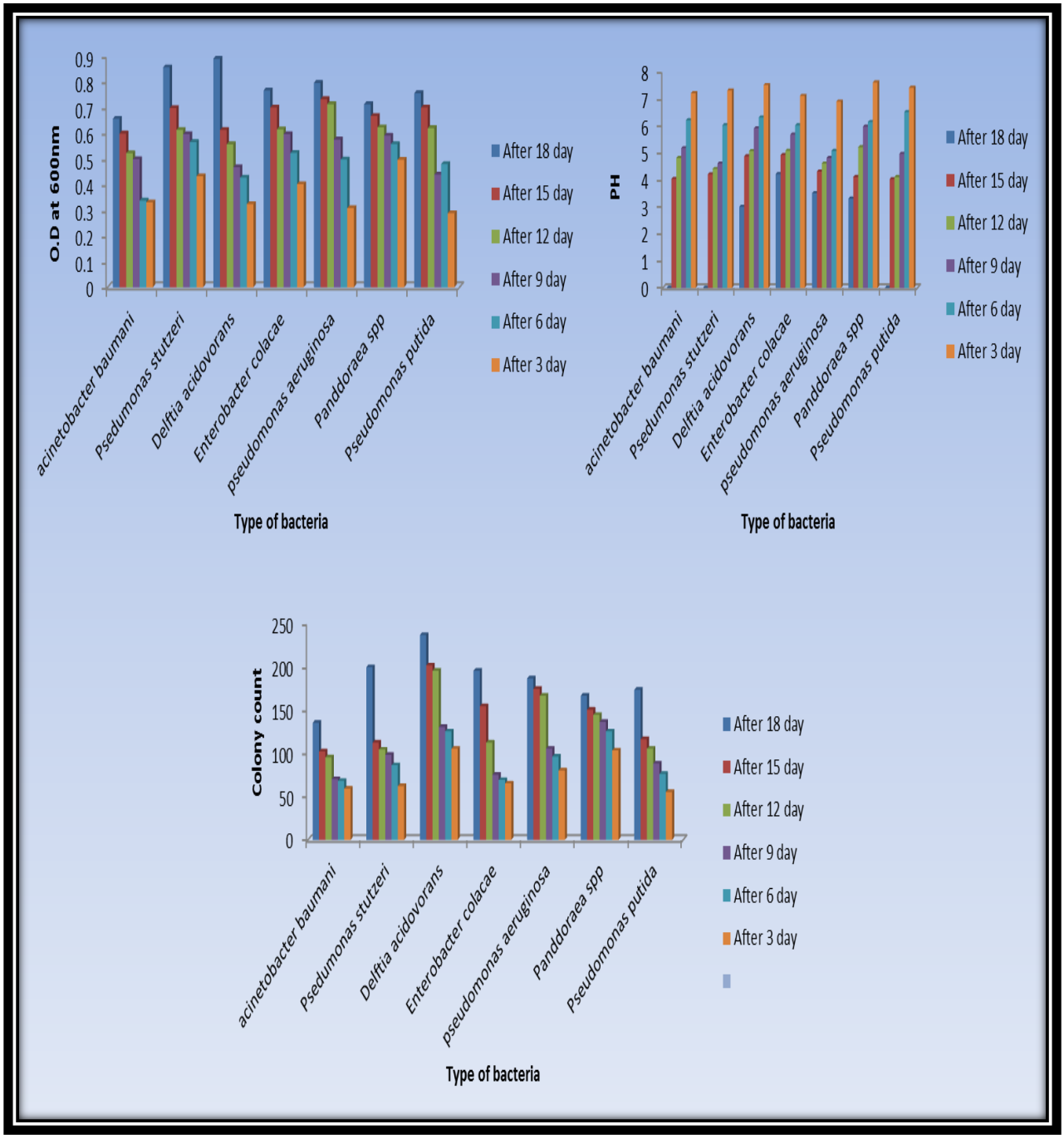



**Figure 4-2 : Optical density, pH, and plate count for Gram negative bacteria (*Ps.aeruginosa, Ps.Stutzeri, Pa.spp, E.cloacae, D.acidovorans, Ps. putida, and Ac.baumanni)*.**

**Table 4-2 parameters for Determining which isolate is best for degrading**

| | | Treatment period (days) | | | | | | LSD (0.05) |
|---|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 9 | 12 | 15 | 18 | |
| | | Mean±S.D | | | | | | |
| ***Kocuria. kristinae*** | **pH** | 7.2±0.1 | 6.2±0.1 | 5.4±0.01 | 4.1±0.02 | 3.7±0.4 | 3.0±0.1 | **0.319** |
| | **O.D.** | 0.35±0.01 | 0.44±0.01 | 0.55±0.02 | 0.69±0.01 | 0.84±0.10 | 0.99±0.01 | **0.080** |
| | **count** | 87.3±0.6 | 91.3±0.5 | 101.3±0.6 | 119.3±0.4 | 173.8±3.2 | 222.0±1.4 | **23.906** |
| ***Staphylococcus. warneri*** | **pH** | 7.01±0.01 | 6.85±0.13 | 5.07±0.05 | 4.02±0.01 | 4.01±0.01 | 3.61±0.02 | **0.103** |
| | **O.D.** | 0.28±0.01 | 0.39±0.07 | 0.53±0.02 | 0.64±0.0 | 0.72±0.01 | 0.97±0.03 | **0.046** |
| | **count** | 67.5±0.7 | 83.0±10.0 | 100.3±0.6 | 114.7±0.5 | 192.7±0.7 | 200.7±0.5 | **7.337** |
| ***Stapyhlococcus.haemolyticus*** | **pH** | 7.0±0.1 | 5.98±0.6 | 4.65±0.01 | 4.47±0.05 | 4.17±0.06 | 3.80±0.10 | **0.508** |
| | **O.D.** | 0.28±0.01 | 0.36±0.05 | 0.46±0.01 | 0.59±0.01 | 0.70±0.02 | 0.90±0.02 | **0.046** |
| | **count** | 54.0±1.4 | 72.5±1.2 | 84.7±0.6 | 109.7±0.5 | 190.3±0.7 | 195.0±1.0 | **9.038** |
| ***Micrococcus .luteus*** | **pH** | 6.85±0.1 | 5.91±0.6 | 5.35±0.01 | 5.17±0.06 | 5.00±0.1 | 4.40±0.2 | **0.485** |
| | **O.D.** | 0.27±0.01 | 0.31±0.03 | 0.35±0.02 | 0.53±0.01 | 0.61±0.02 | 0.89±0.01 | **0.021** |
| | **count** | 45.0±1.4 | 66.5±1.4 | 78.67±0.6 | 99.33±1.2 | 120.67±0.8 | 167.00±1.0 | **10.517** |
| ***Staphylococcus. lugdunebsis*** | **pH** | 6.95±0.1 | 5.76±0.7 | 5.17±0.02 | 5.07±0.01 | 4.47±0.2 | 4.13±0.06 | **0.586** |
| | **O.D.** | 0.20±0.01 | 0.22±0.01 | 0.35±0.02 | 0.47±0.01 | 0.59±0.01 | 0.66±0.01 | **0.065** |
| | **count** | 27.0±2.8 | 40.8±7.8 | 45.0±2.3 | 75.3±0.6 | 101.7±0.5 | 128.0±1.0 | **5.881** |
| ***Staphylococcus. lentus*** | **pH** | 6.75±0.06 | 6.19±0.40 | 5.23±0.06 | 5.01±0.01 | 4.91±0.01 | 4.17±0.06 | **0.302** |
| | **O.D.** | 0.18±0.02 | 0.20±0.01 | 0.33±0.01 | 0.46±0.02 | 0.58±0.01 | 0.66±0.01 | **0.061** |
| | **count** | 16.0±1.4 | 31.0±1.7 | 37.67±1.5 | 68.00±1.0 | 93.33±0.6 | 118.7±1.2 | **7.925** |
| ***Delftia.acidovorans*** | **pH** | 7.45±0. | 6.58±0. | 5.83±0. | 5.06±0. | 4.86±0. | 4.10±0.0 | **0.453** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 6 | 6 | 02 | 03 | 2 | |
| | **O.D.** | 0.32±0.01 | 0.42±0.06 | 0.50±0.01 | 0.52±0.02 | 0.79±0.02 | 0.89±0.01 | **0.044** |
| | **count** | 104.5±0.7 | 119.8±9.9 | 129.7±0.6 | 194.7±0.5 | 214.0±1.1 | 221.0±4.2 | **7.282** |
| ***Pseudomonas .stutzeri*** | **pH** | 7.30±0.1 | 6.33±0.6 | 4.63±0.1 | 4.37±0.1 | 4.23±0.1 | 3.83±0.02 | **0.478** |
| | **O.D.** | 0.30±0.01 | 0.36±0.04 | 0.48±0.02 | 0.51±0.02 | 0.67±0.01 | 0.86±0.02 | **0.046** |
| | **count** | 61.50±2.1 | 79.75±11.8 | 97.67±0.6 | 104.0±1.0 | 200.67±0.6 | 213.67±0.5 | **8.702** |
| ***Pseudomonas .aeroginosa*** | **pH** | 6.89±0.04 | 5.52±0.9 | 4.77±0.1 | 4.67±0.2 | 4.27±0.3 | 3.85±0.4 | **0.667** |
| | **O.D.** | 0.30±0.01 | 0.36±0.03 | 0.48±0.02 | 0.50±0.01 | 0.56±0.01 | 0.80±0.02 | **0.080** |
| | **count** | 45.0±0.2 | 69.0±16.0 | 85.7±0.6 | 93.3±1.5 | 187.0±1.0 | 201.0±1.2 | **11.711** |
| ***Enterobacter .cloacae*** | **pH** | 7.1±0.01 | 6.28±0.5 | 6.7±0.02 | 5.1±0.02 | 4.91±0.01 | 4.20±0.01 | **0.398** |
| | **O.D.** | 0.27±0.1 | 0.33±0.04 | 0.40±0.01 | 0.43±0.01 | 0.52±0.01 | 0.77±0.01 | **0.078** |
| | **count** | 35.5±2.1 | 57.3±1.4 | 76.3±1.5 | 86.3±1.5 | 145.3±0.6 | 194.3±0.6 | **10.920** |
| ***Pseudomonas .putida*** | **pH** | 7.35±0.1 | 6.73±0.4 | 4.96±0.01 | 4.06±0.2 | 4.02±0.03 | 3.87±0.4 | **0.332** |
| | **O.D.** | 0.25±0.01 | 0.27±0.02 | 0.34±0.01 | 0.42±0.01 | 0.48±0.01 | 0.76±0.02 | **0.113** |
| | **count** | 26.5±0.7 | 42.75±1.6 | 66.67±0.6 | 74.67±0.8 | 120.67±1.3 | 156.33±2.6 | **7.698** |
| ***Pandoraea . sp*** | **pH** | 7.55±0.1 | 6.50±0.7 | 5.96±0.02 | 5.17±0.1 | 4.07±0.2 | 3.76±0.6 | **0.537** |
| | **O.D.** | 0.20±0.01 | 0.21±0.01 | 0.32±0.01 | 0.40±0.03 | 0.47±0.01 | 0.71±0.04 | **0.041** |
| | **count** | 20.50±0.7 | 30.75±7.1 | 56.0±1.0 | 65.67±0.6 | 117.0±1.2 | 145.3±0.6 | **5.323** |
| ***Acinetobacter .baumanni*** | **pH** | 7.05±0.1 | 6.38±0.6 | 5.12±0.1 | 4.70±0.1 | 4.02±0.02 | 4.01±0.01 | **0.414** |
| | **O.D.** | 0.13±0.01 | 0.18±0.03 | 0.32±0.02 | 0.35±0.03 | 0.45±0.01 | 0.61±0.01 | **0.122** |
| | **count** | 16.50±0.7 | 26.50±7.6 | 46.3±0.6 | 54.67±1.8 | 104.0±1.2 | 132.3±1.9 | **5.659** |

## 4.4 Screening the bacteria for biosurfactant production

Based on their ability to utilize crude oil as their sole carbon source, a total of 13 G-ve and G+ve bacterial species were isolated from oil-contaminated soils, were cultured on MSM medium. The colony shape, colony color, and development levels of these isolates were differed .Because of their ability to lower the surface tension of crude oil , biosurfactants play an essential role in oil recovery .Blood hemolysis, blue agar plate, oil spreading technique, drop collapse assay, Bacterial adhesion to hydrocarbons (BATH), emulsification behavior, and foaming activity were used to screen all of the pre-identified isolates for biosurfactant production .

### 4.4.1Hemolysis test

Biosurfactant production is frequently tested using the blood agar method. A primary way for screening a biosurfactant manufacturer is to use a hemolytic activity assay. Blood agar plates were used to screen all isolates. In Figure (4-3) 8 isolates *(K.kristinae, S.warneri, S.haemolyticus, M. luteus ,D. acidovorans, Ps.putida, Ps.aeruginosa, and, Ps.stutzeri*,) showed positive results for hemolytic activity by forming a clear zone. It is unclear therefore whether blood agar lysis should be used to screen for biosurfactant production, though such screening can be used as a quick method if positive results are then verified in the emulsification index assay. In the hunt for biosurfactant-producing bacteria, hemolytic activity appears to be an effective screening criterion (Rashedi *et al.*,2005). The isolates utilized in this research showed strong hemolytic activity, which is a key method for identifying bacteria that produce biosurfactant. In addition, Karthik *et al.*, (2010) suggested that the efficacy of the blood agar lysis in

predicting biosurfactant production was not entirely reliable. Hemolysis gave 16% false positives and rejected many potential biosurfactant makers in the study by Youssef *et al*., (2004), thus they advised screening the cultures first using the drop collapse method, then utilizing spreading techniques and the emulsification index. Only 13.5% of the 200 marine bacteria that Maneerat and Phetrong discovered in 2007 were hemolytic strains; the rest were tested as biosurfactant makers using the drop collapse method and the oil spreading method. According to Ogbulie *et al.*, (2014), other microbial products, such as virulence factor, lyse blood agar, and biosurfactants that are poorly diffusible might not lyse blood cells. In contrast, Govindammal and Parthasarathi (2013) noted that the ability of surfactant-producing strains to produce biosurfactants in a liquid medium was shown to be correlated with hemolysis activity and came to the conclusion that this made hemolysis activity a useful screening criterion.

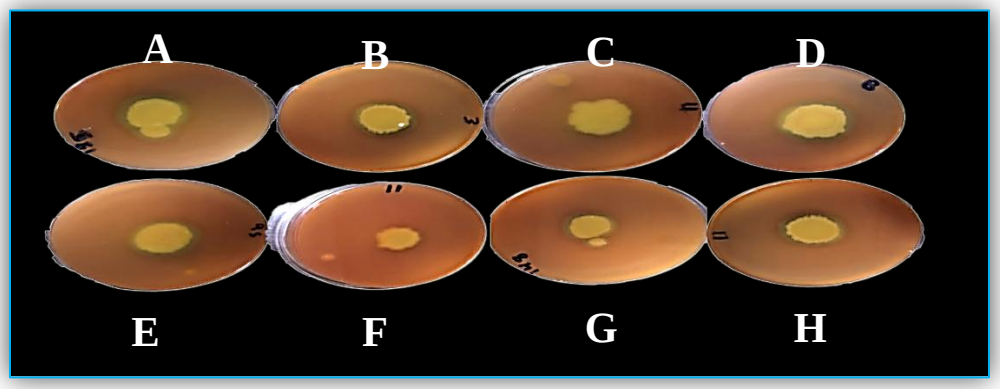



**Figure 4-3: Biosurfactant producing isolates on blood agar medium (A: *K. kristinae, B: S.warneri, C: S.haemolyticus, D:M.luteus, E:D.acidovorans, F:Ps.putida, G: Ps.aeruginosa ,and H: Ps.stutzeri).***

## 4.4.2 CTAB agar plate method

Cetyltrimethylammonium bromide (CTAB) agar plate method is employed to detect glycolipid or other anionic surfactant-producing microorganisms. The blue agar plate method is a semi-quantitative agar plate method that uses the cationic surfactant CTAB and the basic color methylene blue to create an insoluble ion pair of anionic surfactants. All isolates were screened for biosurfactant production. Only 6 isolates ,both G+ve and G-ve, generated dark blue halos on CTAB agar, indicating biosurfactant production, (Figure 4-4),. The CTAB agar plate method is a semi-quantitative test for extracellular glycolipids and other anionic surfactants . These results were obtained in this study compatible with another study that suggested 8 isolates to be positive for CTAB agar plate test, and 52.8%of other isolates were found to be positive for CTAB agar plate test (Primeia *et al.*,2020). In this technique, the microorganisms are grown in a medium consisting of CTAB and methylene blue. The production of anionic surfactant by the microbial strains is indicated by the formation of dark blue halos around the colonies. This technique is simple, specific for anionic biosurfactants and can be applied directly on agar plates or liquid broth using different substrates or temperatures. However, CTAB is noxious and inhibits the growth of some microorganisms (Soltanighias *et al.*, 2019).

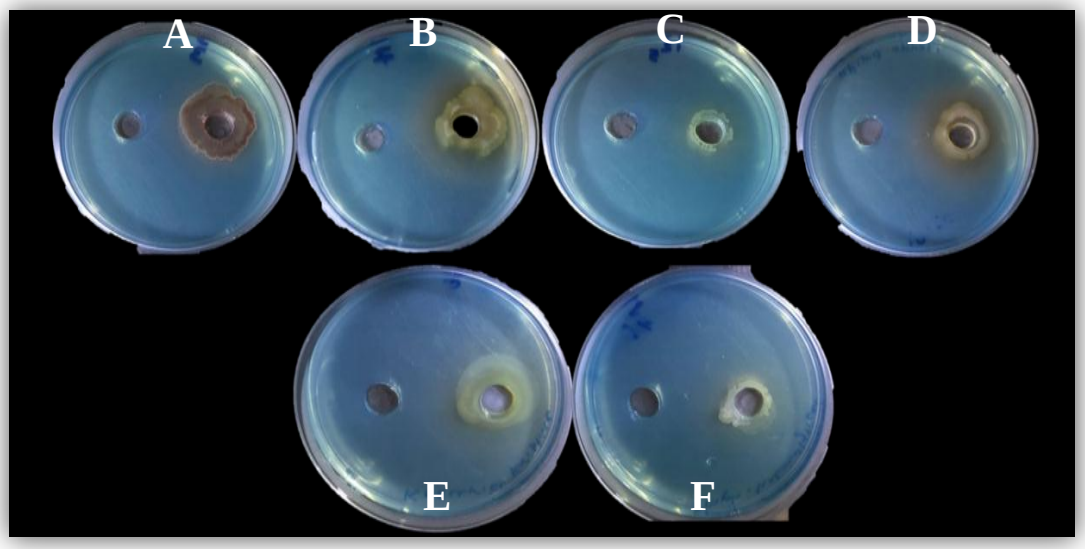



**Figure 4-4: Biosurfactant producing by gram negative and gram positive bacteria (A: *D.acidovorans, B:Ps.putida, C:Ps.aeruginosa ,D: Ps.stutzeri, E:K.kristinae ,and F: S.warneri,*). bacterial isolate on CTAB agar medium**

## 4.4.3 Oil spreading test:

The bacterial culture was introduced to a plate that contained crude oil for the oil spreading test. The biosurfactant-producing organism would dispense with the oil and form a clear zone in the middle of the plate, demonstrating its capacity to do so. This study, however, only performed a qualitative assessment to check for the presence of surfactant. In the oil spreading assay, the cell free supernatant's capacity to displace the oil and create a higher clean zone is another sign of biosurfactant production. According to the findings (Figure 4-5,Table 4-3), Gram positive (*S.lentus, S.lugdunebsis*) and Gram negative (*A.baumanni, E.cloacae, and Pa.sp*.) bacteria gave negative results in the oil spreading assay, whereas *K.kristinae, S.warneri, S.haemolyticus, M.luteus, D.acidovorans, Ps.putida , Ps.aeruginosa, and Ps.stutzeri)* gave positive result with a diameter of 3.3 ,2.9,2.6,2.2,3.2,2.7,2, and 1.9mm respectively. Other results were obtained in other

study have reported the presence of biosurfactant-producing microorganisms in environments polluted with hydrocarbons (Walter *et al.*,2010). The oil displacement area is proportional to the amount of surface-active chemical in the solution. The stability of the drops on the liquid droplets affects the biosurfactant concentration. Contrary to Ndigbe *et al.*,(2018) .'s findings, where 54.5% of their isolates tested positive, this study had a low number of isolates with positive drop collapse tests.

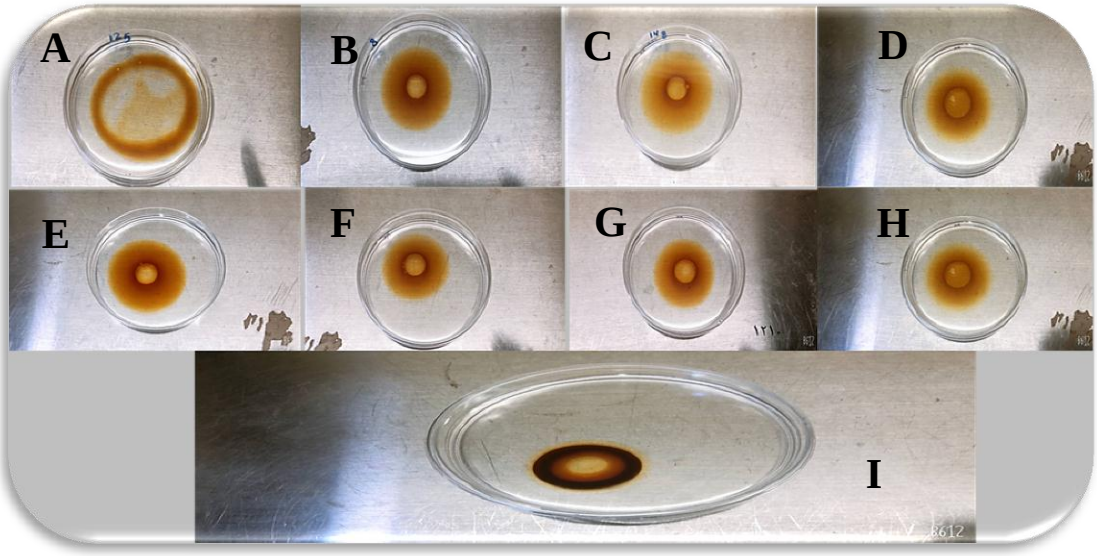



**Figure 4-5: Oil displacement assay for Gram positive and Gram negative *bacteria* (A: *K.kristinae ,B: S.warneri, C: S.haemolyticus, D: M.luteus , E:D.acidovorans, F:Ps.putida, G:Ps.aeruginosa , and H: Ps.stutzeri*) Producing biosurfactant, (I) negative control using only oil**

**Table 4-3: Oil spreading, Drop collapse, Emulsification ,and BATH assay**

| Bacterial isolates (Gram positive) | Oil spreading test (mm) | Drop collapse test(mm) | Emulsification index(%) | BATH assay % |
|---|---|---|---|---|
| **Kocuria kristinae** | **3.3** | **2.2** | **98.6%** | **95.2%** |
| ***Staphylococcus warneri*** | **2.9** | **1.8** | **96.3%** | **71.5%** |
| ***Staphylococcus haemolyticus*** | **2.6** | **1.3** | **88.5%** | **66.8%** |
| ***Micrococcus letus*** | **2.2** | **1.1** | **81.8%** | **56.7%** |
| **Bacterial isolates(Gram negative)** | | | | |
| ***Delftia acidovorans*** | **3.2** | **2.9** | **96%** | **97.3%** |
| ***Pseudomonas putida*** | **2.7** | **1.7** | **92%** | **83.2%** |
| ***Pseudomonas aeruginosa*** | **2** | **1.1** | **87.5%** | **77.3%** |
| ***Pseudomonas stutzeri*** | **1.9** | **0.8** | **76%** | **53.2%** |

## 4.4.4 Drop collapse assay:

This experiment was focused on the destabilization of a liquid drop by a cell free extract containing biosurfactant. All 13 isolates were used to revealed the production of biosurfactant. Eight isolates (*K.kristinae, S.warneri, S.haemolyticus, M.luteus, D.acidovorans, Ps.putida, Ps.aeruginosa, and Ps.stutzeri*) showed good results in the drop collapse test (Table 4-3 ,Figure 4-6).The drop collapse method is rapid and easy to use, as it just requires a small sample volume and no special equipment. Culture supernatant dropped on an oil-coated solid surface as a result

of this. The polar water molecules were repelled off the hydrophobic surface if the liquid did not contain surfactants, and the drops remained stable. When liquid drops contain surfactants, the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced, causing the drops to spread or even collapse. Drop stability is influenced by surfactant concentration, which is linked to surface and interfacial tension. According to Erum *et al.,* (2012), positive cultures for oil drop collapse resulted in better biosurfactant production and were likely involved in lowering surface and interfacial tension between oil and water. The drop-collapse technique is a sensitive and straightforward test with a number of benefits, including the need for a small number of samples, speed and ease of execution, and the need of specialized equipment (Shoeb *et al.*,2015).

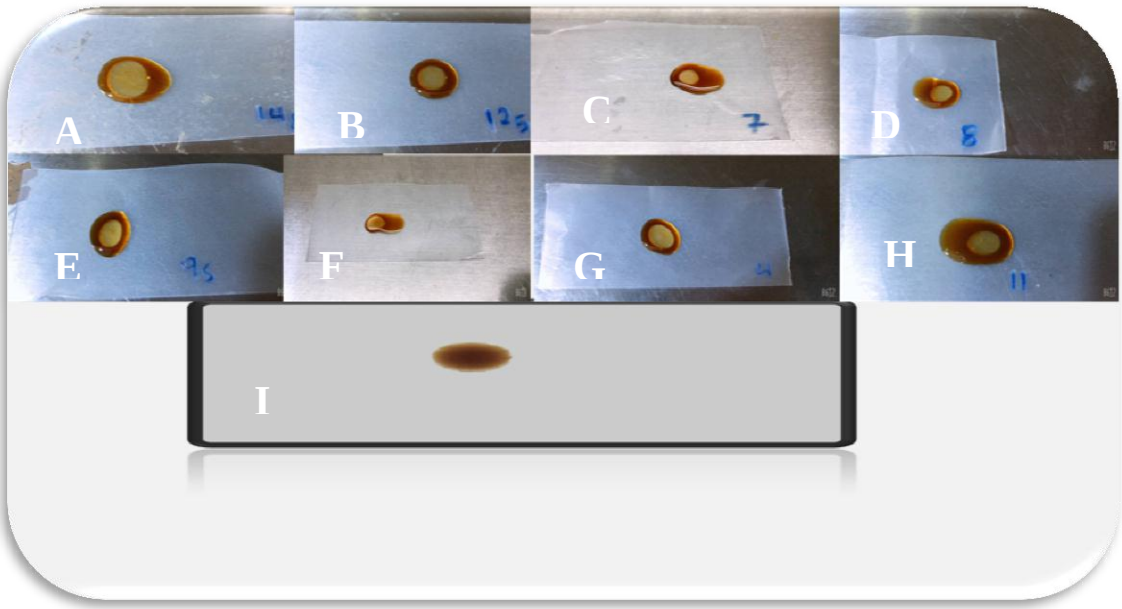



**Figure 4-6: Drop collapse assay for Gram positive and Gram negative bacteria Producing biosurfactant (A: *K.kristinae* ,B: *S.warneri, C: S.haemolyticus, D: M.luteus* , *E:D.acidovorans, F:Ps.putida, G:Ps.aeruginosa* , *and H: Ps.stutzeri*), (I) negative control using only oil**

## 4.4.5 Emulsification method:

An indirect method for screening biosurfactant synthesis is the emulsification assay. It was assumed that if the cell free culture broth contained biosurfactant, the hydrocarbons would be emulsified. Four Gram positive isolates and four Gram negative isolates showed the most emulsification. Gram positive isolates (*K.kristinae, S.warneri, S.haemolyticus, and M.luteus*) had Emulsification index values of 98.6, 96.3, 88.5, and 81.8 %, respectively. Gram negative bacteria (*D.acidovorans, Ps.putida, Ps.aeruginosa, and Ps.stutzeri*) have Emulsification index values of 96 ,92, 87.4, and 76 %, respectively, Table (4.3), Figure (4-7) and Figure (4-8) ,Figure (4-9) and Figure (4-10) were demonstrated these results. The hydrophobic substrate in this case was crude oil. Emulsification ability was regarded as a reliable test for identifying isolates that produce biosurfactant (Goswami and Deka ,2019). Femi-Ola *et al.,* (2015) assessed the Ei24 value of isolates for strain detection and found 53,13% emulsification activity with kerosene. For emulsification, Ann and Parthasarathi (2014) used a variety of hydrocarbon and vegetable oils. They discovered that PBSC1, a biosurfactant isolated from *P. aeruginosa*, had the best emulsification activity against crude oil. Velmurugan *et al.,* (2015) reported a 44 % emulsification index of biosurfactant produced by isolate H11 against kerosene. Using an emulsification test, biosurfactant potential isolates were also confirmed. As opposed to surface tension measurement, which is laborious and time-consuming, making it difficult to screen a large number of isolates, this test was chosen for its accuracy, speed, and ease of use. The results of the emulsification activity differ from those of Ndigbe *et al.,* (2018), who found lower values of 54.8%, which may have been caused by changes in the medium's pH, temperature, and salinity. However, this research employees can make with the findings of some other researchers, who also

identified *Pseudomonas sp*. as a reliable source of biosurfactants. These results were showed that using crude oil as a substrate was crucial due to the availability of inexpensive growth substrates, cost-effective industrial production, and the employment of efficient microorganisms for biosurfactant production (Marchut *et al.*, (2018).

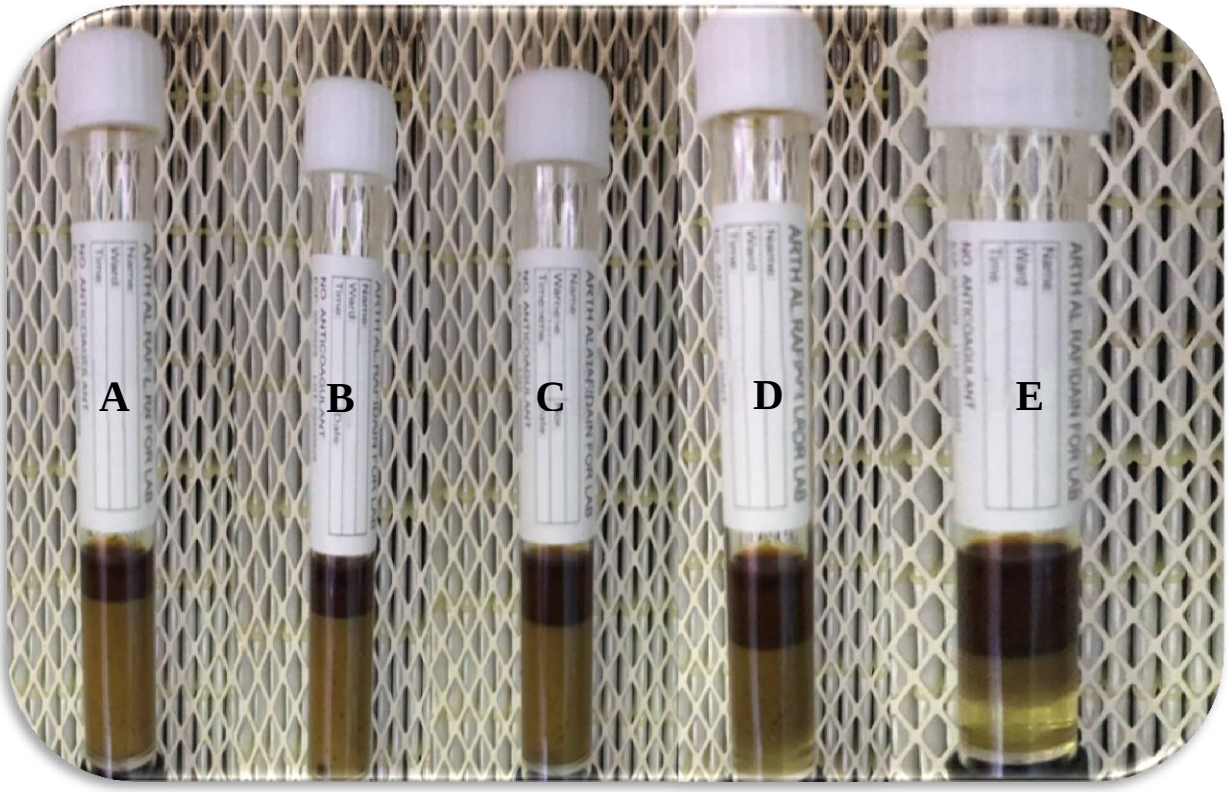



**Figure 4-7: Emulsification assay for Gram positive bacteria*(A: K.kristinae, B:S.warneri, C:S.haemolyticus, and D:M.luteus* . Producing biosurfactant, E: negative control using only oil**

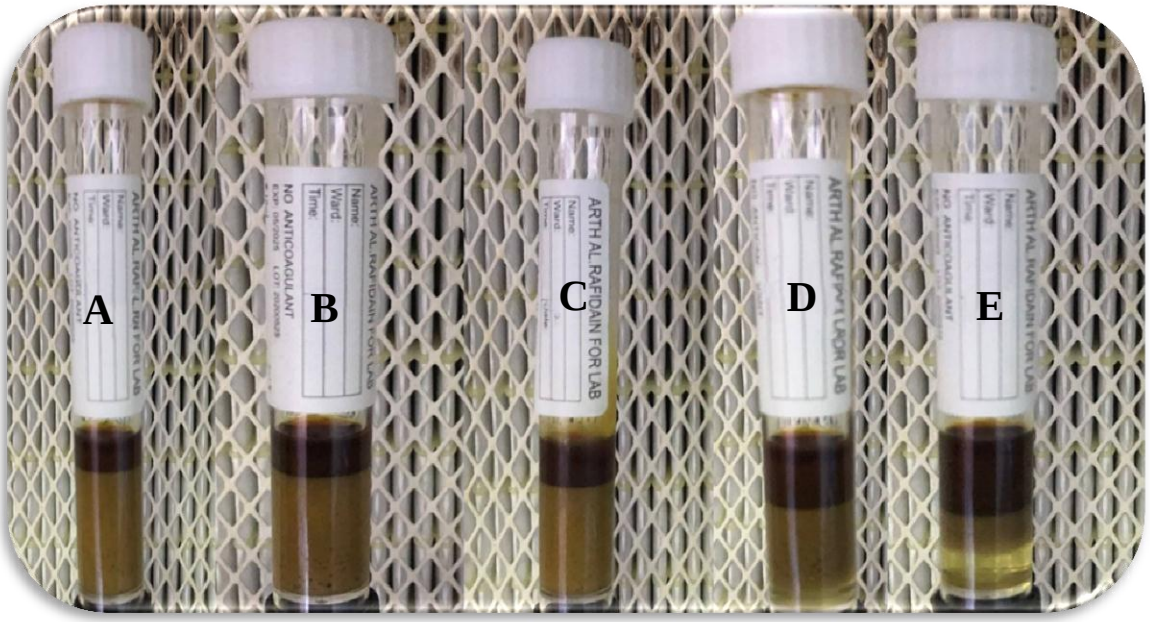



**Figure 4-8: Emulsification assay for gram negative bacteria*(A: D.acidovorans, B:Ps.putida , C:Ps.aeruginosa, and D:Ps.Stutzeri*. Producing biosurfactant, E: negative control using only oil**

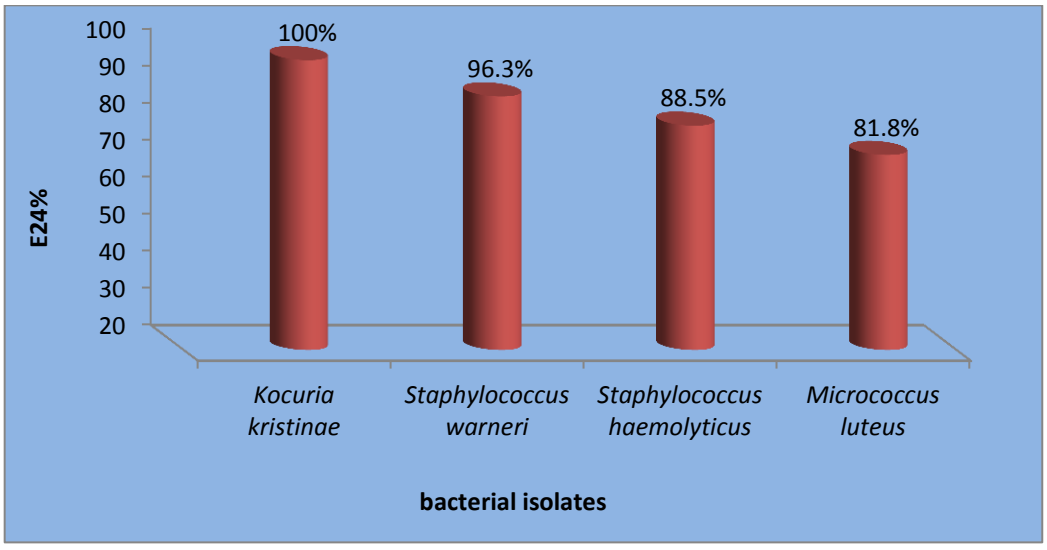



**Figure 4-9: Emulsification index (E24%) of Gram positive , grown in MSM (pH 7) with 1% crude oil, at 35 °C in shaker incubator (150 rpm) for 7 days**

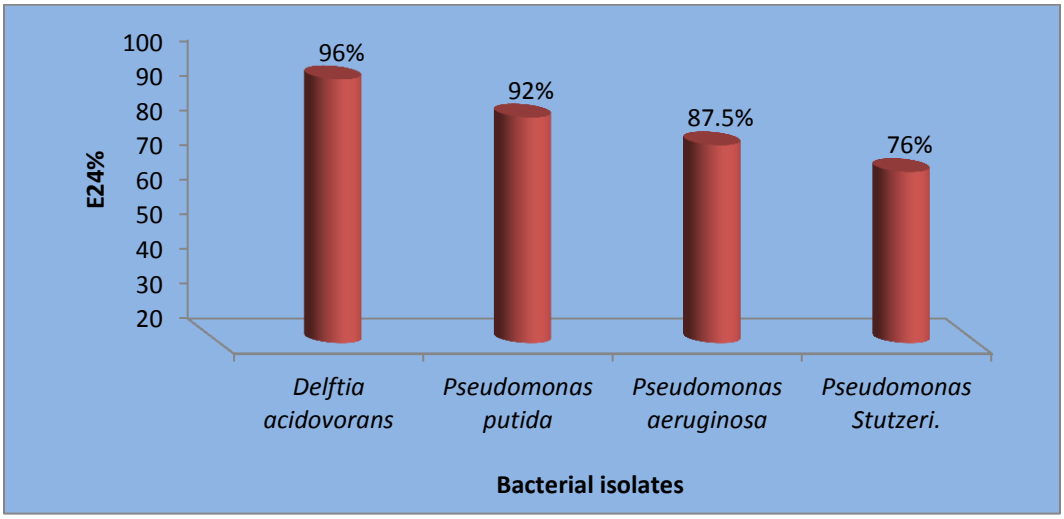



**Figure 4-10: Emulsification index (E24%) of gram negative , grown in MSM (pH 7) with 1% crude oil, at 35 ºC in shaker incubator (150 rpm) for 7 days**

## 4.4.6Bacterial adhesion to hydrocarbons (BATH):

Cell adhesion with hydrophobic materials like crude oil is considered an indirect technique to screen bacteria for biosurfactant production because cells adhere themselves to oil droplets by producing surface active molecules termed biosurfactant. The BATH test demonstrated that all the isolates used in this study, were positive, indicating that the bacteria preferred hydrophobic substrate. Cell adhesion to crude oil for positive isolates ( *K. kristinae, S.warneri, S.haemolyticus, and M.luteus)* that showed 95.2, 71.5, 66.8 and 56.7% ,respectively, while gram negative bacteria (*D.acidovorans, Ps.putida , Ps.aeruginosa, and Ps.stutzeri*) that gave best of bacterial adhesion 97.3,83.2,77.3, and 53.2% ,respectively, Table (4-3). The BATH assay demonstrated that *K.kristinae* and *D.acidovorans* cells with crude oil had a high cell adhesion of more than 93.3 %, which was directly connected with the biodegradation capacity observed in this study for this isolate. Cell adhesion with hydrophobic materials like diesel oil is considered an indirect technique to screen bacteria for biosurfactant production because cells adhere

themselves to oil droplets by producing surface active molecules termed biosurfactant. Sauvageau *et al.*,(2012) found high cell hydrophobicity and degradation for *P. aeruginosa*, which supports the findings of this investigation. Surfactants produced by microorganisms (biosurfactants) are surface-active amphiphilic molecules made up of a variety of structurally different biomolecules, including phospholipids, glycolipids, lipopeptides, lipoproteins, and lipoproteins (Walter *et al.*,2010). When grown on mostly water immiscible substrates, they are collected by microbial cells or discharged in the extracellular environment by some microbes (Makkar and Rockne,2003). Biosurfactants' capacity to reduce surface tensions at the interface between liquids and substrates means that these bacteria have access to nutrients, facilitating nutrition uptake and metabolism as well as development on water-immiscible substrates. In order to decontaminate the environment, bioremediation processes which employ microorganisms to break down soil and water pollutants need surfactants (Kosaric,2001). Together with bioremediation, bio-stimulation the injection of nutrients to speed up the biological removal of toxins by bacteria that break down hydrocarbons is frequently carried out (Magdalena *et al.*,2011). Surfactants improve the bioavailability of hydrocarbons and the growth of microbes, which improves the degradation of contaminants (Silva *et al.*,2017).

## 4.4.7 Foaming activity:

The foaming capacity test was performed on the 8 isolates under examination. Isolates were revealed the highest foaming activity.8 isolates for Gram positive and Gram negative (*K.kristinae, S.warneri, S.haemolyticus, M.luteus, D.acidovorans, Ps.putida , Ps.aeruginosa, and Ps.stutzeri*) respectively that gave the highest foaming activity. Figure (4-11). The aqueous solution of the partially recovered biosurfactant showed good foaming ability more than 50% and

stability for more than 6 h. This result, is in accordance with (El-Shahawy and Hussien ,2014).

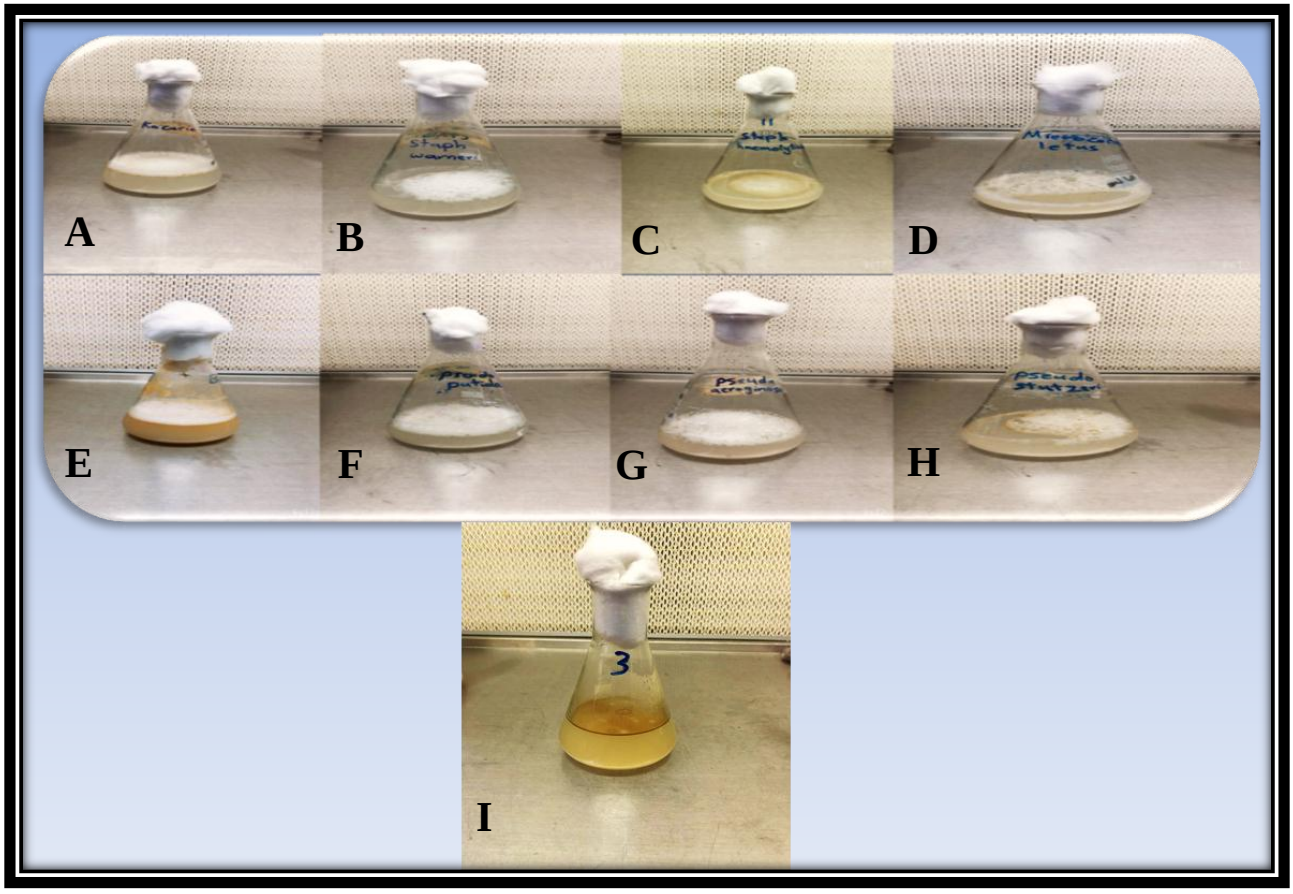



**Figure 4-11: Foaming activity for Gram positive and Gram negative bacteria (A: *K.kristinae* ,*B: S.warneri, C: S.haemolyticus, D: M.luteus* , *E:D.acidovorans, F:Ps.putida, G:Ps.aeruginosa* , *H: Ps.stutzeri,* and I: control)**

## 4.5 Optimization condition for biosurfactant production:

The optimization of the synthesis of biosurfactants depends heavily on process variables such the medium's pH, incubation temperature, nutritional content (such as nitrogen, carbon, and mineral sources), and salinity (Mnif *et al.*,2013; Vijayaraghavan *et al.*,2019).). *K. Kristinae* and *D. acidovorans* were selected as more active isolates to find the ideal circumstances for biosurfactant production.

## 4.5.1 Effect of pH on biosurfactant production

The production of biosurfactant by *K.kristinae* and *D.acidovorans* were studied by growing this bacteria on mineral salt medium with various pH (3, 4, 5, 6, 7 and 8). This results demonstrated that the optimum pH for the production of biosurfactant was 7 that recorded maximum Ei24%, 90 and 80% for *K.kristinae* and *D.acidovorans* ,respectively (Figure 4-12) .These results were obtained in this study was agreement with other research that gave pH 7 was ideal for both bacterial growth and the synthesis of biosurfactants (Mahdi, 2013).The values of pH range from 5-7.5 was an indicator of biosurfactant production, highlighting the significance of maintaining it throughout the fermentation process (AlKaabi *et al.*,2020). Through their impacts on cellular development or activity, environmental elements and growth parameters like pH have an impact on the creation of biosurfactants. According to research by Saharan *et al.*, (2011), *Candida antarctica* and *Candida apicola* produce the most glycolipids when the pH is kept at 5.5. At pH 7, *Pseudomonas species* produced the most rhamnolipids. The synthesis of biosurfactants is significantly influenced by the pH of the medium. Jagtap *et al*.,(2010) research demonstrated that bioemulsifier synthesis was at its highest at pH 7, with decreased activity at pH 6, 8, and 9 ,these results indicated that pH 7 was ideal for *A. chrococcum* to produce biosurfactant is compatible with results obtained in this study. The yield was recorded and the emulsifying index was continuously monitored to track how pH affected the synthesis of biosurfactants. It was previously mentioned that the production of precipitates caused the yield of biosurfactants to decrease at acidic pH. According to *Pseudomonas putida MTCC 2467*, pH 8.0 is ideal for the creation of biosurfactants demonstrating how environmental factors like pH have a substantial

impact on the production of biosurfactants in different microorganisms. (Ghasemi *et al.*, 2019).

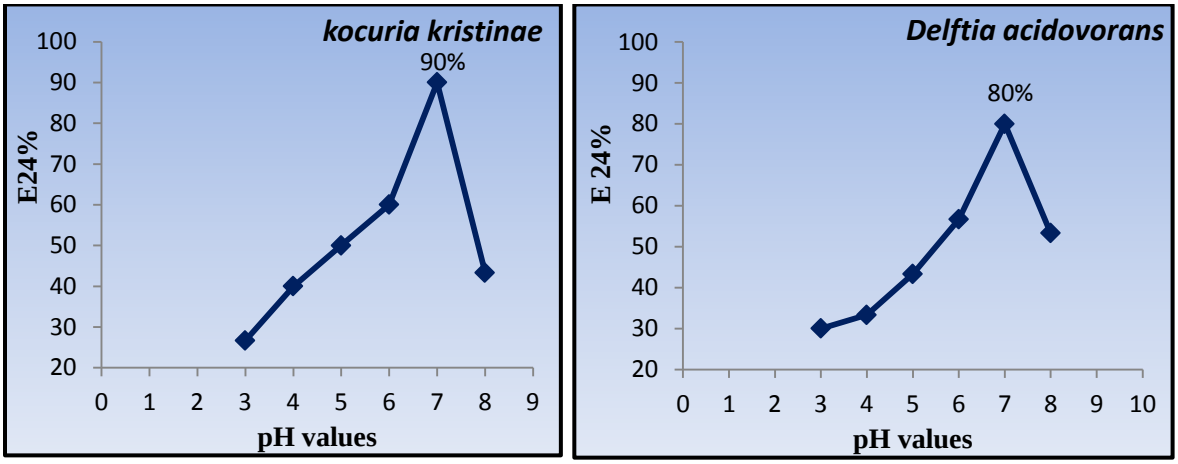



**Figure 4-12:- Effect of pH on biosurfactant production**

## 4.5.2 Effect of temperature on biosurfactant production

The results of this experiment showed the effect of various temperatures (20,25,30,35,40, and 45) on biosurfactant production **(**Figure 4-13). Maximum Ei24% in biosurfactant 93.33% and 86.66% for *K.kristinae* and *D.acidovorans* respectively was obtained at 35˚C.But there was a reduction in Ei24% (76.33, and 46.66%) in 40, and 45 ˚C in *K.kristinae* .Also, the Ei24% in *D.acidovorans* decreased in higher temperature values (40 and 45˚C) to (66.66 and 56.66%) respectively. Although considerable biosurfactant production was also observed below this temperature, the production sharply dropped at 40°C in all isolates. The metabolism of microorganisms has direct relationship with culture temperature. Maximum enzymatic activation can only be obtained at an optimum temperature. A lower culture temperature might make strain *Azotobacter chrococcum* hibernate partially, and its enzyme system for biosurfactant production couldn't be activated completely (Qomarudin *et al.*,2012).Both *Pseudomonas aeruginosa MR01* and *Pseudomonas aeruginosa S2*, which were isolated from diesel-contaminated soil

and oil excavation locations, performed best when cultivated at 37°C (Chen *et al.*, 2007;Lotfabad *et al.*, 2009). A different study by Praveesh *et al.*, (2011) revealed that different bacterial species produced the most rhamnolipid at 35°C, while at 40°C, bacterial growth and the production of biosurfactants were inhibited. For example, *Pseudomonas aeruginosa* was revealed to be a mesophilic bacterium that cannot survive at temperatures higher than 40°C.Among the other factors, a temperature of 37°C appeared to be most suitable for the selected isolates to produce biosurfactants. The optimal temperature was close to the soil temperature (approx. 30°C) during isolation which indicates a direct correlation of biosurfactant production to the growth of the microbes under suitable temperature, i.e.higher production as the cell density increases. Similar growth conditions were observed in several other bacteria, many species also showed optimum yield for different temperatures and pH, either higher or lower (Ventorino *et al.*,2017; Gichuhi *et al.*,2020; Abdelshafy *et al.*,2020).

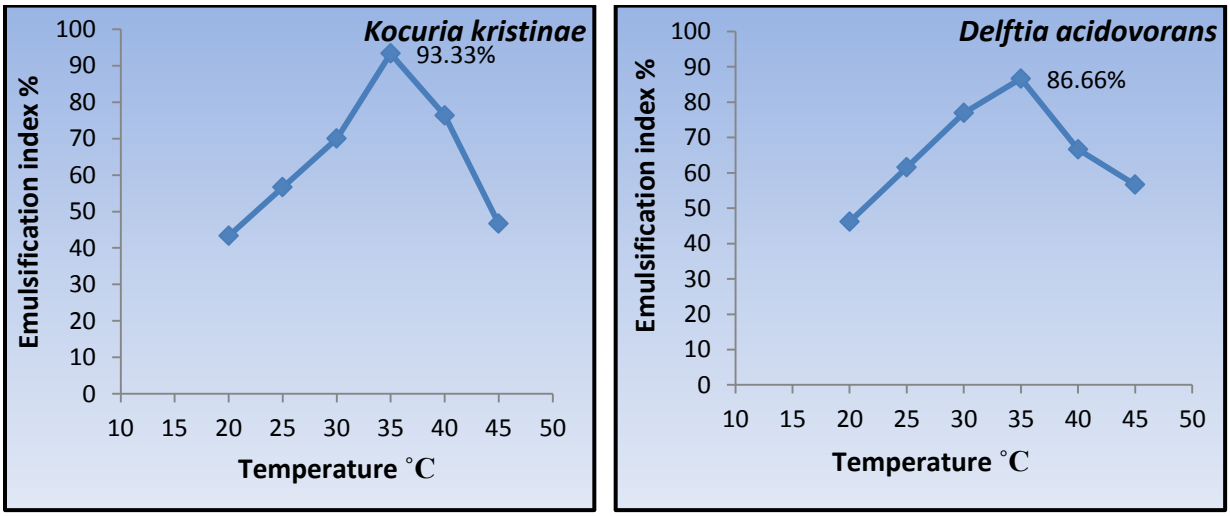



**Figure 4-13:- Effect of temperature on biosurfactant production**

## 4.5.3 Effect of carbon source on biosurfactant production

A variety of carbon sources (glucose,fructose,manitol,crude oil, sucrose, and date extract) were investigated into in order to biosurfactants production because there is a correlation between the development of biosurfactants and growth on hydrocarbons. The results shown in Figure (4-14) , the Ei24% (93.33 and 88.33%) were obtained when crude oil was used as the a sole source of carbon and energy for *K. kristinae* and *D. acidovorans,* respectively. This was followed by sucrose with an Ei24% (72.3%) in *K. kristinae* and manitol with an Ei24% (77.66%) in *D. acidovorans.* While other carbon sources were showed low Ei24%. These findings showed the bacterium's capacity to break down a variety of carbon sources and produce biosurfactants. The emulsification index of the crude oil, which was 74.320.52% and had a surface tension of 30.98 mN/m, increased the biosurfactant production from *Pseudomonas aeruginosa PBSC1* (Joice and Parthasarathi, 2014). The *Bacillus licheniformis PTCC* produced biosurfactant when grown on almond, castor, and olive oil as the only carbon source, although Noudeh *et al.,* (2007) discovered that olive oil produced the highest yield. Additionally, according to Gudia *et al,.* (2015), the surfactin synthesis of *Bacillus subtilis 573* was examined using maize steep liquor as a substitute low-cost culture medium. On the other hand, Solaiman *et al.,* (2004) utilized soy molasses and oleic acid as co-substrates for *Candida bombicol* to produce sophorolipids. The characteristics of the carbon substrate have an impact on and influence both the quantity and quality of biosurfactant production (Rahman and Gakpe, 2008). In contrast to these findings, Kiran *et al.,* (2010) found that using glucose as a carbon source resulted in the greatest synthesis of glycolipid. According to Manivasagan *et al.,* (2014), *Streptomyces sp. MAB36* produced the most glycolipid biosurfactant

when fructose (78.81%), starch (68.32%), and glucose (64.76%) were present. According to Zhang *et al.*,(2005) research, *Pseudomonas aeruginosa* produced the most biosurfactant when it was cultivated in 30 g/L glycerol as opposed to glucose, vegetable oil, and paraffin oil. This amount exceeds the levels discovered in this study by a factor of 3. Another study by Wei *et al.*, (2008) discovered that *Pseudomonas aeruginosa J16* produced more rhamnolipid when cultured in medium with 0.32 M glycerol than it did when grown in soy bean oil, sunflower oil, or manitol. Other researcher was concluded of 3% (v/v) glycerol for *P. aeruginosa UCP0092* to produce biosurfactants. Glycerol is a simple fatty acid precursor that is highly soluble in medium, making it simple for bacteria to use as a source of carbon and energy (Silva *et al.*, 2010)**.**

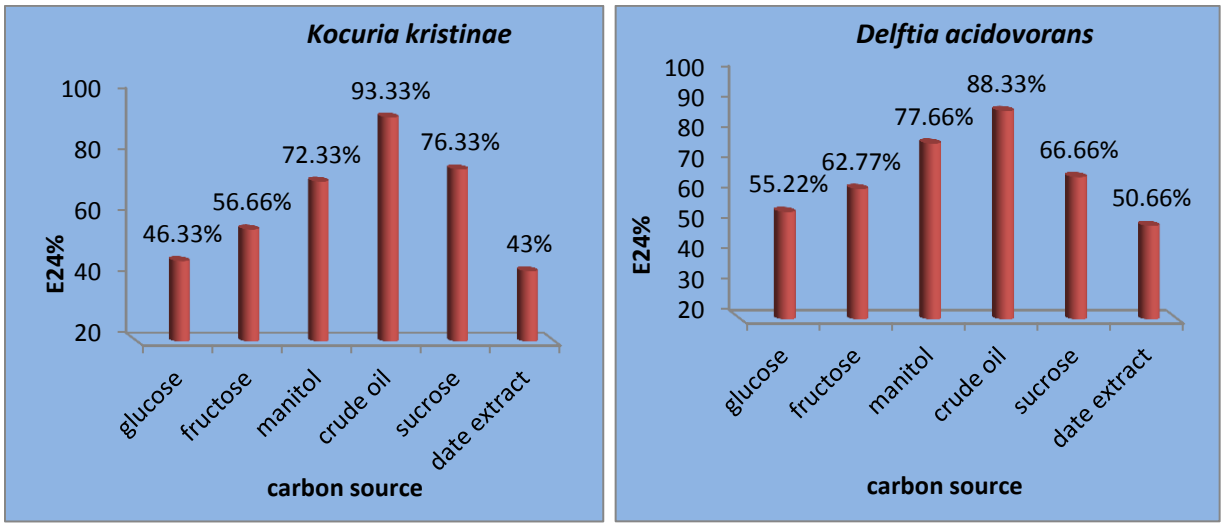



**Figure 4-14:- Effect of carbon sources on biosurfactant production**

### 4.5.4 Effect of carbon source concentration on biosurfactant production

The best conditions for the biosurfactant production of *K. kristinae* and *D. acidovorans* were found using various concentrations(0.5,1,1.5,2,2.5,and 3%) of the ideal carbon source (crude oil ). The results shown that the carbon source concentration reached its optimum level, there were dramatic changes in the emulsification index, which reached to its best values of 93.33 and 96.55% at a concentration of 1%. The emulsification index was an indicator of biosurfactant production. Figure (4-15)

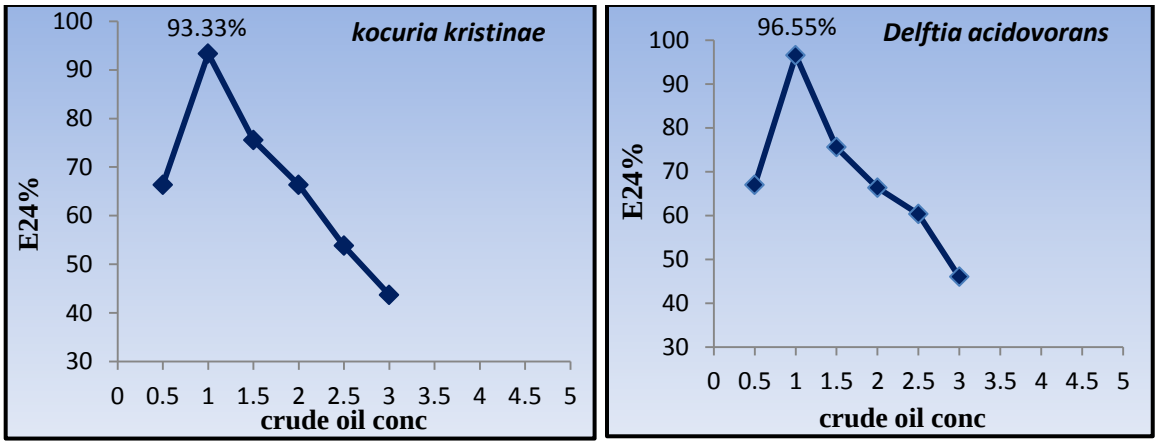



**Figure 4-15: Effect of different concentrations of crude oil on biosurfactant production by *K.kristinae* and *D.acidovorans***

While the higher concentration of crude oil may have an adverse effects on the bacterial development and/or fail to stimulate the bacterium to generate a biosurfactant, the low concentration of crude oil may support the growth of this bacterium and induce it to produce a biosurfactant. The availability of carbon sources and the equilibrium between carbon and other nutrients are key factors in the synthesis of biosurfactants. The growth and generation of biosurfactants by

diverse microorganisms depend on carbon sources, which vary from species to species. Ascomycetes are mostly produced using soybean oil, crude oil, agricultural industrial waste, hydrocarbons, and glucose as carbon sources (Reis *et al.*,2018; Adnan *et al.*,2018; da Silva *et al.*,2021).

### 4.5.5 Effect of different nitrogen sources on biosurfactant production

Six nitrogen sources($(NH_4)_2SO_4$, peptone, $NH_4Cl$, yeast extract, urea, and $NH_3NO_3$) were employed to test the impact of various nitrogen sources on *K. kristinae and D. acidovorans'* production of biosurfactants. Results have shown that the production of biosurfactant were differed depending on the nitrogen source .The highest Ei24% (93.66 and 95.33%) for *K. kristinae* and *D. acidovorans* were obtained when an ammonium sulfate $(NH_4)_2SO_4$ was used, and this may attributed to the simplicity of $(NH_4)_2SO_4$ as a nitrogen source and easy to uptake by bacterium (Figure 4-16). In contrast to other nitrogen sources, peptone and $NH_4NO_3$,urea,$NH_4Cl$,and yeast extract had the lowest emulsification activity when compared with ammonium sulphate for two isolates. In addition to being necessary for the bacteria's metabolic pathways to be completed, nitrogen is also crucial for microbial development because it is used in the synthesis of proteins and enzymes (Faqri *et al.*,2019).

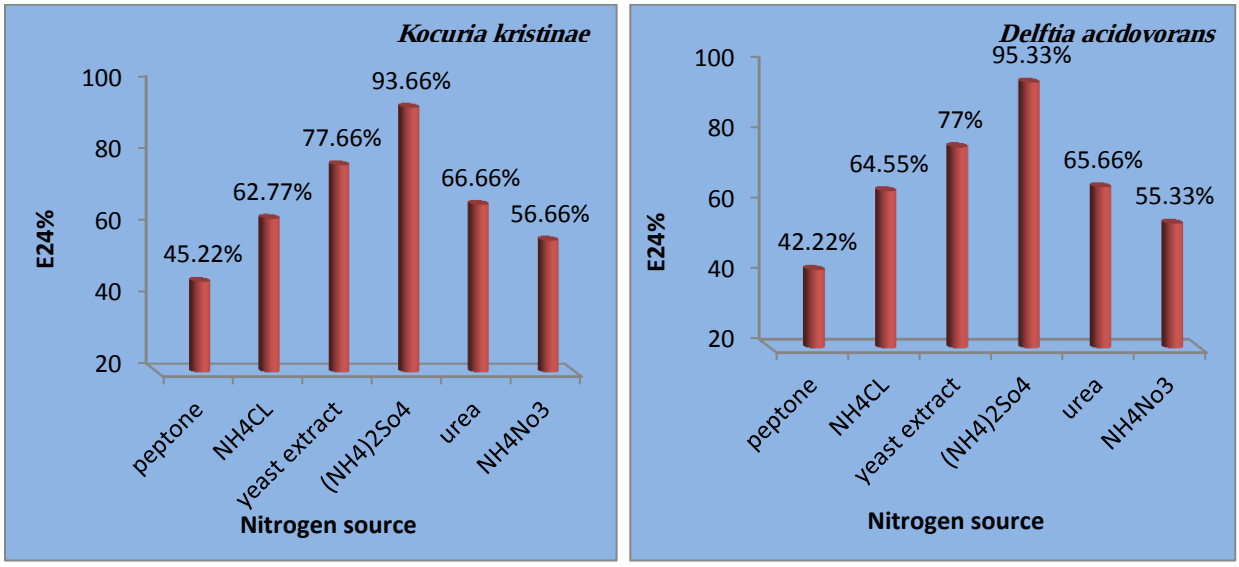



**Figure 4-16:Effect of nitrogen sources on biosurfactant production**

According to Okoliegbe and Agarry (2012), nitrogen is necessary for bacteria to complete their metabolic pathways and is also crucial for microbial growth because it is required for the synthesis of enzymes and proteins. A similar phenomenon was observed in the strain *Bacillus subtilis* RI4914, for which NH4NO3 was the best nitrogen source for biosurfactant production (Fernandes *et al.*, 2016). The fermentative production of biosurfactant, acid matter, and gas during the metabolism of microorganisms can decrease the viscosity and increase the fluidity of crude oil (She *et al.*, 2019). The main nutrient required by bacteria to produce biosurfactants is nitrogen. The creation of biosurfactants as a mineral medium has utilized a variety of organic and inorganic nitrogen sources. Yeast extract, a nitrogen source, also has a significant role in the formation of biosurfactants, which is in contrast to our findings (Cicatiello, *et al.*,2019).

## 4.5.6 Effect of nitrogen source concentration on biosurfactant production

*Kocuria kristinae* and *Delftia acidovorans* experimented with various ammonium sulphate concentrations (0.1,0.2,0.3,0.4,0.5,and 0.6) to find the one that produced the most biosurfactant. Results were indicated that (0.4%) (w/v) of $(NH_4)_2SO_4$ the best concentration that given Ei24% (93.33 and 96.55%) for *K. kristinae* and *D. acidovorans*, respectively ,Figure (4-17). Additionally, the results demonstrated that when the concentration of $(NH4)_2SO4$ was less than 0.4%, the emulsification index decreased. Nitrogen is important in the biosurfactant production medium because it is essential for microbial growth as protein and enzyme syntheses depend on it. Different nitrogen compounds have been used for the production of biosurfactants such as urea peptone, yeast extract, ammonium sulphate, ammonium nitrate, sodium nitrate, meat extract and malt extracts. Though ammonium sulphate is the most used nitrogen source for biosurfactant production, its usage with respect to concentration is organism and culture medium dependent. Ammonium salts are preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffineus* whereas nitrate supports maximum surfactant production in *Pseudomonas aeruginosa* (Varjani and Upasani, 2017). Different substrates can be employed to achieve microbial production of biosurfactants, however the composition and properties of the biosurfactants are determined by the types of carbon and nitrogen sources utilized (Fontes *et al.*, 2008). Therefore, investigation into the nutritional needs and production parameters of microorganisms is crucial. The effects of nitrogen supply and inducer type on the synthesis of biosurfactants using microorganisms isolated from diesel oil-contaminated soil (soybean oil and diesel oil). The greatest results were

achieved without the use of ammonium sulfate as a nitrogen source and 1% soybean oil as an inducer (Decesaro *et al.*, 2013).

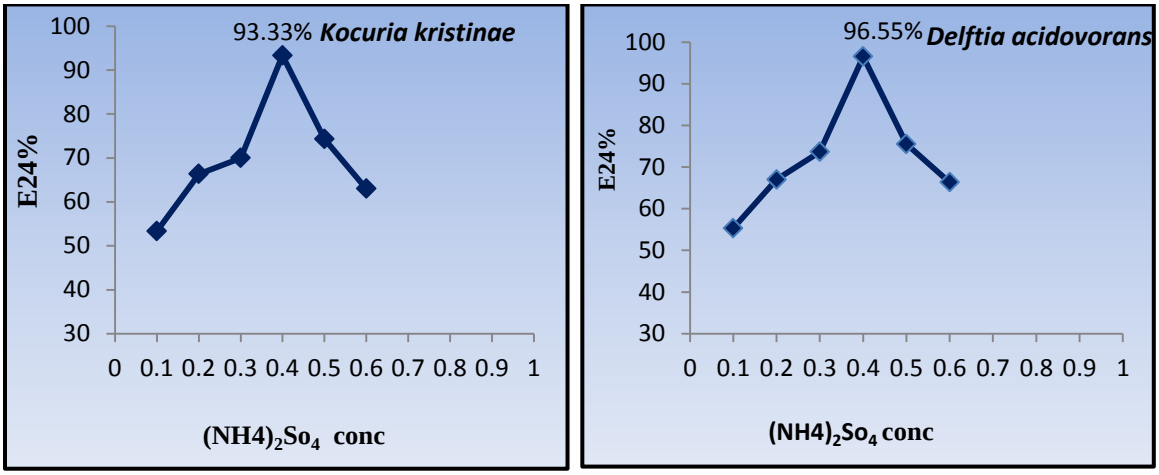



**Figure 4-17: Effect of ammonium sulphate concentration on biosurfactant production by *K.kristinae* and *D.acidovorans***

## 4.5.7 Effect of incubation period on biosurfactant production

*Kocuria kristinae* and *Delftia acidovorans* were grown on MSM medium for different incubation period(24,48,72,96,120,144,168,and 192 hrs.). The results were showed incubation for 7 days (168 hours) the most suitable for maximum biosurfactant production, in which, the highest Ei24% for these species was 98.88 and 96.33% ,respectively (Figure 4-18). Then it was reduced sharply in 192 (8 days) for both *K.kristinae* and *D.acidovorans*

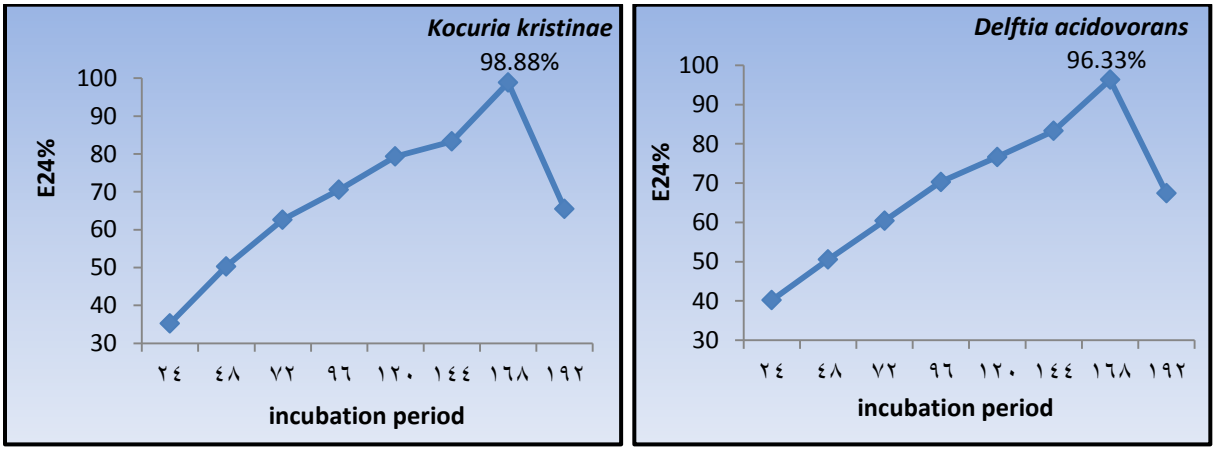



**Figure 4-18: Effect of incubation period on biosurfactant production**

In late exponential growth or stationary phase, biosurfactants are typically formed as secondary metabolites. They could either be expelled extracellularly into the surrounding media or maintained intracellularly (Kazim *et al.*, 2017). Through solubilization and desorption, the biosurfactants created help the microbe access insoluble substrates (Mahdy *et al.*, 2012). Additionally, they increase the hydrophobic surfaces' surface area and modify the attachment or exclusion of microbes from surfaces (Saravanan and Vijayakumar, 2015). According to the research provided by Khopade *et al.*, in (2012), biosurfactants are the main metabolites linked to the expansion of cell biomass. Moreover, Amaral *et al.*, (2006) found that while some species can produce biosurfactants during the exponential phase, most of them are produced during the stationary period of culture growth. On the other hand, some biosurfactants are reportedly created as secondary metabolites, according to Barakat *et al.*, (2017). The period of microbial growth is crucial for this since it affects how their metabolites are produced. In the current investigation, biosurfactant production was assessed during the course of an 8-day incubation period at various stages. The activity of the biosurfactant gradually decreased after the ninth day. Following an incubation period of ten

days, the activity reduction was clearly identified. Depending on the strain, the maximum biosurfactant production in the current study was discovered at day 7.

In several other analyses, a comparable incubation time of maximum output was also recorded (Yaraguppi *et al.*,2020). According to Bidlan *et al.*, (2007), *Serratia. marcescens DT-1P* produced more biosurfactant during the course of the incubation period, starting at an early stationary phase and peaking on day 8 after which both growth and biosurfactant production decreased. On the other hand, *Geobacillus pallidus* needed 14 days to produce biosurfactant (Zheng *et al.*, 2011). The greatest emulsification index (Ei24%) for the biosurfactant synthesis by *Pseudomonas sp. strain LP1* was 80.33 1.20 on day 8 of incubation, indicating that the biosurfactant production is growth-associated (Obayori *et al.*, 2009). The biosurfactant biosynthesis was reported to have occurred by Bonilla *et al.*, (2005), perhaps as a result of the creation of secondary metabolites that might have inhibited the formation of emulsions and the adsorption of surfactant molecules at the oil-water interface. *Acenitobacter calcoaceticus RAG-1* produces the most emulsan when in the stationary growth phase (Ali *et al.*,2016). While Astuti *et al.*, (2019), shown that the biosurfactant biosynthesis utilizing olive oil took place mostly during the exponential growth phase, indicating that the biosurfactant was created as a main metabolite accompanying the creation of cellular biomass (growth-associated kinetics). After 108 hours of incubation, Faqri *et al.*, (2019), achieved the highest level of biosurfactant production, 10.6 g/L. The biosurfactant production increased over time.

## 4.6 Extraction of biosurfactant compound:

*Kocuria kristinae* and *Delftia acidovorans* were cultivated on a MSM made at the optimum conditions which were pH7, 1% crude oil as a sole source of carbon and 0.4% ammonium sulphate as the source of nitrogen, at 35°C, with

shaking (150 rpm) for 7 days. After that, biosurfactant was extracted using three different methods (Chloroform, Ethyl acetate, and HCl) .The extraction with Ethyl acetate for *D.acidovorans* gave a better biosurfactant activity, which showed (Ei24% 95.6%) as in Figure(4-19), while *K.kristinae* was gave the best biosurfactant activity (Ei24% 96.3%) with HCL that showed in Figure (4-20).

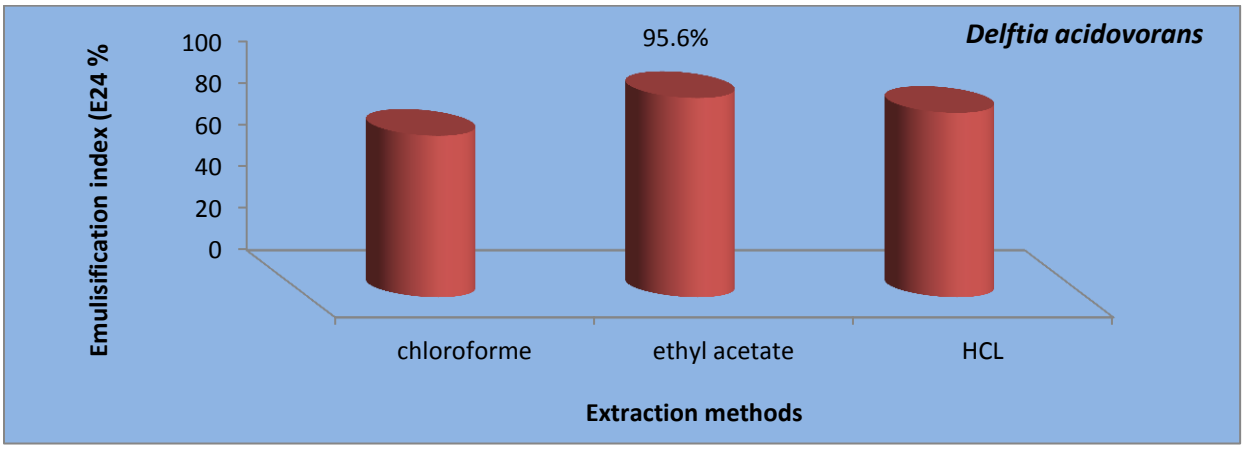



**Figure 4-19: Extraction of biosurfactant produced by *D.acidovorans* using different methods**

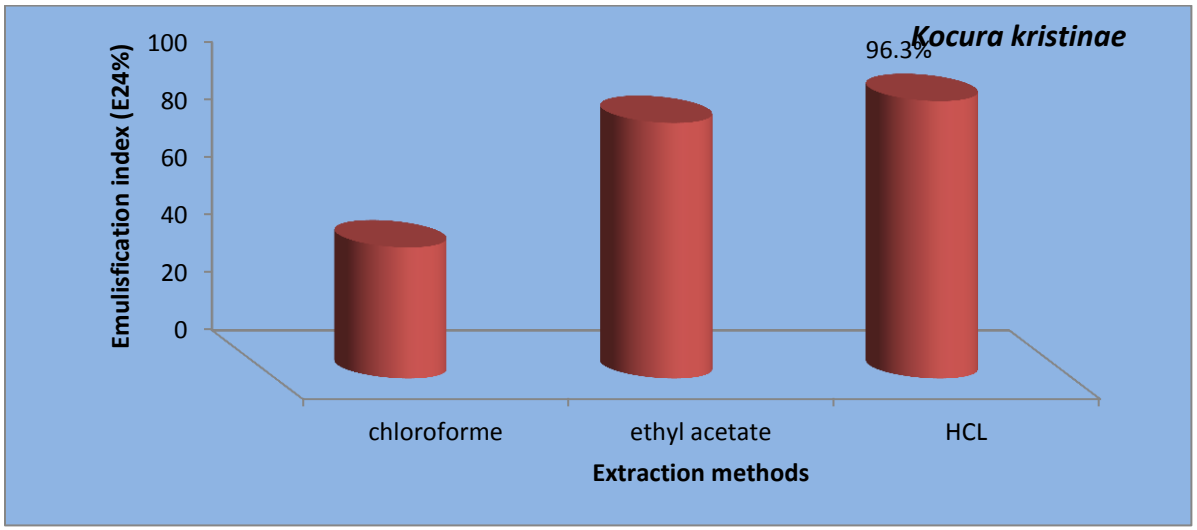



**Figure4-20 :Extraction of biosurfactant produced by *K.kristinae* using different method**

## 4.7 Purification of biosurfactant by using RP-HPLC

High performance liquid chromatography (HPLC) is one of the best methods for identifying and removing biosurfactants .The purity of the surfactin-type biosurfactant generated by *D. acidovorans* and *K. kristinae* was demonstrated by the appearance of a distinct single peak during HPLC. *K. kristinae* and *D. acidovorans* were purified and their biosurfactant material was analyzed using a high-performance liquid chromatography (HPLC) system; the results were revealed two peaks for biosurfactant from *K.kristinae* while *D.acidovorans* gave single peak Figures (4-21) and (4-22), respectively. The pure peak that acquired in this study after preparative HPLC indicate that the extract method is very selective to biosurfactant by bacteria. Also, this referred that one pure isolate bacteria usually produced one to few kind of biosurfactant. The primary reason that reversed phase chromatography is employed is because it separates proteins based on their polarity (Symmank *et al.,* 2002; Aguilar, 2004). The emulsification index for each fraction was then calculated after collecting all of the fractions. Zhao *et al.*, (2013) employed silica gel column chromatography to separate rhaminolid, a biosurfactant chemical produced by *P. aeruginosa*, from other biosurfactant compounds. In addition, Thanomsub *et al.*, (2006) used silica gel column chromatography in conjunction with progressive washings in hexane, chloroform, ethyl acetate, and methanol to purify the rhamnolipids generated by *P. aeruginosa B189*. Bacterial biosurfactants are often peptides with a tiny lipid component, and methods including gel permeation, hydrophobic interaction, and ion exchange are typically used to remove various bacterial species from cell-free fermentation broth. Ion exchange chromatography has been described for the purification of biosurfactants in earlier investigations (Tene *et al.*,2018). Using DEAE anion

exchanger chromatography, another biosurfactant that resembles lipopeptides was purified before being run through an HPLC or a HiTrap Q system. It was previously observed that microbial communities' rates of hydrocarbon breakdown increase as a result of their adaption to hydrocarbons (Phulpoto *et al.*,2020). Previous investigations have shown that biosurfactants in oil-polluted soil can emulsify the oily hydrocarbon molecules by improving solubility and reducing surface tension. In earlier reports, Sephadex was utilized as the matrix and molecular sieve chromatography was also used to resolve the low molecular mass biosurfactant (Moyne *et al.*,2001). The antibiotic biosurfactant peak was separated from other chromatographic peaks using ion exchange chromatography, which is also particularly effective at removing colored contaminating compounds from the biosurfactant fraction. Multiple techniques were utilized to verify a biosurfactant molecule's purity (Moghimi *et al.*,2017). In comparison to earlier results, the pure surfactin biosurfactant molecule showed more engine oil breakdown capacity (Pathak *et al.*,2012).

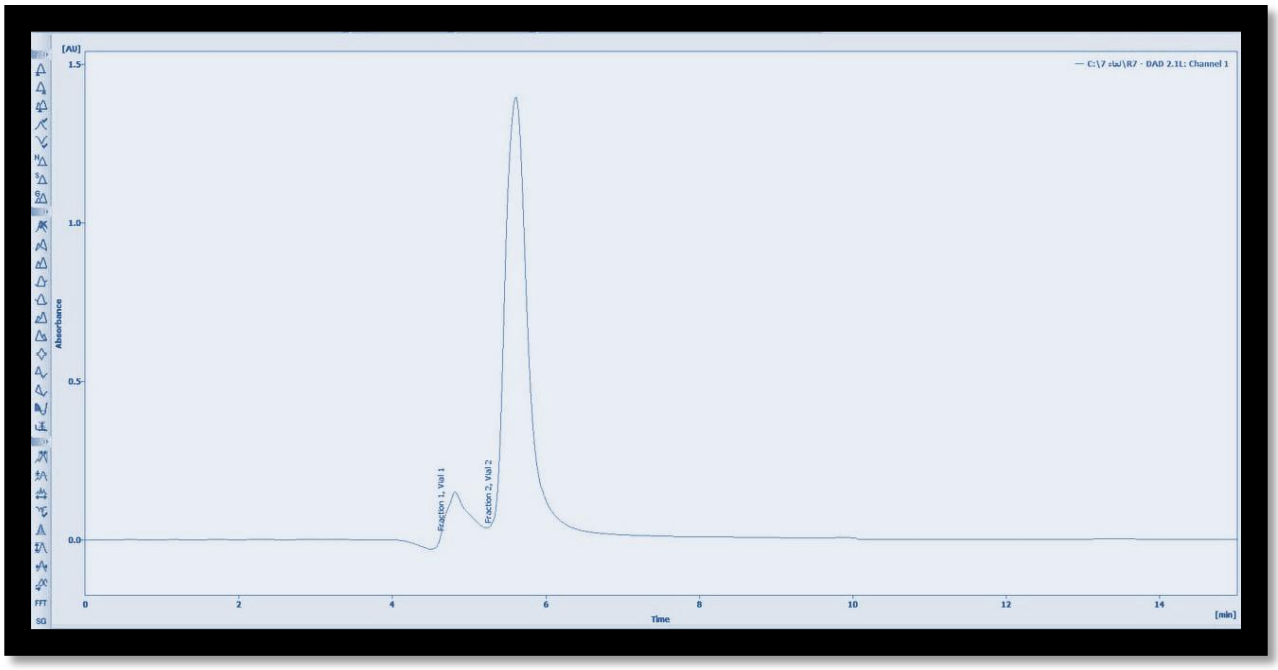



**Figure 4-21: Purification of biosurfactant by using HPLC chromatography from *K.kristinae***

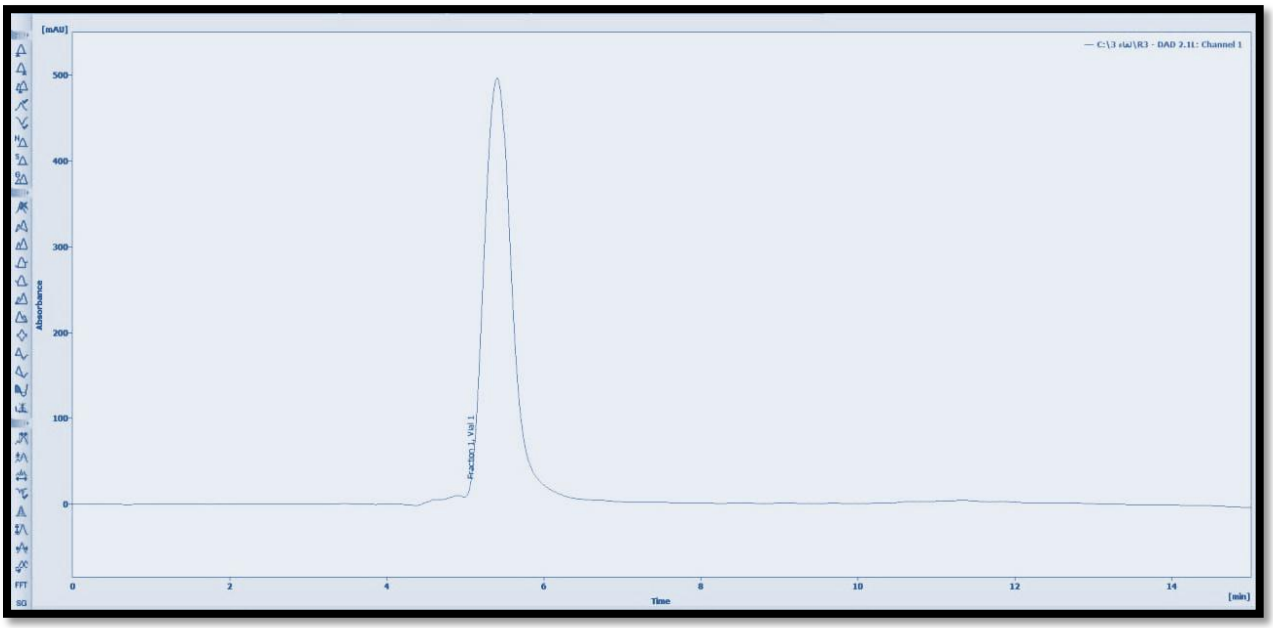



**Figure 4-22: Purification of biosurfactant by using HPLC chromatography from *D.acidovorans***

## 4.8 Biochemical Composition of the Isolated Biosurfactant

The biosurfactant produced in partial-purified form by *K. kristinae* and *D. acidovorans*, respectively. According to the findings, the partially purified biosurfactant had no carbohydrate content. Using a Bradford standard curve to measure the protein's absorbance, the analysis showed that the protein concentration was (71.066 mg/ml) and small amount of lipid. The findings demonstrated that protein and lipid constituted the majority of the biosurfactants generated by *K. kristinae and D. acidovorans*(Lipo glycoprotein).According to these findings, which are in line with those of Jagtab et al. (2010), 46% of *Bacillus stearothermophilus's* content is made up of protein. Additionally, Amaral *et al.,* (2006) showed that Yansan from *Yarrowia lipolytica IMUFRJ 50682* has a low lipid content polysaccharide-protein complex, and that the protein content of this polymer is crucial to the polymer's ability to emulsify. In the presence of glucose as a carbon source, Sarrubbo *et al.,* (2001) generated a bioemulsifier from *Y. lipolytica* that contained 47% protein, 45% carbohydrate, and 5% lipids. According to Zheng *et al.,* (2011), the bioemulsifier created by *Geobacillus pallidus* was primarily composed of carbohydrates (68.6%), followed by lipids (22.7%), and protein (8.7%). The chemical study of the bioemulsifier from *Acinetobacter* was discovered by Jagtap *et al.,* (2010) to be a proteoglycan comprising protein (53%), polysaccharide (43%), and lipid (2%). Compared to other bioemulsifiers, Kokare *et al.*, (2007) noted that marine *Streptomyces sp. S1* produced one that was 82% protein, 17% polysaccharide, and 1% reducing sugar.

## 4.9 Polyacrylamide gel electrophoresis of purified biosurfactant

In order to investigate the purity of biosurfactant which is purified from *K.kristinae* and *D.acidovorans*, polyacrylamide gel electrophoresis(PAGE) under

denaturing conditions was done. The results were exhibited the sample which produced from HPLC has molecular weight 70KD.This samples belong to *D.acidovorans*. Figure (4-23).

On the other hand, the purity of biosurfactant which is purified from *K.kristinae* which represent other samples (two samples), polyacrylamide gel electrophoresis was done under denaturing conditions. The electrophoresis involve two samples produced from HPLC chromatography, first purified sample was given two protein bands seemed with different molecular weight (72 and 65 KD) while one band appeared in the other sample, with molecular weight 71KD as shown in Figure (4-23).The two potent isolates *K.kristinae* and *D.acidovorans* were grown under different conditions. Results showed that slightly changing in protein between two isolates ,this means that the two isolates had metabolic system to maintain with extreme environmental conditions. Gel electrophoresis of biosurfactant-producing bacteria revealed that SDS-PAGE analysis was used to determine the purity of biosurfactant obtained after purification (Hu *et al.,* 2015).

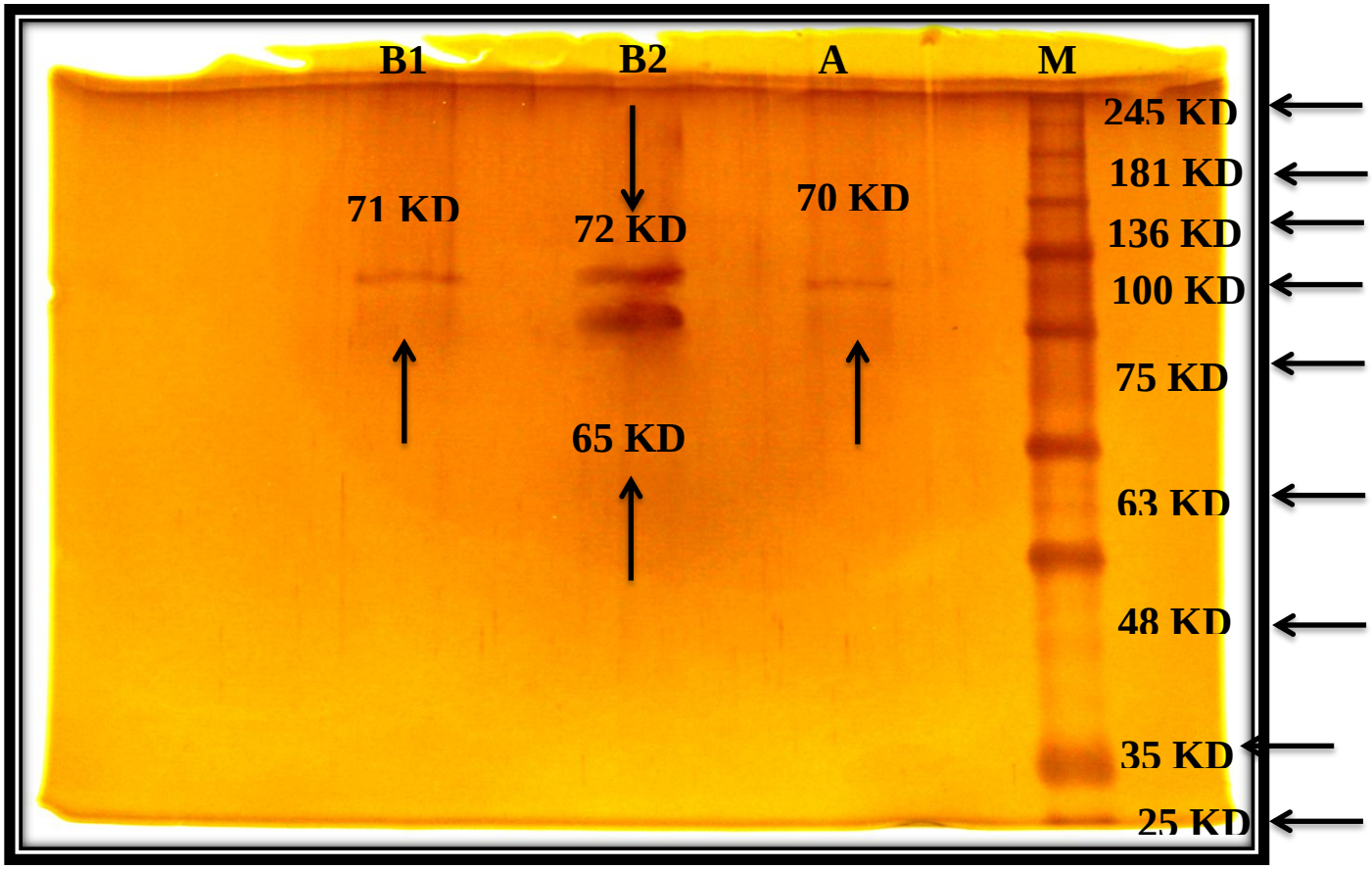



**Figure 4-23: Polyacrylamide gel electrophoresis of the biosurfactant under denaturing conditions, since M=molecular weight marker, A= purified biosurfactant produced from HPLC chromatography of *D.acidovorans*, B1= represent first portion of purified biosurfactant produced by *K.kristinae* . B2= represent second portion of purified biosurfactant produced by *K.kristinae***

## 4.10Characterization of partial purified biosurfactant compound:

The partial and/or pure biosurfactant was subjected to FTIR analysis in an effort to finish the chemical characterization of the biosurfactant produced by *K. kristinae* and *D. acidovorans*.

### 4.10.1 Analysis with Fourier Transform Infra-Red (FTIR)

Fourier Transform Infra-Red is an effective tool for identifying chemical compounds by their functional groups. Both samples (*K. kristinae and D. acidovorans*) were characterized by using FTIR. By identifying the functional groups that are present in the substance, the chemical nature of the bioactive compound, or protein, produced by these isolates has been identified, Figure (4-24 and 4-25).At various wave length, many bands were seen. There was an asymmetric stretch between the C-H of the CH3, CH2 group, and a symmetric stretch between the C-H of the CH2 group, as evidenced by the broad absorption peaks at 2952 cm-1, 2852 cm-1, and 2359 cm-1, respectively. Asymmetric stretch of CH3 and CH2, which occurs more frequently than symmetric stretch of CH2, indicated the presence of an aliphatic chain. The presence of the carbonyl group (C=O) of the functional group of esters and carboxylic acid, respectively, was reflected by the peaks 1716 cm1 and 1652 m1,1541. Peaks between 722 cm1 and 1258 cm1 revealed C-C stretch of skeletal vibrations, while the peak at 1455 cm1 proved the presence of C=H stretch. The O-H band was found between 1440 and 1395 cm 1 and 950 and 910 cm 1. Strong absorption is visible in FTIR spectra between 2952.42 cm1 and 2852.42 cm1. This could be explained by the polysaccharide's O-H groups, which show the presence of a lengthy aliphatic chain congaing -CH, -CH2, and -CH3, like the fatty acid chain of the lipopeptide. At

various wavenumbers, many bands were seen. The carbonyl stretch (C=O) emerged between 1760 and 1690 cm1, the C-O stretch appeared between 1320 and 1010 cm1, the O-H band was found between 1440 and 1395 cm1 and between 950 and 1010 cm1, and the C-H bending appeared between 2800 and 3100 cm1. The COOH group was then detected (Liu *et al.,* 2015). The C=O sharp band was deformed by CH3 stretching brought on by the presence of 2937 cm-1 and 1629 cm-1. the CH3 bending mode mixed with the 1400 and 1109 cm1 from the stretching vibration mode of C-O, respectively. The existence of glycolipid biosurfactants is confirmed by the presence of an aliphatic hydrocarbon and a glucose moiety (Silva *et al.,* 2014).

The protein and lipid *from K. kristinae* and *D. acidovorans* had similarities in their FTIR spectra to lipopeptides made by *Bacillus amyloliquefaciens SAS-1* and *B. subtilis BR-15* that had been previously reported (Sharma *et al.,* 2018). The amide group (C=O) is located between 1680 and 1630 cm 1, although the major amides' N-H group (-NH2) spans two bands close to 3350 and 3180 cm 1. N-H bending occurs for both primary and secondary amides between 1640 and 1550 cm 1. The secondary amides contain one band at 3300 cm 1. Oleic acid esters showed C-O esters carboxylic acids at 1300-1000, while normal aliphatic esters showed C=O stretch at 1750-1735 cm 1. As a strong band, the carbonyl stretch (C=O) of carboxylic acid could be seen from 1760 to 1690 cm 1, the C-O stretch could be seen between 1320 and 1210 cm 1, the O-H band could be seen between 1440 and 1395 cm 1 and 950–910 cm 1, and the C–H bending bands could be seen between 2800 and 3100 cm 1. At 1351 cm 1, a CH2 group near to a carboxyl ester vibrated in a similar manner (Singh and Tiwary, 2016). By observing peaks in the range of 2850-2950 cm 1, -C-H stretching in the form of CH3 and CH2 groups in alkyl chains was discovered, proving the existence of

aliphatic chains. The portion's aliphatic chains (-CH3, -CH2-) are reflected in the distortion ambiances between 1411 and 1270 cm 1. This distinctively identified fatty acid present as lipopeptide. In the range of 3000-2700 cm 1, -CH2 and -CH3 group C-H stretching bands were seen. Alkyl groups were also proven to exist by the deformation vibrations at 1467 and 1379 cm 1 (El-Sheshtawy *et al.*, 2015). The FTIR spectrum also displayed a band at 1027 cm-1 that belonged to the polysaccharide. It was discovered that polysaccharide (C-O-C) stretching is related to the area between 1200-950 cm1 (Dean *et al.*, 2010). The most noticeable bands in the lipoprotein biosurfactant made by *Pediococcus dextrinicus SHU1593* were also visible, according to Ghasemi *et al.*, (2019), and they correspond to the NH group, C=O stretching in proteins (AmI band), and NH bending in proteins (AmII band), respectively, at wavelengths of 3000-3600 cm-1, 1640–1700 cm-1, and 1500–1620 cm-1. By using mass spectrometry, Abdalsadiq *et al.*, (2018) determined the structure of biosurfactants made from *Lactobacillus acidophilus* to be a lipopeptide complex with nine amino acids and C12–C17 -hydroxyl fatty acids. Only a small number of lactobacilli-produced lipopeptide biosurfactants have been examined using FTIR and X-ray photoelectron spectroscopy (Kaur and Kaur,2019).

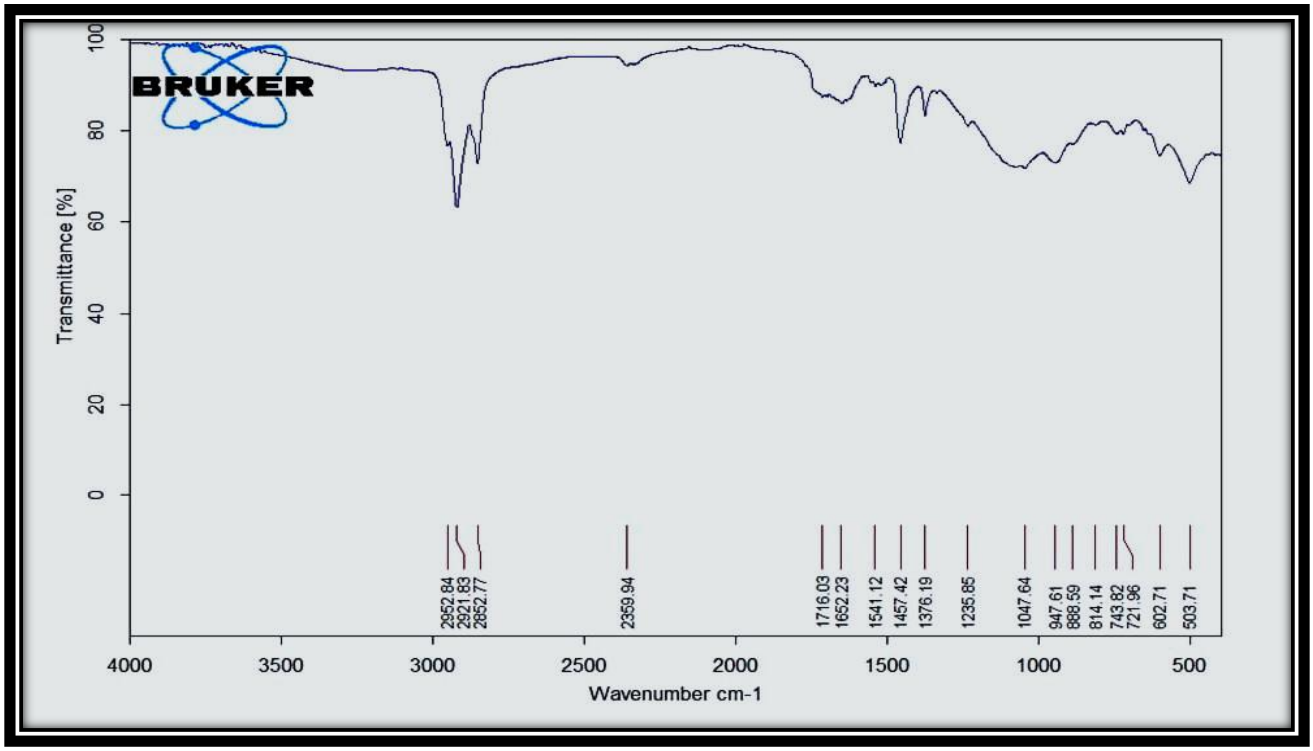



**Figure 4-24:FTIR analyses of biosurfactant produced by *K.kristinae***

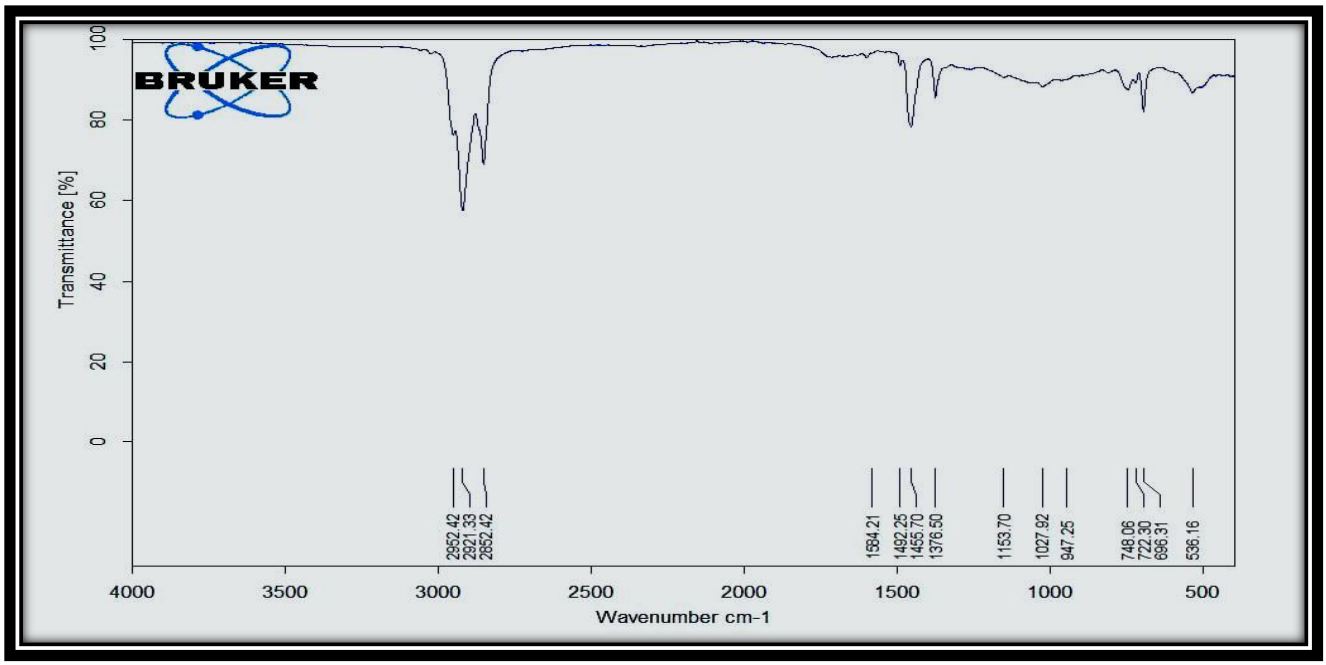



**Figure 4-25:FTIR analyses of biosurfactant produced by *D.acidovorans***

## 4.11 Molecular study

### 4.11.1 Detection of target genes responsible for contaminant biodegradation

In an attempt to amplify and characterize the gene responsible for biodegradation of the 7 Gram negative bacteria (*A.baumanni, P.putida, D. acidovorans, E.cloacae, P.aeruginosa, P.stutzeri, and P.spp*.) and 6 Gram positive bacteria (*M.luteus, S.haemolyticus, S.warneri, S.lentus, S.lugdunebsis* and *K.kristinae),* primers for P34O, C230, NidA, C12O, C23O, NahAc,Cat1,and sMO gene coded for biodegradation produced by isolates was studied.

### 4.11.2 Extraction of genomic DNA

Genomic DNA of 7 Gram negative bacteria and 6 Gram positive bacteria were extracted using method described in (3.11.1.1**)** PCR technique was used to amplify the gene coded for biodegradation. Based on the extinction coefficients of nucleic acids at 260 nm and 280 nm, a pure DNA preparation predicted an A260/A280 ratio of 1.8. (Green and Sambrook , 2012). In addition, these outcomes were shown when the DNA samples were examined using gel electrophoresis, where distinct DNA bands were found, indicating purified DNA samples as shown in Figure (4-26).

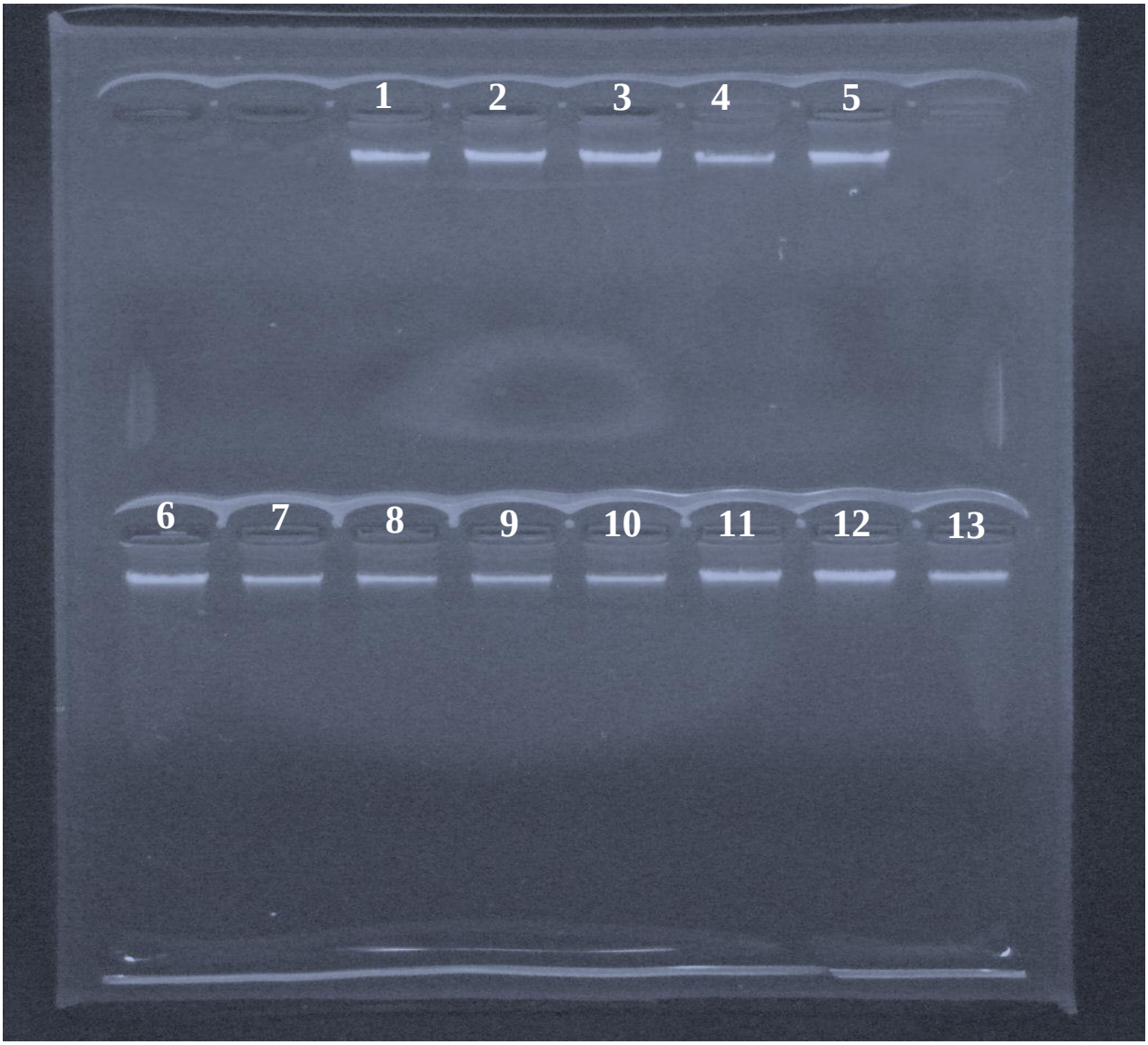



**Figure 4-26:gel electrophoresis for genomic DNA of *1.Ac.baumanni, 2.Ps.putida, 3.D.acidovorans, 4.E.cloacae, 5.Ps.aeruginosa, 6.Ps.stutzeri, 7.Pa.spp ,8.M.luteus,9. S.haemolyticus, 10.S.warneri, 11.S.lentus, 12.S.lugdunebsis.* and *13.K.kristinae* gel electrophoresis was performed on 1% agarose gel and run with 100 V for 60 minutes**

### 4.11.3 Detection of biodegradation genes by polymerase chain reaction technique (PCR)

The genomic DNA of Gram negative bacterial isolates (*Ac.baumanni, P. putida, D.acidovorans, E.cloacae, Ps.aeruginosa, Ps.stutzeri,* and *Pa.spp*.) and 7 Gram positive bacterial isolates (*M.luteus, S.haemolyticus, S.warneri, S.lentus, S.lugdunebsis , S.haemolyticus and K.kristinae*) were isolated and eight genes coding for biodegradation of PAH compound were amplified by PCR. The products of PCR were electrophoresed by agarose gel electrophoresis. Genomic DNA was used to amplify the gene coded for the biodegradation using a specific primer (Table 3-9). The results were showed in (Figure4-27,4-28,4-29,4-30,4-31,and 4-32) respectively, **gene *C23O*** that positive in 4 isolates (*K.kristinae, E.cloacae, Pa.spp,* and *Ps.stutzeri*) that have molecular weight 900 pb. **The second gene *C230*** that gave positive results in 6 isolates (*D.acidovorans, K.kristina, E.cloacae, S.haemolyticus, Pa.sp.* and *Ps.putida*) with molecular size 450 pb. **Third gene *C12O*** that have molecular size 350 pb which showed positive result in 5 isolates (*D.acidovorans, M.luteus, S.lugdunebsis, Ps.aeruginosa,* and *S.warneri*). **The fourth gene *Cat1*** that have molecular size 650 pb, this gene give positive result in 3 isolates (*S.warneri, Pa.spp,* and *Ps.aeruginosa).* **The fifth gene *nahAc*** *with molecular* 487 bp was showed negative results for all isolates. **The sixth gene *NidA*** with molecular size 1400 pb that showed positive result in 3 isolates (*D.acidovorans, K.kristinae, and E.colacae).***The seventh gene *P34*** gene that have molecular size 800 pb, isolates were positive in this gene 6 (*D.acidovorans, S.lugdunebsis, K.kristinae, E.colacae, Pa.spp, and Ps.putida).* **Finally, last gene *sMO*** with molecular size 374, all isolates are negative. The specific activities of a bacterial strain can be tracked down using the catabolic genes. In current study,it

was focused on monooxygenase and dioxygenase, two important enzymes involved in the mineralization of aliphatic and PAH chemicals. Based on PCR amplification, the existence of genes in these isolates was discovered. It was established that the genes for catechol dioxygenase (C12O and C23O), monooxygenase (alkB and alkB1), and dioxygenase (NahAc) exist. Degradation of PAHs in the environment is becoming more important and fascinating since PAHs are severe pollutants and health risks that appear as complex mixes with both low and high molecular weights. High molecular weight is more difficult to decompose and requires specialized microbes, whereas low molecular weight is easier to do so. The creation of synthetic consortia employing a variety of recognized PAH degraders has not been successful in maximizing cooperation across various species (Ghazali, *et al.*,2004). Therefore, using enrichment techniques is the most effective strategy to find the promising strain that might use PAHs as the only carbon source (Hesham *et al.*,2006).

The fact that these discovered genes are largely conserved among many Gram-negative bacteria could be one explanation. However, since the PAH-RHD gene is conserved among Gram-positive bacteria, no signal for this gene was found (Cebron *et al.*,2008). Because the enzyme that this gene encodes not only degrades naphthalene but also mediates the degradation of other PAHs compounds, the naphthalene dioxygenase (NahAc) gene is of special importance as a marker for PAHs degradation (Lu *et al.*,2011). Because they cleave aromatic C-C bonds at either the ortho or meta position, *C12O* and *C23O* dioxygenase are essential to the metabolism of aromatic rings by bacteria. On the other hand, because short chain n-alkanes are immediately poisonous and serve as solvents for cellular membranes and lipids, it is important to look into the existence of catabolic genes (alkB and alkB1) (Seo *et al.*,2012). This findings showed that genes were present in certain

isolates but not all, indicating that this bacteria was crucial to the breakdown of PAH compounds and would be useful for petroleum chemical bioremediation in the environment.

Thirteen Gram-negative isolates had the NahAc gene found, and the sequences of Nah Ac-like genes were found in strains of *Pseudomonas brenneri*, *Enterobacter*, *Pseudomonas entomophila*, *Pseudomonas koreensis*, and *Stenotrophomonas* (Folwell *et al.*,2016). *Mycobacterium gilvum PYR-GCK* contains the four aromatic ring cleavage dioxygenase genes Phd F, Phd I, Pea G, and Pca H that are essential for the biodegradation of pyrene (Badejo *et al.*,2013). The following bacteria were found in mangrove sediment: *Microbacterium BPW*, *Novosphingobium PCY, Ralstonia BPH, Alcaligenes SSKIB*, and *Achromobacter SSK4*. Within two weeks, these strains break down more than 50% of 100 ugml-1 of phenanthrene. Pyrene is fully degraded by strains PCY and BPW. The presence of the pyrene dioxygenase gene's -subunit (NidA) in the capacity to degrade pyrene (Liang *et al.*,2019). Once identified by clone library analysis following polymerase chain reaction (PCR) detection of one or more of these catabolic genes from Gram-negative or Gram-positive bacteria, they operate as biomarkers for PAH breakdown and detoxification (Yang *et al.,* 2015). Previous research had examined the functional gene diversity in PAH-degrading bacteria, demonstrated the relationship between PAH-RHD genes and the level of PAH contamination, and explored the mechanisms of PAH degradation (Lu *et al.,* 2011;Song *et al.*,2015).

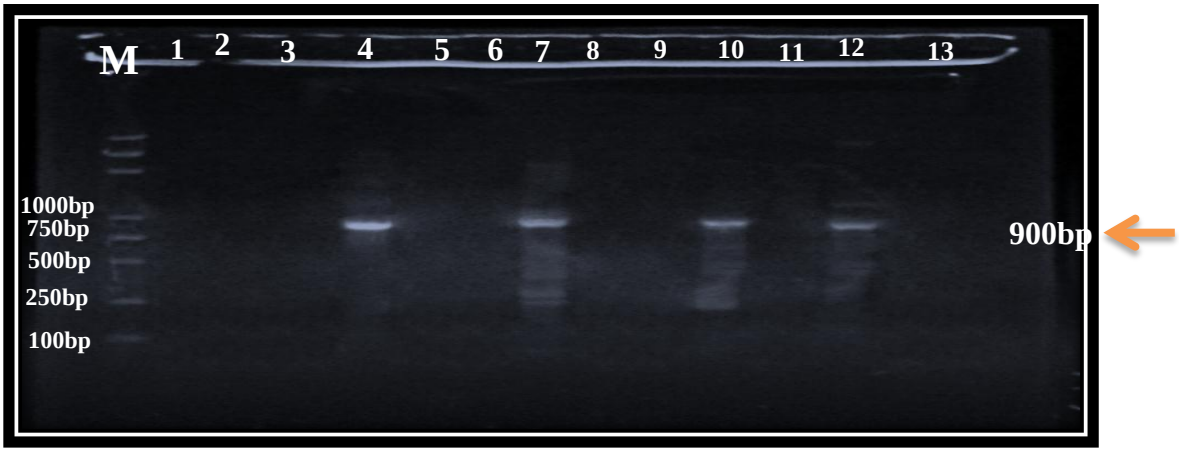



**Figure 4-27: Agarose gel electrophoresis of PCR amplified products of 13 bacterial isolates M= DNA ladder with molecular weight (100-5000bp),4 positive results for gene *C23O* with M.wt 900bp 4.*K.kristinae*, *7.E.cloacae*, *10.Pa.spp*, and *12.Ps.stutzeri***

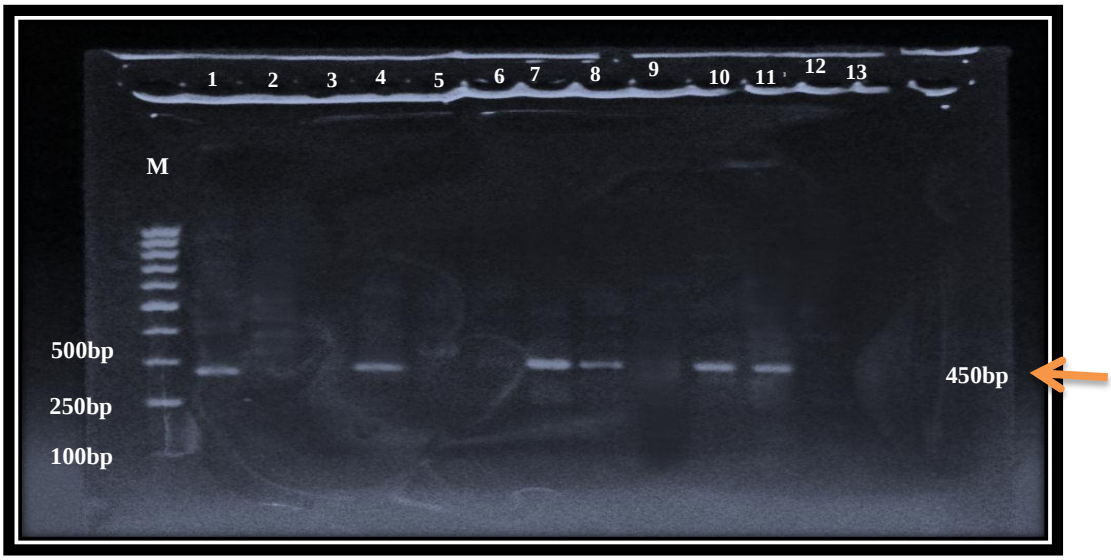



**Figure 4-28:Agarose gel electrophoresis of PCR amplified products of 13 isolates, M= DNA ladder with molecular weight (100-5000bp),6 positive results for gene *C230* with M.wt 450 bp (*1.D.acidovorans*, *4.K.Kristina*, *7.E.cloacae*, *8.S.haemolyticus*, , *10.Pa.sp.* and 11.*Ps.putida*)**

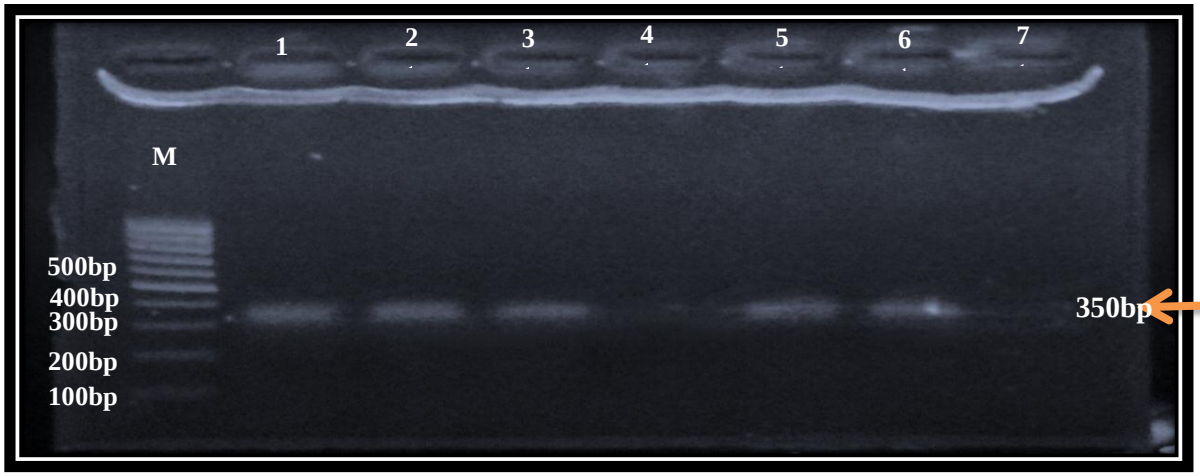



**Figure 4-29: Agarose gel electrophoresis of PCR amplified products of 13isolates, M= DNA ladder with molecular weight (100-2000bp),5 positive results for gene *C120* with M.wt 350bp (*1.D.acidovorans, 2.M.luteus, 3.S.lugdunebsis,5.Ps.aeruginosa, and 6.S.warneri***


**Figure 4-30: Agarose gel electrophoresis of PCR amplified products of 13 isolates, M= DNA ladder with molecular weight (100-5000bp),3 positive results for gene *Cat1* with M.wt 650bp (*6.S.warneri, 10.Pa.spp* and 12. *Ps.aeruginosa***

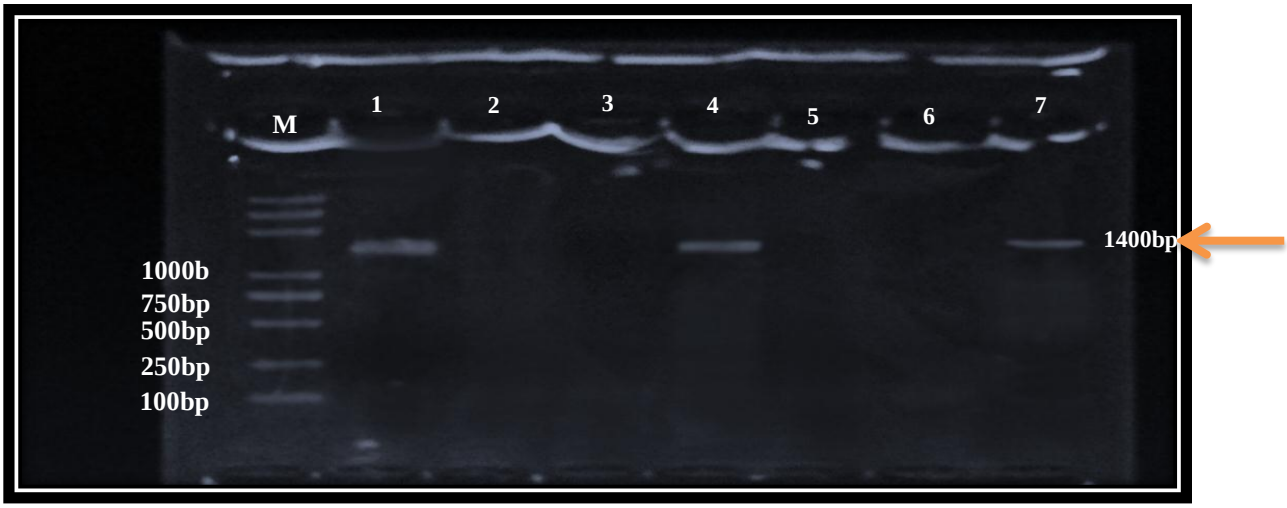



**Figure 4-31 : Agarose gel electrophoresis of PCR amplified products of 13 isolates, M= DNA ladder with molecular weight (100-5000bp),3 positive results for gene *Nid* with M.wt 14bp00 (*1.D.acidovorans*, *4.K.kristinae*, and 7.*E.cloacae***

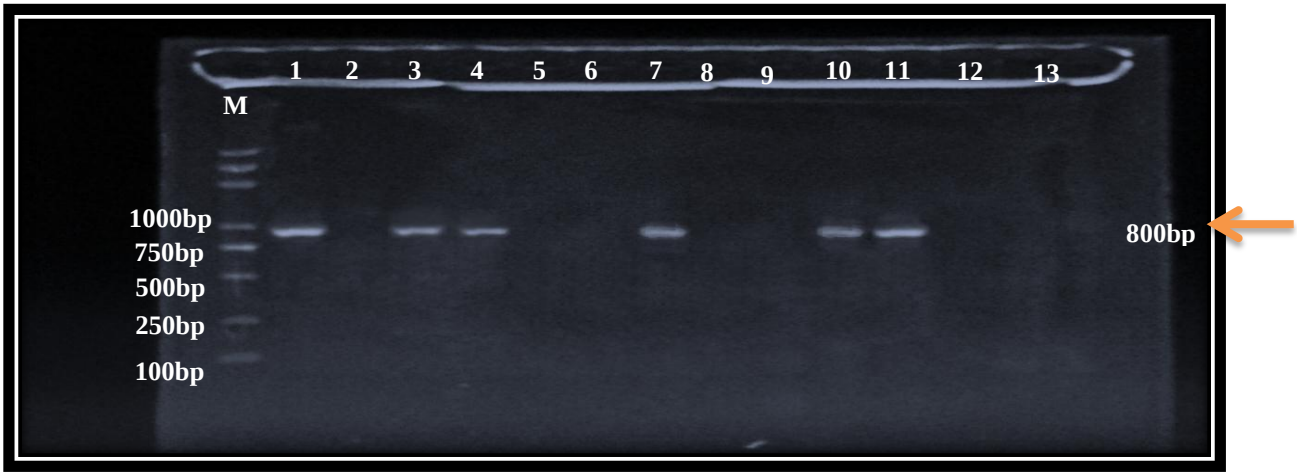



**Figure 4-32: Agarose gel electrophoresis of PCR amplified products of 13 isolates, M= DNA ladder with molecular weight (100-5000bp),6 positive results for gene *P34* with M.wt 800 bp00 (1.*D.acidovorans*, *3.S.lugdunebsis*, *4.K.kristinae*, *7.E.Colace*, *10.Pa.spp*, and *11.Ps.putida***

## 4.11.4 Molecular Identification and Phylogenetic Analysis Using 16S rRNA Gene Sequence.

The 16S rRNA gene sequences of all isolates were aligned and compared to the published 16S rRNA gene sequences in the GenBank database by BLAST search. Genomic DNA was then extracted from the isolated bacteria. Two different sets of PCR primers were used in this study. The first set of primers were 5′-CTACGGGGGGCAGCAG-3′ and 5′-GGACTACCGGGGTATCT-3′(Mori *et al.*,2014) .The second set of primers were 5′-GTTTGATCCTGGCTCAG-3′ and 5′-AAGGAGGTGATCCAGCC-3′(Kane *et al.*,1993). Within the first set of primers, three samples were included in the present study. These samples were screened to amplify the 16S rRNA sequences of *D. acidovorans, M.luteus,* and *S.lugdunensis* (Figure 4-33).

Other isolates were screened by second sets of primer (Figure 4-34).Thus, the variation of the 16S rRNA sequences can be used for characterization for these bacterial organisms due to its possible ability to adapt to variable genetic diversity as it was seen in different bacterial cases. After conducting NCBI BLASTn for these PCR amplicons, the sequencing reactions confirmed the specific identification. The NCBI BLASTn engine revealed around 99 to 100% sequence similarity between the sequenced samples and the intended reference target sequences for both sets of primers (465 bp and 1500 bp amplicons),these results was showed in Table (4-4) .Two comprehensive phylogenetic trees were generated in the present study according to nucleic acid variations observed in the amplified 465 bp and 1500~ bp of the 16S rRNA sequences amplicons. The first one was contained S1 to S3 samples to represent the amplified 465 bp , while the second tree was generated to represent S4 to S13 samples. Concerning the 465 bp–based

tree, S1 – S3 samples were aligned alongside other relative nucleic acid sequences of *S.lugdunebsis*, *M.luteus*, and *D.acidovorans* sequences(Figure 4-35). Within this tree, the investigated S1 – S3 samples were incorporated alongside other relative sequences to constitute three major clades of incorporated sequences within the cladogram. The S1, S2, and S3 were suited in the *D.acidovorans*, *M.luteus*, and *S.lugdunebsis* clades respectively. No other related sequences were found to be highly correlated to these incorporated sequences.

This data indicated the highly specific ability of 16S rRNA sequence-based amplicons to detect these bacterial sequences without including any noticeable homology with other sequences of other species. In this extensive tree, there were 69 aligned nucleic acid sequences altogether. The analyzed samples were grouped into three phylogenetic clades of three different phylogenetic sequences within the included bacterial sequences of this tree, according to the cladogram that was created. The capacity of the employed 16S rRNA sequences-based amplicons to classify the *D.acidovorans, M.luteus, and S.lugdunebsis* sequences into these reported phylogenetic distributions is connected with the most intriguing finding in our researched bacterial isolates. Within the clade of *D.acidovorans*, twenty-five sequences of the same species were incorporated, in which the S1 sample was suited. Within the clade of *M.luteus*, twenty-three sequences of the same species were incorporated, in which the S2 sample was suited. Within the clade of *S.lugdunebsis*, twenty-one sequences of the same species were incorporated, in which the S3 sample was suited. These sequences were exerted close positions from each other within the same major clade, and one phylogenetic position was observed among them. However, no nucleic acid variation was observed in S1. It seems that the utilized 16S rRNA sequences -based amplicons provide high accuracy in the detection and discrimination of this bacterial organism in high

accuracy . However, the incorporated sequences within in the vicinity to S1 – S3 samples within this clade showed the presence of various strains of the *D.acidovorans*, *M.luteus*, and *S.lugdunebsis* sequences with one variable origin. However, no obvious tilt was observed for the investigated S1 – S3 samples with respect to the incorporated reference sequences. This style of the positioning of the S1 – S3 samples indicated the absence of any evolutionary effect of the observed genetic variations in inducing any possible deviation in the evolutionary positioning of these bacterial samples.

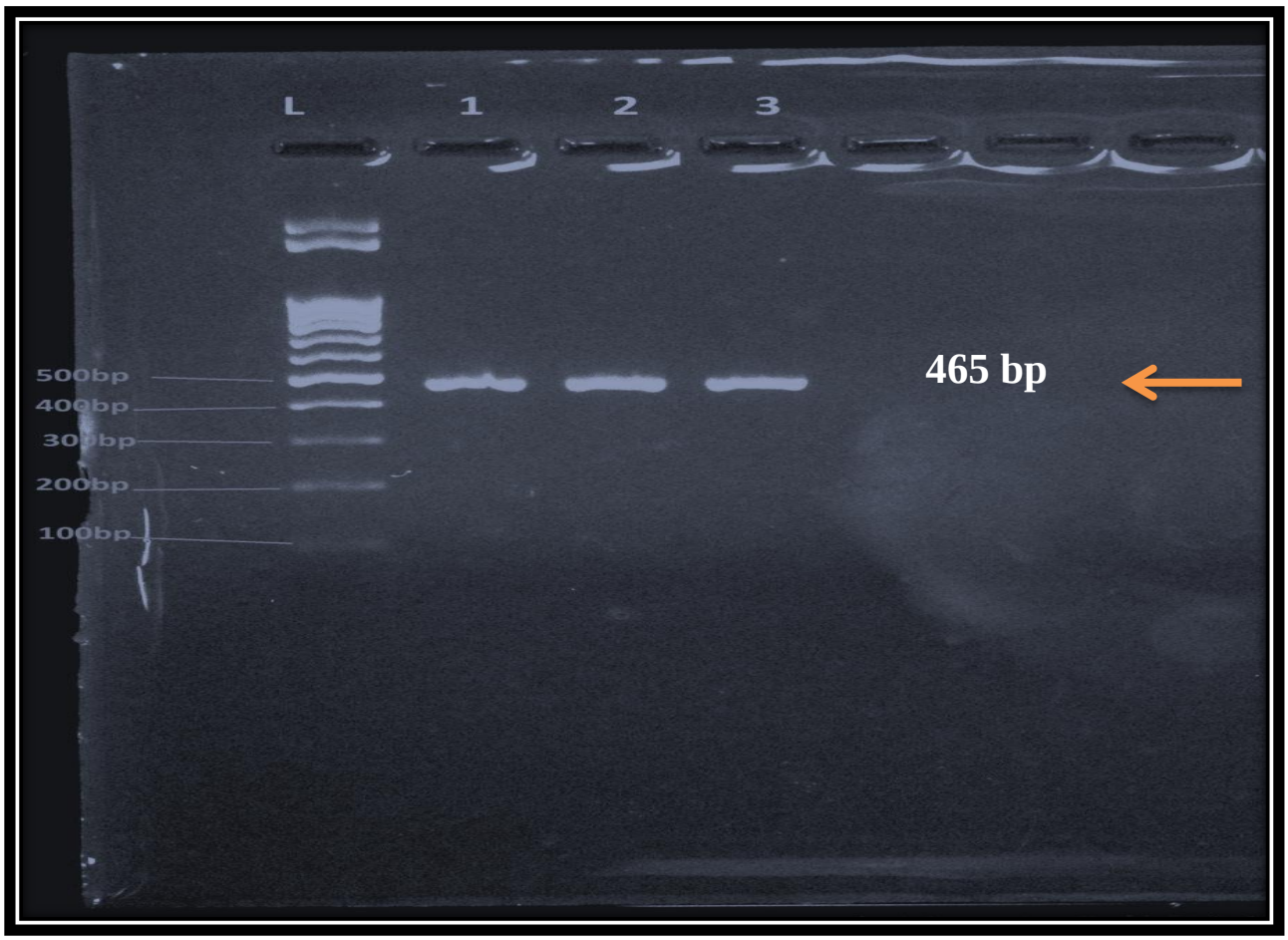



**Figure 4-33:Agarose gel electrophoresis to amplify the 16S rRNA gene sequences of *D.acidovorans, M.luteus,* and *S. lugdunensis* .**

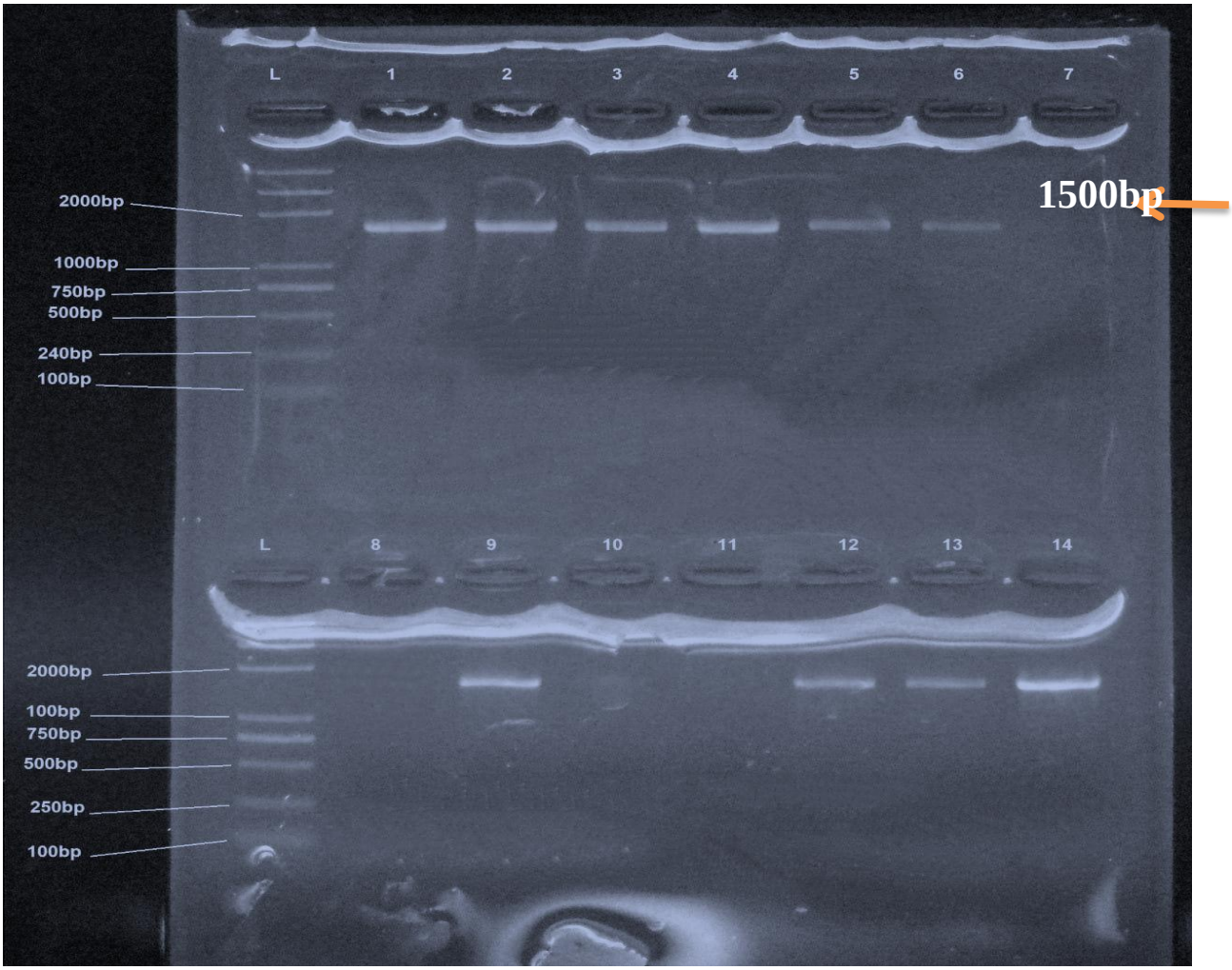



**Figure 4-34: Agarose gel electrophoresis to amplify the 16S rRNA gene sequences of *K.kristinae, S.warneri, S.lentus, S.heamolyticus, E.colacae, Ps.putida, Ps.aeroginosa, Ps.stutzeri, Pa.spp. and Ac.baumanni***

**Table 4-4: Phylogenetic affiliation of 16S rRNA partial sequences of Thirteen bacterial isolates**

| Isolate code | Closet related sequence (GenBank accession number) | Identity |
|---|---|---|
| S1 | *Delftia acidovorans* (OM838393.1) | 100% |
| S2 | *Micrococcus luteus*(OM838394.1) | 99% |
| S3 | *Staphylococcus lugdunebsis*(OM838395.1) | 99% |
| S4 | *Kocuria kristinae*(OM838480.1) | 100% |
| S5 | *Pseudomonas aeroginosa*(OM838481.1) | 99% |
| S6 | *Staphylococcus warneri*(OM838482.1) | 99% |
| S7 | *Enterobacter cloacae*(OM838483.1) | 99% |
| S8 | *Staphylococcus haemolyticus*(OM838484.1) | 99% |
| S9 | *Staphylococcus lentus*(OM838485.1) | 99% |
| S10 | *Pandoraea spp* (OM838486.1) | 99% |
| S11 | *Pseudomonas putida*(OM838487.1) | 99% |
| S12 | *Pseudomonas stutzeri* (OM838488.1) | 99% |
| S13 | *Acinetobacter baumanni*(OM838489.1) | 99% |

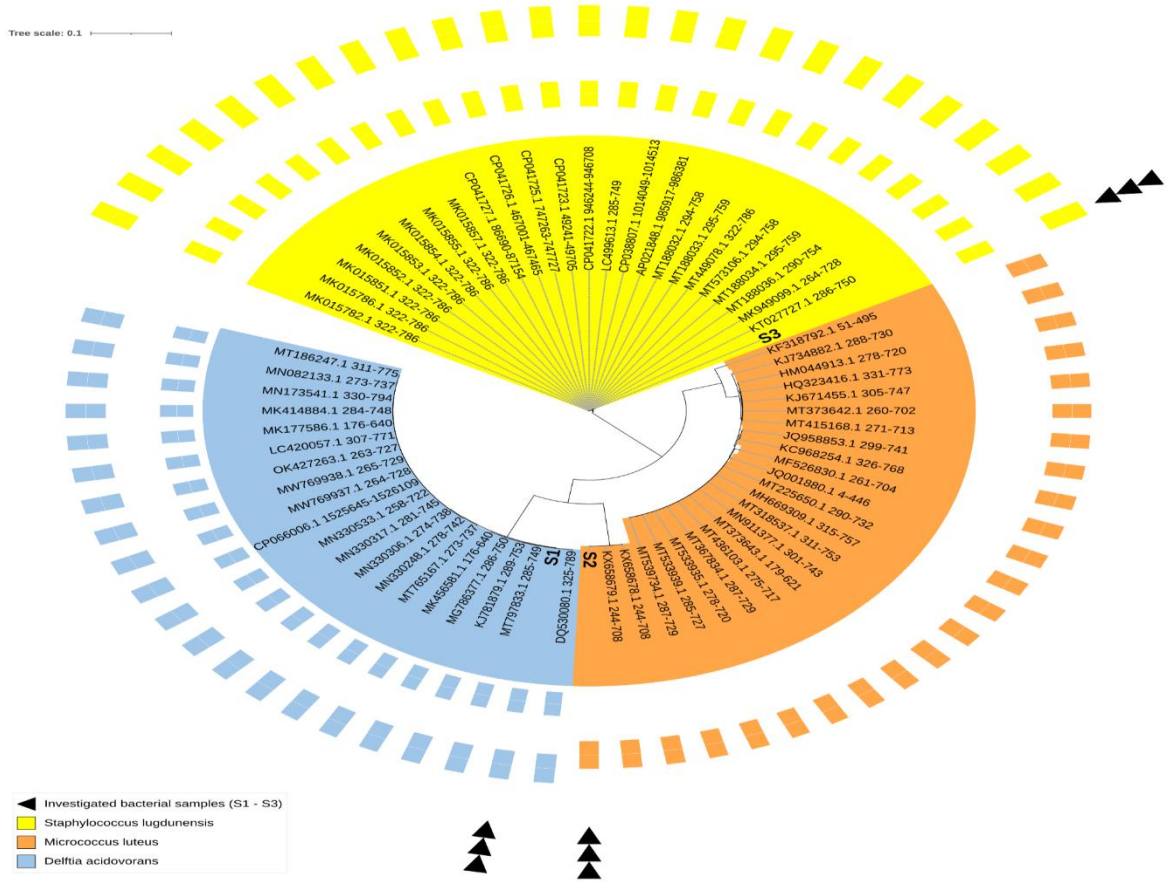



**Figure 4-35 . The comprehensive cladogram phylogenetic tree of genetic variants of the 16S rRNA sequences fragment of *D.acidovorans, M.luteus,* and *S.lugdunebsis* samples. The black-colored triangle refers to the analyzed bacterial variants. All the mentioned numbers referred to GenBank accession number of each referring species. The number "0.1" at the top portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The letter "S#" refers to the code of the investigated samples.**

The second tree was contained S4 to S13 samples to represent the amplified 1500~ bp to represent S4 to S13 samples,(Figure 4-36). The S4 – S13 samples were aligned alongside other relative nucleic acid sequences of their reference sequences. Within this tree, the investigated S4 – S13 samples were incorporated alongside other relative sequences to constitute ten major clades of incorporated sequences within the cladogram. The S4, S5, S6, S7, S9, S10, S11, S12, and S13 were suited in the *K.kristinae, Ps.aeruginosa, S.warneri, E.cloacae, S.lentus, Pa.sp., Ps.putida, Ps.stutzeri,* and *Ac.baumanni* clades respectively. Whereas S8 was suited in the *S.haemolyticus* clade. As it was found in the previous tree, no other related sequences were found to be highly correlated to these incorporated sequences. This data indicated another highly specific ability of 16S rRNA sequence-based fragments to detect these bacterial sequences without including any noticeable homology with other sequences of other species. The total number of the aligned nucleic acid sequences in this comprehensive tree was 94. In the constructed cladogram, the investigated samples were clustered into ten phylogenetic clades of ten distinct phylogenetic sequences within the incorporated bacterial sequences of this tree. The most interesting fact observed in our investigated bacterial isolates is related to the obvious ability of the utilized 16S rRNA sequences -based amplicons to categorize the *K.kristinae, P.aeruginosa, S.warneri, E.cloacae, S.lentus, Pa.sp., Ps.putida, Ps.stutzeri, Ac.baumanni, and S.haemolyticus* sequences into these observed phylogenetic distributions .Within the clade of *K.kristinae*, fifteen sequences of the same species were incorporated, in which the S4 sample was suited. Within the clade of *Ps.aeruginosa*, eight sequences of the same species were incorporated, in which the S5 sample was suited. Within the clade of *S.warneri*, ten sequences of the same species were incorporated, in which the S6 sample was suited. Within the clade of *E.cloacae,*

eight sequences of the same species were incorporated, in which the S7 sample was suited. Within the clade of *S.lentus*, ten sequences of the same species were incorporated, in which the S9 sample was suited. Within the clade of *Pa.* sp., ten sequences of the same species were incorporated, in which the S10 sample was suited. Within the clade of *Ps.putida*, eight sequences of the same species were incorporated, in which the S11 sample was suited. Within the clade of *Ps.stutzeri*, two sequences of the same species were incorporated, in which the S12 sample was suited. Within the clade of *Ac.baumanni*, seven sequences of the same species were incorporated, in which the S13 sample was suited. Within the clade of *S.haemolyticus*, fifteen sequences of the same species were incorporated, in which S8 sample was suited. These suited sequences were exerted close positions from each other within the same major clade, and variable phylogenetic positions were observed among them. However, no nucleic acid variation was observed in S4, S6, S9, S12, S13 samples. It also appears that the employed 16S rRNA sequences - based amplicons provide high precision in the detection and discrimination of these bacterial organisms in high accuracy.

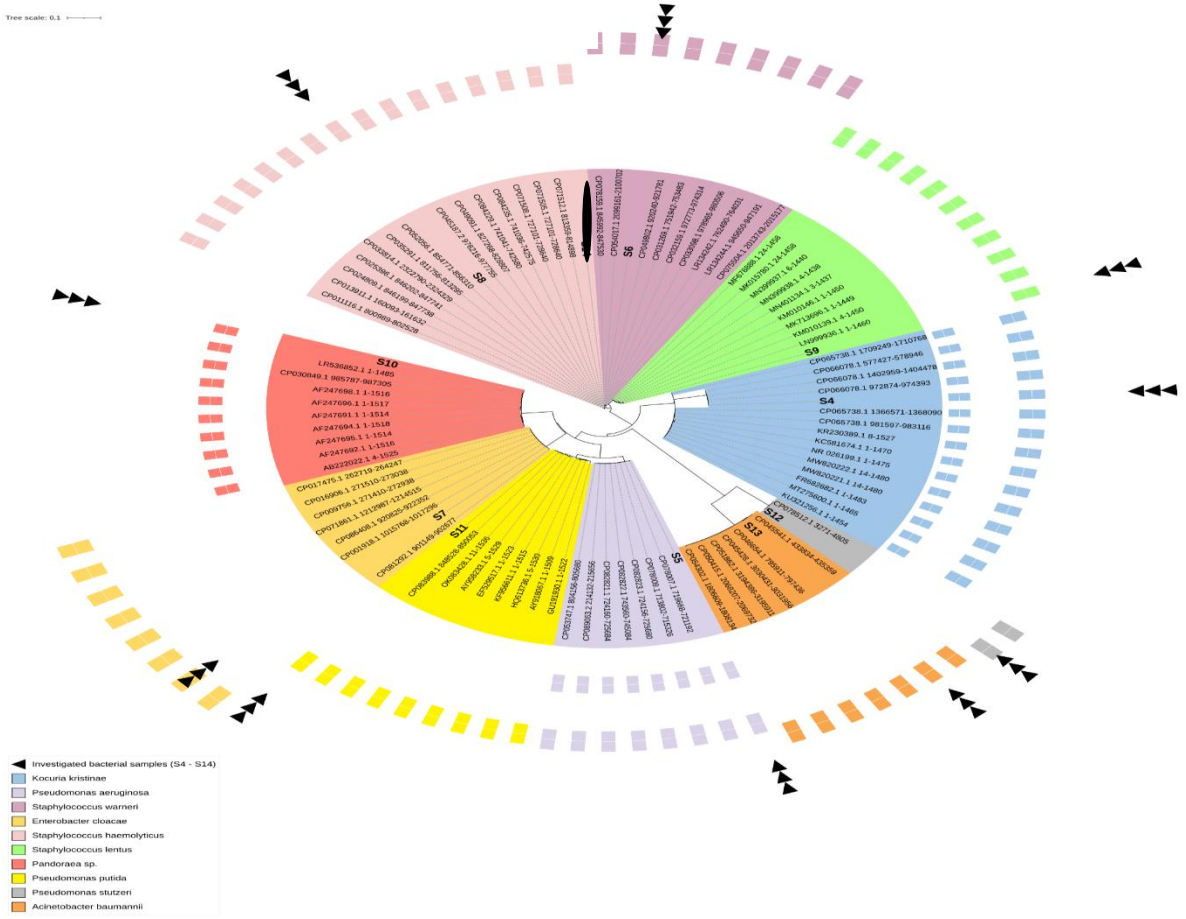



**Figure 4-36 . The comprehensive cladogram phylogenetic tree of genetic variants of the 16S rRNA sequences fragment of *K.kristinae, Ps.aeruginosa, S.warneri, E.cloacae, S.lentus, Pa.sp., Ps.putida, Ps.stutzeri, Ac.baumanni,* and *S.haemolyticus* samples. The black-colored triangle refers to the analyzed bacterial variants. All the mentioned numbers referred to GenBank accession number of each referring species. The number "0.1" at the top portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The letter "S#" refers to the code of the investigated samples.**

However, the incorporated sequences within in the vicinity to S4-S13samples within this clade showed the presence of various strains of the *K.kristinae, Ps.aeruginosa, S.warneri, E.cloacae, S.lentus, Pa.sp., Ps.putida, Ps.stutzeri, Ac.baumanni, and S.haemolyticus* sequences with various geographical distribution. Despite the nucleic acid variations detected in S5, S7, S8, S10, and S11, no obvious tilt was observed for these investigated samples with respect to the incorporated reference sequences. These patterns of the positioning of the S5, S7, S8, S10, and S11 samples indicated the absence of any noticeable evolutionary impact of the observed genetic variations in inducing any possible deviation in the evolutionary positioning of these bacterial samples. The current observation of this tree has confirmed sequencing reactions because it explained the actual neighbour-joining-based positioning in such observed variations. Interestingly, the multiple origins of our investigated samples could not be ignored. This was due to their positioning in the vicinity to different strains that deposited from various geographical distributions worldwide.

Based on the currently analyzed 16S rRNA sequences, it was found that the most related sequences to incorporated sequences was S8 and S6 samples, which respectively represented the *S.haemolyticus* and *S.warneri* due to their high similarity ratio in these ribosomal sequences. This is due to the belonging of both species to the same genus. Interestingly, the utilization of the 16S rRNA sequences in this study has given further indication for the presence of the precise identification of the actual identity of these bacterial organisms. Consequently, these observations are in line with each other to support our observation of the divergence of these bacterial sequences from close multiple geographical sources. However, this complete tree based on 16S rRNA sequences has provided extensive proof of the great competency of such genetic elements to accurately detect this

type of phylogenetic distribution. The capacity of the currently used 16S rRNA sequence-specific primers to describe the examined bacterial sequences and their precise phylogenetic positions is further demonstrated by this. The bacterial isolates and the blast share a significant percentage of similarities, indicating a strong relationship between them. Some of the bacteria found in this study had already been isolated using conventional methods by other researchers, including Ojo (2006) and Boboye *et al.*, (2010). It has not been mentioned that Ewingella Americana, which was discovered to have the highest degradative activity in the three oils, is engaged in oil degradation.

Therefore, it is indicated that these native bacteria are potential oil degraders in contaminated locations and might be used during in situ bioremediation or bioaugumentation. This is in line with the recommendations of (Ghazali *et al.,* 2004 ;Das and Mukherjee 2006). According to past investigations, the phylum Proteobacteria typically exhibits traits that are closely related to those of organisms that break down aliphatic and aromatic hydrocarbons (Lima *et al.*,2019;Bamitale and Ayomikun,2020). The labor-intensive processes of cultivating and isolating specimens are reduced thanks to the genomic DNA analysis (Czaplicki and Gunsch, 2016). In order to identify and define community structures, genomic molecular techniques are applied. The methodology is based on investigations of 16S rRNA genes (Brito *et al.,* 2006).The 16S rRNA gene is the most frequently used species proxy in microbial community analysis Hyper variable regions of the 16S rRNA gene and nucleotide barcodes (aid in sample identification) can be combined with primers in the surveying of environmental samples with numerous sequences at a particular time(Cardenas and Tiedje, 2017), as its universally found in bacteria. 16S rRNA genes are known to comprise of 9

hyper variable regions (V1-V9) and flanking conserved regions .The conserved regions are quite similar between bacterial species while the hyper variable region possess varying sequence diversity in bacteria species and can be targeted in the taxonomy identification of bacteria. Due to the sequence diversity within 16S rRNA hyper variable regions, a single hyper variable cannot be utilized in distinguishing all bacteria , and its believed more than one hyper variable region should be targeted(Chakravorty *et al.,* 2007).

The primers utilized can be specific and used in targeting certain taxa whilst others normally classed as universal primers can target all prokaryotic genes (Beale *et al.,* 2017). These hyper variable regions, which are between 100-350bp, possess information, which have been utilized in microbial classification by databases such as Ribosomal Database Project (RDP), Green genes and Silva (Cardenas and Tiedje, 2017).. The use 16S rRNA genes has its limitations which include low evolution rate, inability to link organism functions and variation in copy numbers, however it's the only molecular marker located in all prokaryotic organisms that possess enough genetic information which could be utilized in differentiating closely related microorganisms (Schloss *et al.,* 2011).

## 4.12 Immobilization of Microorganisms

### 4.12.1 Beads formation

The bacterial isolates *K.kristinae* and *D.acidovorans* which previously identified as active oil-degrading bacterial isolates and kept in GenBank under accession numbers (ac: OM838480.1 and OM838393.1), respectively were used in this study. These findings indicated that bacterial cells were immobilized into protective carriers to promote cellular survival in the concrete matrix. A promising example of this kind of solution is the encapsulation of bacterial cells within a

polymeric matrix like calcium alginate (Ca-alginate). The beads have a 2mm diameter. Additionally, bacterial cells that have been enclosed using encapsulation may be removed and used again. The permeability and mass transfer capabilities of the beads are strongly influenced by the amounts of Na-alginate and CaCl2 used to create Ca-alginate, which has an impact on the bio mineralization of CaCO3. Bacterial cells can be immobilized in four ways: (1) by adhering to or adhering to solid carrier surfaces; (2) by becoming trapped within a porous matrix; (3) by self-aggregating through flocculation or with the help of crosslinking agents; and (4) by being contained behind barriers (Seifan, *et al.*,2017). Alginates, agar, chitosan, and other polysaccharide gel matrices, as well as other polymeric matrix materials like gelatin, can all be used for cell entrapment. In order to keep the cells from dispersing into the environment, polysaccharide gel entrapment (Ca-alginate) was used in this work as an effective immobilization technique. The bacterial cells were successfully adsorbed into uniformly sized Ca-alginate beads, as seen in Figure (4-37). The bioprocess must be facilitated by the mass transfer of nutrients, and the cells must be shielded from the environment throughout the optimal immobilization method. The defense mechanism works by inducing a stress response, which results in a more resilient cell. The macromolecular level has previously been used to demonstrate this phenomenon (Cray *et al.*,2015). The immobilization carriers should be permeable while also being able to stop cells from diffusing into the fermentation media in order to enhance CaCO3 precipitation. In addition to being trapped inside the beads, bacteria may also move to the beads' outer surface if they are immobilized in a porous matrix, such as polysaccharide gel. However, the immobility may have an impact on water activity, which will then have an impact on metabolism (Okyay, and Rodrigues,2015).

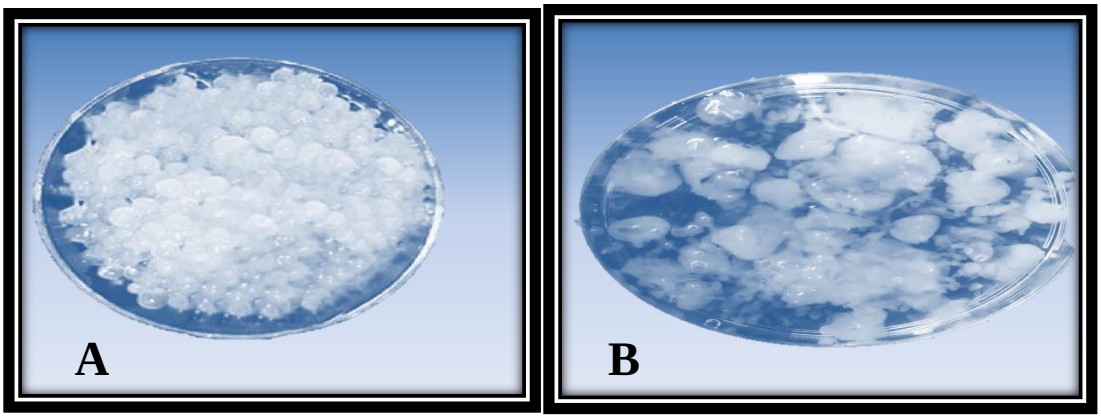



**Figure 4-37: Beads formation in current study A: *D.acidovorans* ;B: *K.kristinae*.**

## 4.13 Soil treatment

### 4.13.1 Measurement By HPLC

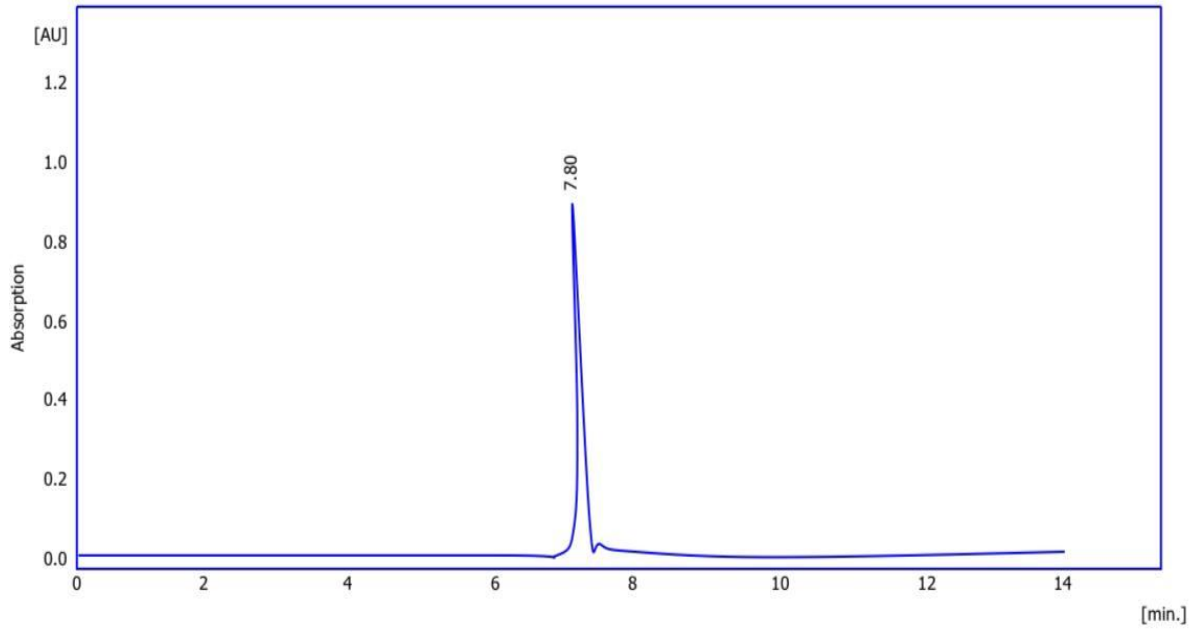
Measurement was carried out by using HPLC system equipped with a binary high pressure gradient pump, sample loop , and Diode array detector . Through the results of the present study, immobilized bacteria (*K.kristinae* and *D.acidovorans*) were used to show their ability to treat soil contaminant oil by using standards concentrations (10) ppm of naphthalene,(Figure 4-38) while Figure (4-39) was showed control concentration for soil contaminated oil was estimated before treatment by using HPLC to compare with immobilized isolates that used for treatment of soil contaminated oil which given concentration (4598.6 ppm). Figure (4-40) and (4-41) were showed the soil concentrations after treatment with these immobilized (*K.kristinae*) and (*D.acidovorans*) were (479.0,and 658.5) ppm respectively,. These results mean the immobilization of bacteria will in general lead to increment soil contamination gathering by biomass and finally increase the removal efficiency of these isolates to remove the pollution from soil .These results agree with results were obtained by Chen and Yuan (2011) that showed the removal percentages were 85–93 % for phenanthrene and 94–98 % for pyrene in

the solution amended with plant residues immobilized bacteria, while for the immobilized-bacteria in bio chars, the corresponding values increased to 92–100 % for phenanthrene and 96–100 % for pyrene because of the higher sorption affinity of the bio char. Also immobilization increases the rate of biodegradation of pollutants through increasing cell loading and this also improve the catalytic stability as well as the tolerance against toxic pollutants (Baskaran and Nemati, 2006; Wang *et al*., 2007). The degradation rates by immobilized cells on Ca-alginate and chitosan were higher than those of free cells (Wu and Wan, 2009). For comparison, the individual removal rates of phenanthrene and pyrene were 22–38% and 39–40% in the solution supplemented with free bacteria, and they increased to 46% and 77% in the absence of carriers (with 2% (w/v) sodium alginate) when amended with immobilized bacteria. These findings suggest that IMT can significantly improve PAH removal from water by utilizing bacteria and adsorption carriers. An essential consideration when choosing a substance as a potential adsorption carrier for IMT is the bioavailability of sorbed PAHs to immobilized bacteria. The physiochemical properties of carriers as well as the characteristics of microbes influence the bioavailability of sorbed PAHs . The bioavailability of sorbed PAHs is influenced by the physiochemical qualities of carriers as well as the traits of bacteria (Yang *et al.,* 2010). It was shown that P400-immobilized beads had a higher bioavailability of related PAHs than P100- and P300-immobilized beads. High pyro lytic temperatures (400 °C) were used to break down the cellulose portions of plant wastes, exposing the aromatic cores produced from the lignin fractions (Chen *et al.,*2011).

**Chromatography Laboratory**
**HPLC**

Sample Info:

| | | | |
|---|---|---|---|
| Sample ID | : st naphthalene ( 10 PPM ) | Amount | 0 |
| Sample | : st naphthalene ( 10 PPM ) | ISTD Amount | 0 |
| Inj. Volume [mL] | : 0.1 | Dilution | 1 |
| Autostop | : 30.00 min | External Start | : Start - Restart, Down |
| Detector 1 | : Detector 3 | Range 1 | : Bipolar, 2000 mAU, 10 Samp. per Sec. |
| Subtraction Chromatogram | : (None) | Matching | : No Change |




Result chromatography Table (Uncal - F:\ st naphthalene ( 10 PPM )

| No | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Compound Name |
|---|---|---|---|---|---|---|---|
| 1 | 7.80 | 356.98 | 921.44 | 100.00 | 100.00 | 0.30 | |
| | Total | 356.98 | 921.44 | 100.00 | 100.00 | | |

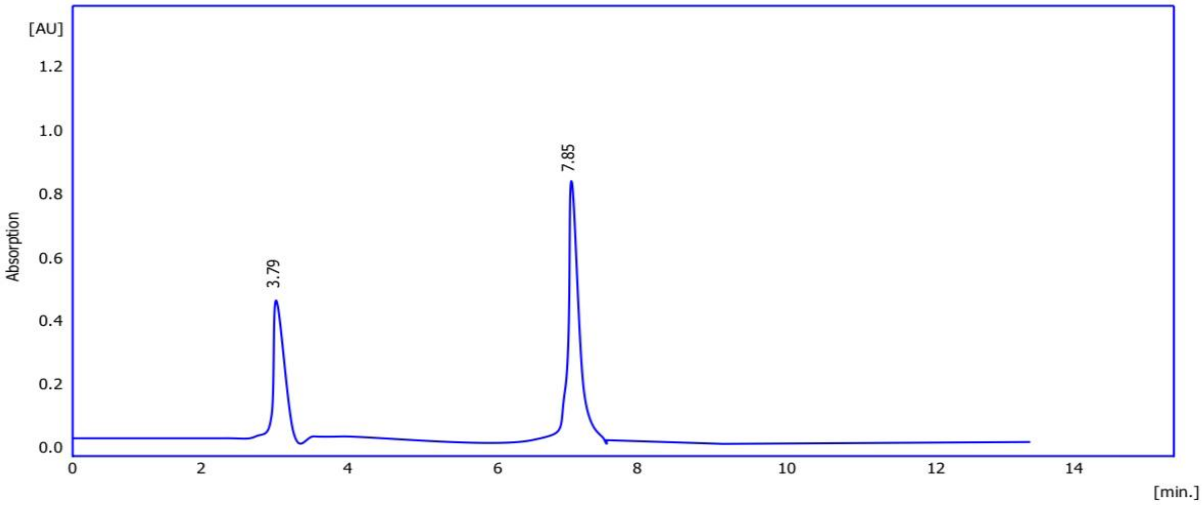
**Figure 4-38 : HPLC estimation of standard concentration of naphthalene(10ppm)**

**Chromatography Laboratory**
**HPLC**

Sample Info:

| | | | |
|---|---|---|---|
| Sample ID | : control | Amount | 0 |
| Sample | : control | ISTD Amount | 0 |
| Inj. Volume [mL] | : 0.1 | Dilution | 1 |

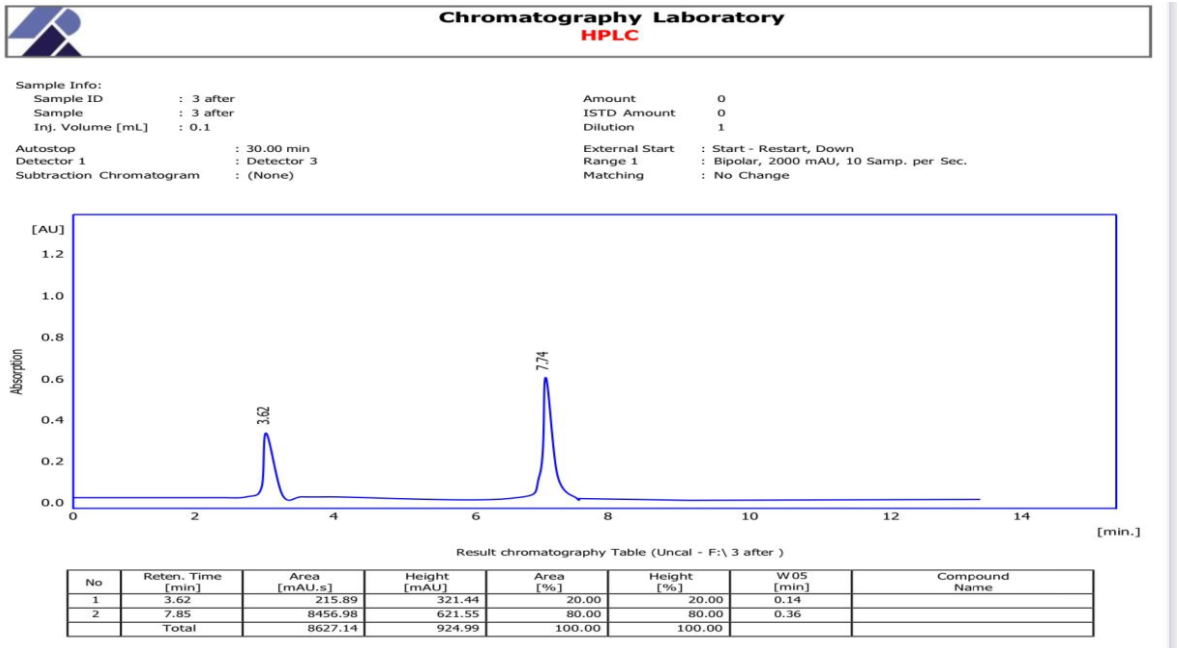
| | | | |
|---|---|---|---|
| Autostop | : 30.00 min | External Start | : Start - Restart, Down |
| Detector 1 | : Detector 3 | Range 1 | : Bipolar, 2000 mAU, 10 Samp. per Sec. |
| Subtraction Chromatogram | : (None) | Matching | : No Change |




Result chromatography Table (Uncal - F:\ control

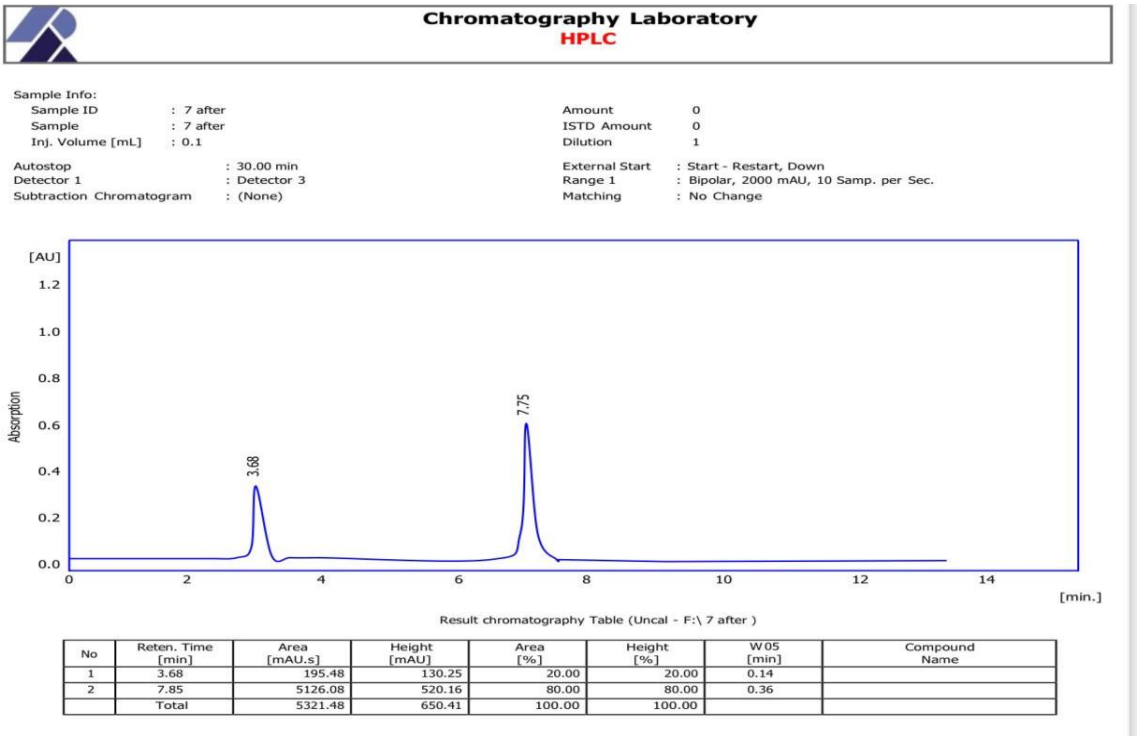
| No | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W 05 [min] | Compound Name |
|---|---|---|---|---|---|---|---|
| 1 | 3.79 | 4589.25 | 598.77 | 20.00 | 20.00 | 0.14 | |
| 2 | 7.85 | 22895.98 | 864.22 | 80.00 | 80.00 | 0.36 | |
| | Total | 27485.33 | 1462.19 | 100.00 | 100.00 | | |

**Figure 4-39 : HPLC estimation of control soil before treatment**




| No | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Compound Name |
|---|---|---|---|---|---|---|---|
| 1 | 3.62 | 215.89 | 321.44 | 20.00 | 20.00 | 0.14 | |
| 2 | 7.85 | 8456.98 | 621.55 | 80.00 | 80.00 | 0.36 | |
| | Total | 8627.14 | 924.99 | 100.00 | 100.00 | | |

**Figure 4-40: HPLC estimation of soil contamination oil after treatment by *D.acidovorans***




| No | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Compound Name |
|---|---|---|---|---|---|---|---|
| 1 | 3.68 | 195.48 | 130.25 | 20.00 | 20.00 | 0.14 | |
| 2 | 7.85 | 5126.08 | 520.16 | 80.00 | 80.00 | 0.36 | |
| | Total | 5321.48 | 650.41 | 100.00 | 100.00 | | |

**Figure4-41 : Figure 4-41 : HPLC estimation of Soil contamination oil after treatment by *K.kristinae***

## 4.13.2 Measurement of reactivity of beads By FTIR

FTIR analysis was carried out for the both samples (produced by *K.kristinae* OM838480.1. and *D.acidovorans* OM838393.1 respectively before and after treatment. First isolate *K.kristinae* OM838480.1 ,results of current study show in (Figure 4-42) were demonstrated that bio adsorption procedure happened because of the existence of hydroxyl, carbonyl, amide, and carboxyl combinations these mean that the hydrocarbons after treatment were bind with the functional group mentioned above, leading to shifting the absorbance from site to another, compared with Figure 4.43, which shows the FTIR for bacterial beads without any care (Nessim *et al*., 2011; Patel *et al*., 2016).FTIR is a powerful technology for the functional group's determination of chemical compounds that determination of treatment according to the functional group that shift/absent/appear was carried out by FT-IR that depend on mixed the KBr(200mg) with dried samples(1mg), the result were read by X-axis and the Y-axis of the spectrum.

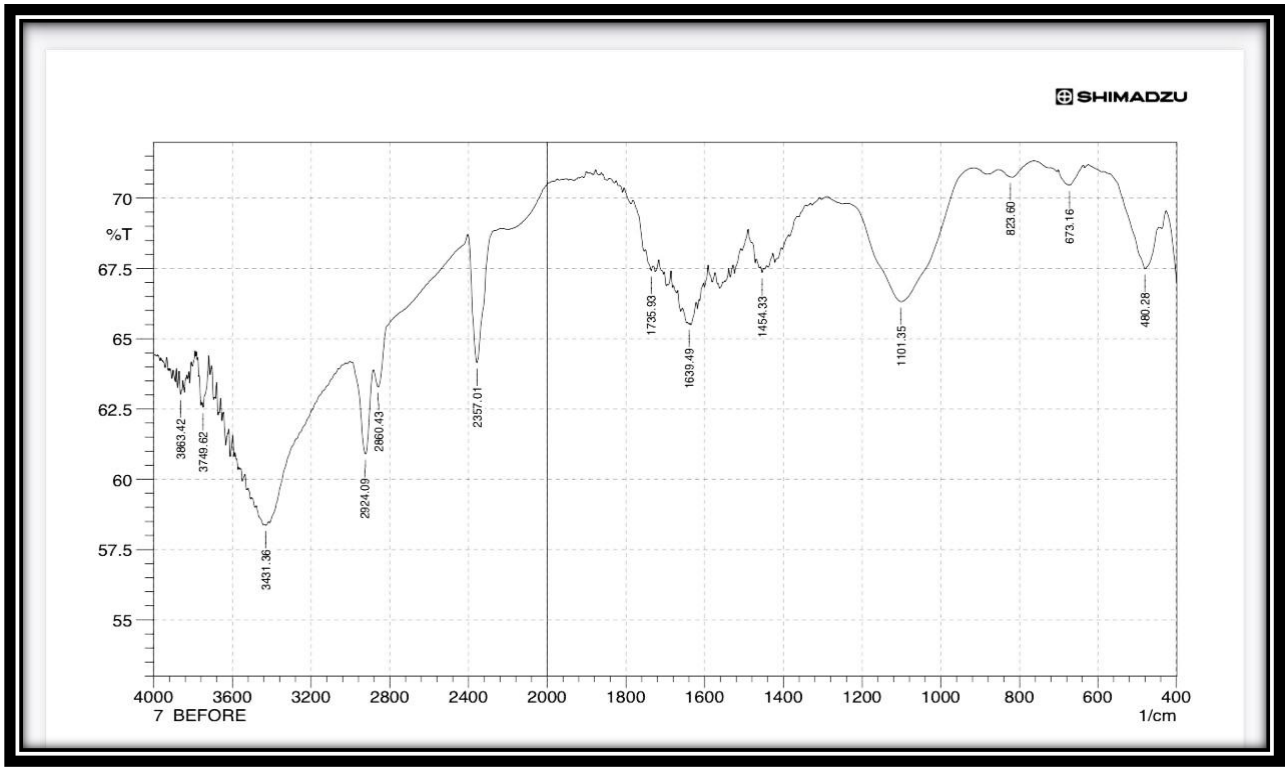



**Figure 4-42:FT-IR for beads from *K.kristinae* without treatment**

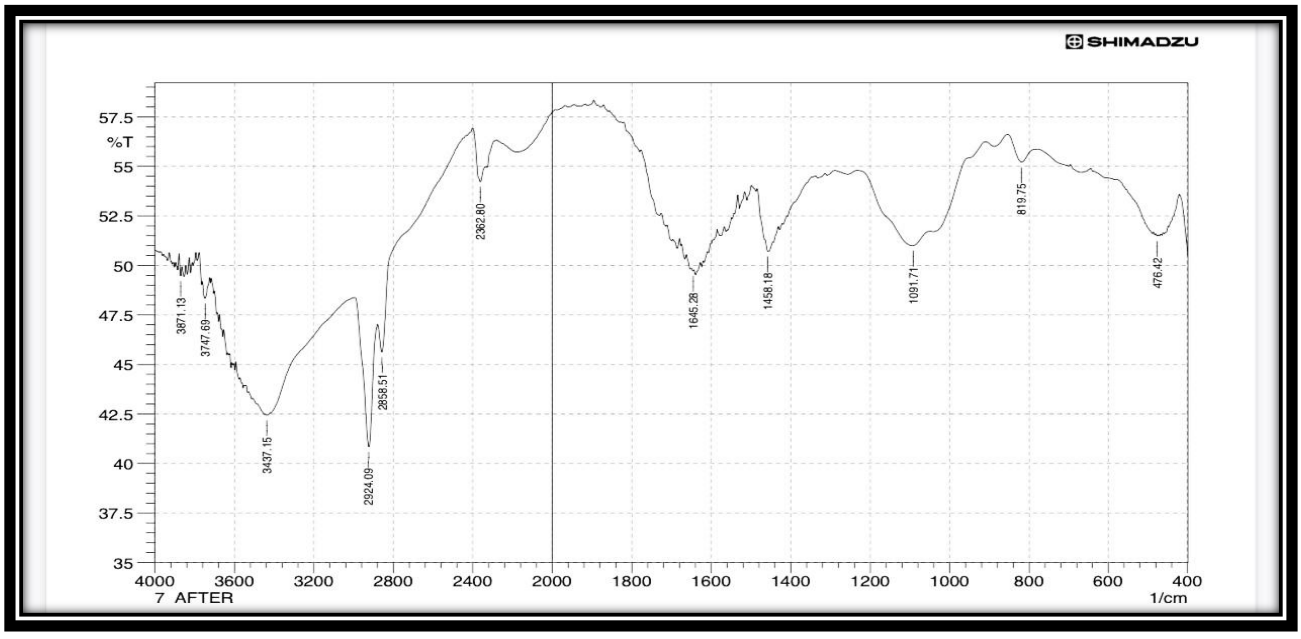



**Figure 4-43:FT-IR for beads from *K.kristinae* with treatment**

*K.kristinae* OM838480.1 was given different functional group before treatment. A typical pattern of absorption bands for this kind of material was visible in the untreated beads. N-H stretching vibrations correspond to the vibrational band at 3863 cm1. C-H asymmetrical and symmetrical stretching vibrations link the peaks in the 3000 to 2800 cm-1 range. The strong bands in the region of 1300-1000 cm1 (asymmetric and symmetric O-C-O stretching vibrations), the double peak in the region of 1680-1740 cm1 (C=O stretching vibrations), the bands above 1500 cm1 (probably N-H bending vibrations and C-N stretching vibrations), and the bands above 1500 cm1 are all connected to the bonds of the urethane group.

The growth of *Microbacterium sp. NA23*, *Paenibacillus urinalis NA26*, *Bacillus sp. NB6*, and *Pseudomonas aeruginosa NB26* on polystyrene film was demonstrated by Asmita *et al.*, (2015). They noted that the area of the absorption peaks in the bacterially treated and untreated films did not grow, and they hypothesized that this would be because there were no appreciable surface changes over the four weeks of incubation with the bacterial isolates. However, after treatment, some of the peaks in the isolate's FTIR spectra vanished. Additionally, a rise in peak intensity of 1640 cm-1 was seen. When compared to the peak's intensity before treatment, there was a decrease at 1091 cm1, 819 cm1, and 476 cm1. FTIR spectra is useful to elucidate chemical and physical structure, hydrogen bonding, and end group detection, degradation reactions, cross linking behavior of molecules and copolymer composition in liquid and solid form of chemicals and polymers. FTIR technique is employed in the degradation studies of polymers to assess the chemical changes due to microbial activity (Umamaheswari, and Murali,2013). The absorption bands are observed in two wave number regions of 3433 - 2931 cm−1 and 1631,1663,1112 - 622 cm−1 before treatment for second isolates *D.acidovorans* . The presence of peaks on the spectra of these samples

coming from different functional groups that corresponds to bands of.(Figure 4-44)before treatment, while differences in absorption bands intensities and appearance of new peaks in spectra of treated samples were observed (Figure 4-45). Identification of the absorption bands is following: The observed peaks in the wave number range of 3842- 2926 cm−1 is characteristic for stretching vibration of O-H and C-H bonds in polysaccharides. The broad peak at 3435 cm−1 is characteristic for stretching vibration of the hydroxyl group in polysaccharides . The band at 2660 cm−1 is attributed to CH stretching vibration of all hydrocarbon constituent in polysaccharides . The peaks located at 1633 cm−1 correspond to vibration of water molecules absorbed in treated sample. The absorption bands at 1428, 1367, 1334, 1027 cm−1 and 896 cm−1 belong to stretching and bending vibrations of -CH2 and -CH, -OH and C-O bonds in cellulose.FTIR spectroscopy has been widely used to provide information on a range of vibrational active functional groups (including O–H, N–H, C=O, =C–H, –CH2, –CH3, C–O–C and >P=O) in biological specimens(Danial *et al.*,2015;Sackett *et al*., 2016).

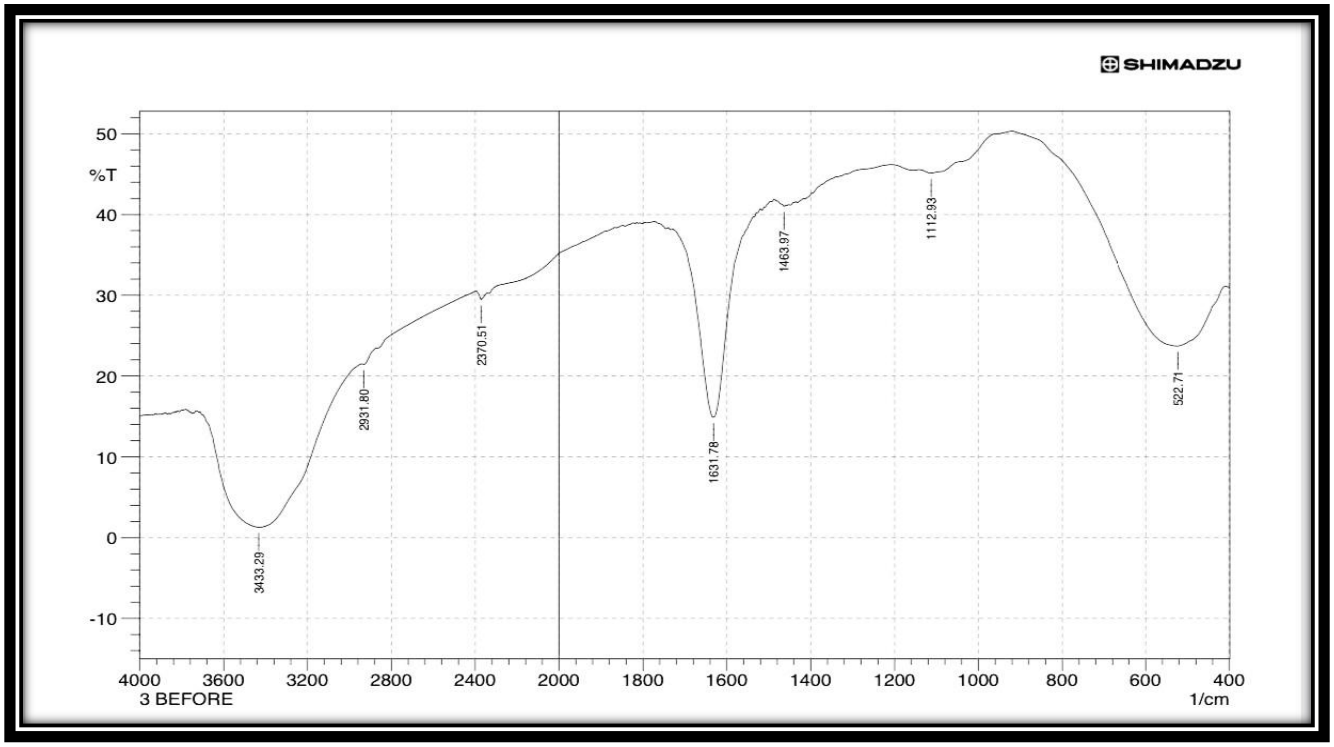



**Figure 4-44: FT-IR for beads from *D.acidovorans* without treatment**

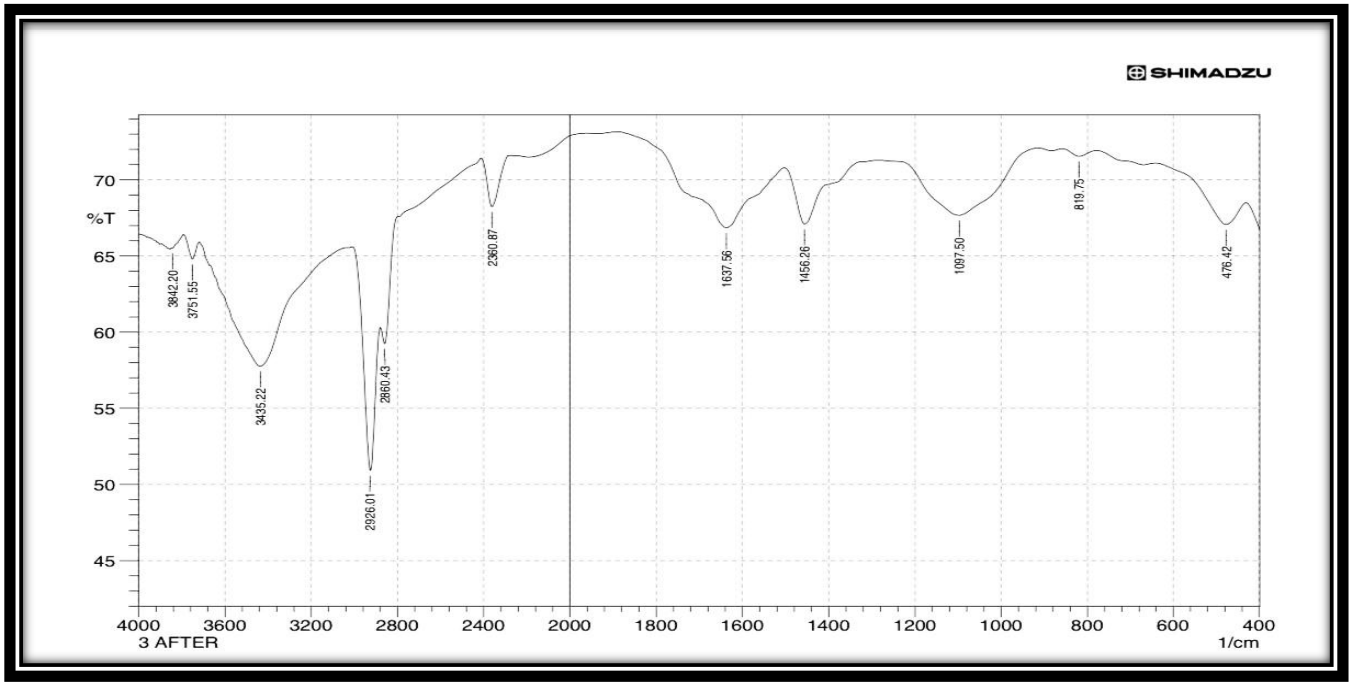



**Figure 4-45 :FT-IR for beads from *D.acidovorans* with treatment**

## 4.14 Development of Consortia of the Selected Bacteria

Thirteen isolates were grown in culture with crude oil. These were used in this experiment as; six gram positive (*Kocuria kristinaeOM838480.1*, *Staphylococcus warneriOM838482.1*,*Staphylococcus haemolyticusOM838484.1*, *Micrococcus luteusOM838394.1*,*S.lugdnebsis OM838395.1*,and *S.lentusOM838485.1)* and seven Gram negative bacteria *(Delftia acidovorans OM838393.1*, *Pseudomonas putidaOM838487.1*, *Pseudomonas aeruginosaOM838481.1* , *Pseudomonas stutzeriOM838488.1*,*E.colacae OM838483.1*,*Pandorea sp.OM838486.1* and *Acinetobacter baumanni OM838489.1).* In this study, The 13 microbial consortium obtained show considerable efficiency in biodegradation of hydrocarbons used as carbon source in their respective enrichment procedures. In order to determine and compare alternative PAH degradation pathways, the current study aims to estimate degrading capacity of G+ve and G-ve isolates in the presence of PAH. The findings are divided into three sections. The first parameter of this study focuses on an experiment employing optical density(O.D), the second parameter, PH, while the third section evaluates plate count for 13 isolates. These results were showed best growth and high different significant after 18 days at optical density 600nm.The different pH curves revealed that the pH in the control flasks stayed nearly constant (7). Regardless of the treatment, the pH of the flasks containing oil decreased after 3 days and continued to decline after the last day of incubation (18 days) that give different significant. After 3 days of incubation, the growth of the bacterial consortium increased, and after 18 days of incubation, it reached a peak and give high different significant, then began to decline at 21 days to evaluate plate count, these results were conducted in Figure (4-46) and Table (4-5).The

extent of the attack on the different fractions of a crude oil by the different consortia was consistent with their origin. Consortium was more efficient in degradation compared with single isolate cultures, these results were showed in Table (4-6). Mixed microbial communities in natural settings are responsible for the biodegradation of crude oil. Natural degradative consortia may not exist or function as effectively in other situations. In contrast to oil-contaminated environments, which may experience physical conditions (such as low temperature) or chemical conditions (such as low oxygen or fixed nitrogen levels), pristine environments devoid of petroleum hydrocarbons typically support low levels of hydrocarbon-degrading microbes. The use of bioaugumentation or bio stimulation may be appropriate in certain circumstances. While the latter refers to the addition of chemicals such as nutrients (such as nitrogen, phosphate, and/or oxygen) or surfactants to stimulate the natural flora, the former refers to the introduction of exogenous organisms (such as those having oil-degradative phenotypes) to the environment to enhance the natural flora.

Crude oil is a very important mineral resource vital to everyday life. However, crude oil spillage is one of the most serious forms of water and land pollution. Oil spillage is the accidental discharge or pouring of crude oil into the environment. It involves the contamination of any part of the environment with any liquid hydrocarbon. These spills put public health at risk, damage drinking water, destroy natural resources, contaminate beaches and shorelines, and have a negative economic impact (Gesinde *et al.*,2008). By introducing oil-degrading bacteria into sediment or water, there are numerous obstacles to overcome, such as nutrient deprivation, which has been identified as one of the causes limiting oil decomposition (Mohsen *et al.*,2014). Although nutrient enrichment aids in bioremediation, oil breakdown is also influenced by other factors. Other factors

that slow down biodegradation include the presence of inhibitory metabolites and the capacity of the microorganisms that can break down oil (Amer *et al.*,2014). A single organism cannot carry out the complex metabolic events needed in biodegradation. Numerous papers emphasized the significance of the bacterial consortium for bioremediation. In comparison to a microbial consortium of bacteria-fungi complex capable of decomposing more than 50% of old polycyclic aromatic hydrocarbons in soil and slurry, multiple studies have demonstrated a consortium of symbiotic bacteria's ability to degrade microbial-resistant turbine engine oil (Arafa *et al.*,2016).

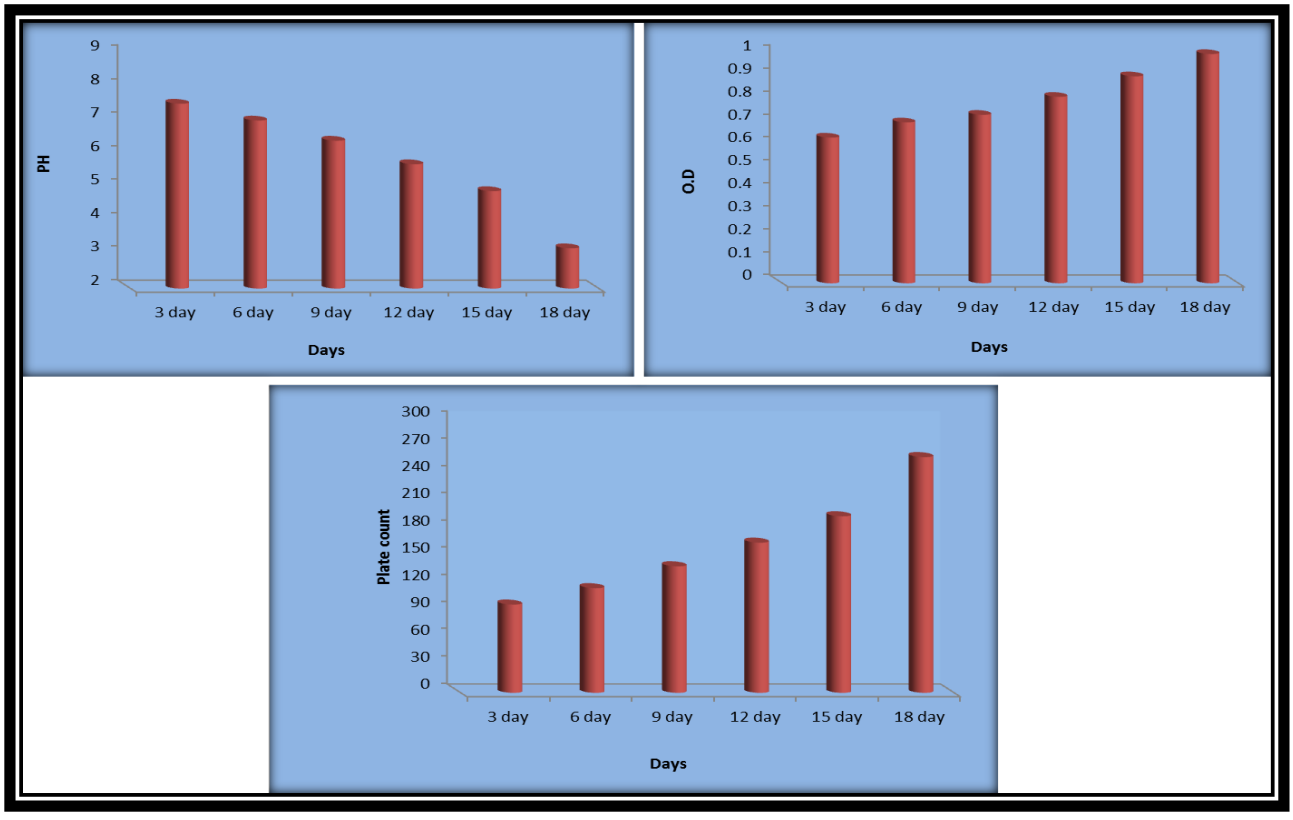



**Figure 4-46: Optical density, pH, and plate count for determination of activity of microbial consortia**

**Table 4-5: Determining pH,O.D and plate count for activity of microbial consortium**

| Parameters<br>Treatments (Days) | O.D | pH | Count*$10^8$ |
|---|---|---|---|
| | Mean±S.D | | |
| 3 | 0.66±0.03 | 7.55±0.11 | 96.33±1.7 |
| 6 | 0.69±0.02 | 7.00±0.34 | 114.00±2.9 |
| 9 | 0.71±0.12 | 6.40±0.21 | 136.33±4.1 |
| 12 | 0.74±0.03 | 5.69±0.06 | 142.33±3.4 |
| 15 | 0.85±0.02 | 4.89±0.20 | 177.67±2.3 |
| 18 | 0.99±0.01 | 3.17±0.10 | 258.67±1.2 |

**Table 4-6 Comparison between microbial consortia and bacterial species alone**

| Bacterial species alone After 18 days | O.D | PH | Plate count *$10^8$ |
|---|---|---|---|
| *K. kristinae* | 0.993 | 3.01 | 223 |
| *S. warneri* | 0.967 | 3.605 | 200 |
| *S.haemolyticus* | 0.899 | 3.9 | 195 |
| *M.luteus* | 0.889 | 4.5 | 167 |
| *S.lugdunebsis* | 0.665 | 4.1 | 127 |
| *S.lentus* | 0.654 | 4.1 | 118 |
| *D.acidovorans* | 0.890 | 4.102 | 222 |
| *Ps.putida,* | 0.755 | 3.9 | 156 |

| | | | |
|---|---|---|---|
| ***Ps.aeruginosa*** | **0.795** | **3.85** | **201** |
| ***Ps.stutzeri*** | **0.856** | **3.852** | **214** |
| ***E.cloacae*** | **0.766** | **4.205** | **195** |
| ***Pa.sp.*** | **0.712** | **3.765** | **145** |
| ***A.baumanni*** | **0.611** | **4.01** | **132** |
| **Microbial consortium After 18 days** | **0.998** | **3.1** | **258** |

## 4.15 Production of biosurfactant by microbial consortium bacteria

Biosurfactant or bioemulsifiers play a key role in emulsifying hydrocarbons. Biosurfactant and bioemulsifiers are thought to be very suitable alternatives to chemical surfactants due to their properties like ecofriendly, less or not toxicity, biodegradability, high specificity, selectivity at temperature, pH, salinity and synthesis from cheaper renewable substrates. In this results, eight bacterial isolates act as consortium that used and screened for production of biosurfactant by using blood hemolysis, drop collapse, oil spreading, emulsification index, CTAB agar, BATH, and foaming activity. Blood hemolysis give positive results when cultured microbial consortia. The results in Figure(4-47)and Table (4-7) showed that among all microbial bacterial consortia were able to displace oil and form a clear zone. This may be due to the presence of biosurfactant which degrade the oil and form a clear zone. Bacterial isolates giving positive haemolytic test were screened on the basis of oil displacement technique (Kaur *et al.,* 2017) .Others parameters (drop collapse give good positive results, oil spreading best positive results, emulsification index also give high results 100%, CTAB agar give clear zone around consortia, BATH give strong positive results

when screened biosurfactant production, and foaming activity also showed good foaming results ) were conducted and also given strong positive results when used microbial consortia compared with results that obtained when estimate biosurfactant by using single bacteria (Table 4-7). Therefore, these consortia were subjected to further screening using oil displacement technique, E24 index, surface tension reduction and atomized oil assay for stronger confirmation of biosurfactant production. A total of eight different consortia were developed involving selected isolates. In order to simultaneously conduct all the metabolic necessary for greater degradation, bacterial cultures must be compatible with one another and not have any animosity toward one another in order to create a successful microbial consortium (Sarkar *et al.*,2013). Each member of a consortium for hydrocarbon cleanup should be able to manufacture biosurfactant (Mnif *et al.*,2015).

By lowering surface and interfacial tensions, biosurfactants enhance the surface areas of insoluble molecules, improving the mobility and bioavailability of hydrocarbons. Therefore, biosurfactants facilitate the removal and biodegradation of hydrocarbons. Therefore, it is reasonable to assume that adding biosurfactant-producing bacteria to a culture system that is already contaminated with hydrocarbons may accelerate the biodegradation of the hydrocarbons through mobilization, solubilization, or emulsification (Das *et al.*,2014). Secondary metabolites known as biosurfactants are primarily created by microorganisms to aid in their survival. These have been discovered to be superior substitutes for their synthetic counterparts and to play a helpful function in the breakdown of hydrocarbons. Previous research has demonstrated the contribution of biosurfactant manufacturers to improved hydrocarbon biodegradation. According to Ti *et al.*, (2020), a moderate dose of biosurfactant can improve intracellular accumulation and extracellular adsorption. According to Mehetre *et al.*, (2019), biosurfactant

production occurs concurrently with biodegradation processes. Yalaoui-Guellal *et al.*,(2020) who focused on the characterization of structurally distinct biosurfactants for oil spill remediation is highlighted in their work from 2020. While Mnif *et al.*, (2017) reported increased biodegradation of diesel oil when biosurfactant producing bacterial inoculations are supplemented with lipopeptides, Khan *et al*., (2017) validated the link between bio- surfactant production and hydrocarbon degradation suggesting their use in in-situ remediation. They can also be used to produce photo catalysts by the use of surfactants, which have better physicochemical qualities and photo catalysis efficiency (Liang *et al.*, 2019).

Due to the numerous potential uses for biosurfactants in the industrial, food, agricultural, and petroleum industries, they have gained commercial significance. These microbial products are useful for wetting, emulsification, and foaming applications due to their diversity in chemical and structural composition (Behzadnia *et al.*, 2020). Microorganisms that naturally produce biosurfactants have active metabolisms that can be used for bioremediation (Dhasayan *et al.*, 2015). Several bacterial species have been combined in previously obtained native bacterial strains to create biosurfactant, including *Bacillus species, Paenibacillus species*, *Pseudomonas species*, *Agrobacterium species*, *Aeribacillus species*, and *Acinetobacter species* (Mehetre *et al.*, 2019; Sharma *et al.*, 2019). Due to the low levels of biosurfactant synthesis, their use is constrained. For the development of bioremediation techniques, it is crucial to examine how local bacteria react to PAH. Glycolipid, lipopeptides, fatty acids, and polymeric biosurfactants are just a few of the many microorganisms that are known to produce chemically unique biosurfactants. Despite the fact that several biosurfactant manufacturers have been

located, only a few number of them are commercially feasible due to the low yields and low stability of the produced biosurfactants relative to their cost of production.

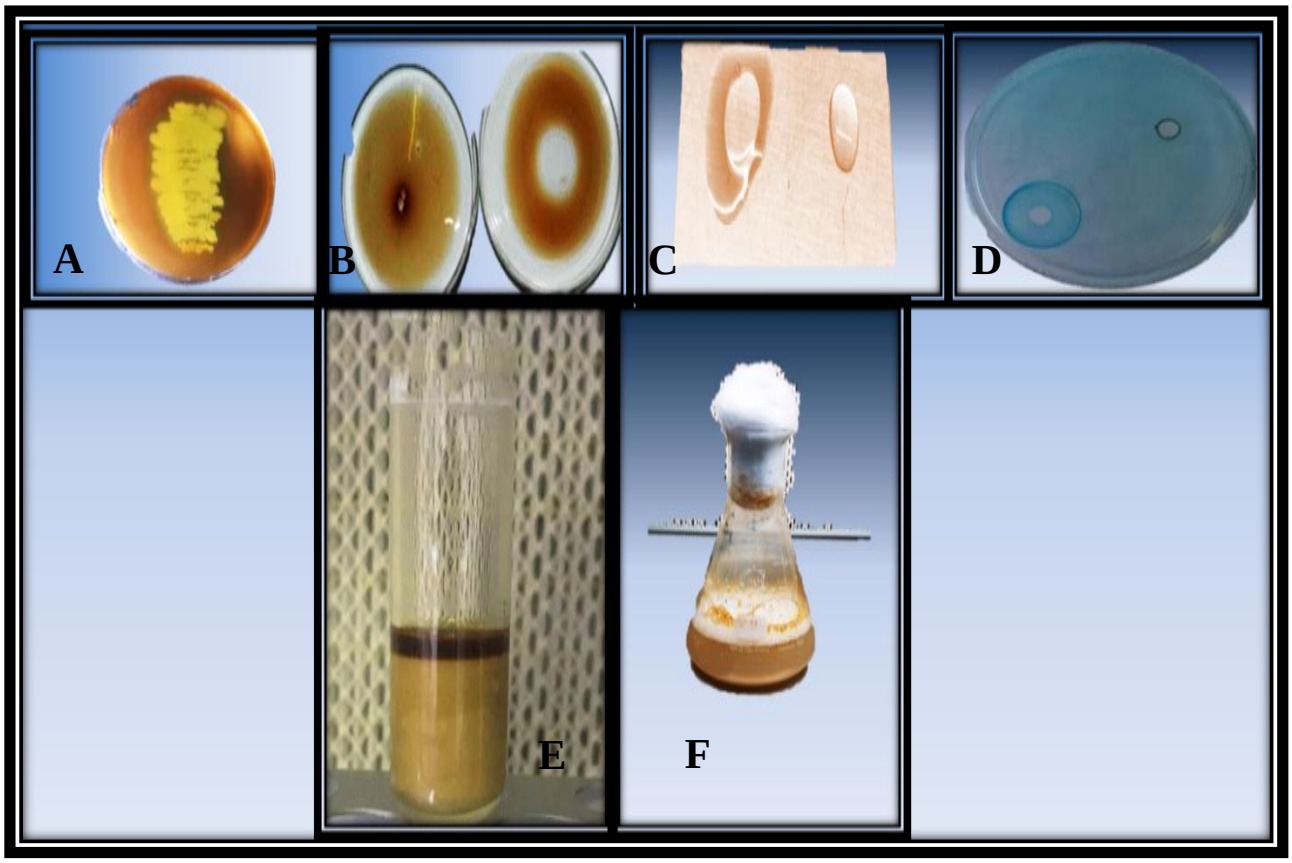



**Figure 4-47 : Screening the microbial consortia for biosurfactant production: A:Blood hemolysis; B:oil spreading; C:Drop collapse ;D:CTAB test; E:Emulisification test; and F: Foaming activity**

**Table 4-7: Oil spreading, Drop collapse, Emulsification ,and BATH assay**

| Bacterial isolates (gram positive) | Oil spreading test (mm) | Drop collapse test(mm) | Emulsification index(%) | BATH assay % |
|---|---|---|---|---|
| Microbial consortium | 3.4 | 3.5 | 100% | 100% |

# *Acknowledgements*

*This Ph.D. journery started with a dream when I was first employed by the research. Dreams alone are not enough and it took hard work, perseverance, encouragement and faith to fulfill this dream. First of all, I would like to thank God Almighty for giving me the opportunity and guidance to achieve my goal and to be successful in this part my life's journey so far. Then I pray all my respects and Praises to the greatest man of the universe, the Holly Prophet* ***Mohammad*** *(Peace Be Upon Him) whose life and wordings are ultimate source of guidance.*

*I express my deep gratitude and special thanks to the Deanship of the College of Science and the presidency of Biology Department for their continued support. A great debt of gratitude and sincere thanks are extended to the Head of Biology Dept. Assist. Prof.Dr.* ***Adi Jassim Abd Al-Rezzaq*** *for his great help.*

*I would like to express my heartiest and sincerest sense of gratitude to my worthy Supervisor* ***Prof. Dr.Eman Mohammad Jarallah*** *,She is one of the most intriguing person I have encountered in my life who had more insight about me than myself. I thank her whole heartedly for providing me this opportunity. I remember being lost and confused. It was her who saw the potential in me and helped me to perceive or at least have a glimpse of my true potential., and Co-Supervisor* ***Prof. Dr. Ayad M.J.Al-***

***mamoori*** *, Who has created the invaluable space for me to do this research and develop myself as a researcher in the best possible way.*

*I express my deep gratitude and special thanks to* ***Dr.Basheer Abd-Alhamza*** *and* ***Dr. Shaker Hammad Mohammad*** *for the valuable guidance enduring support and inspired a kindness.*

*My profound thanks to* ***Wameedh Adil Khadim*** *for the support, and he has been a constant and strong pillar of support during my Ph.D. I thank him for everything.*

*My personal and very sincerest acknowledgment to my friend* ***Rafal Ahmed Lilo*** *for supporting and encouraging me to pursue this dissertation.*

*Finally, special thanks to my family especially my mother, brother(Sadiq), whom I do appreciate their long patience, support and help to everyone who gave hand to complete this work.*

***Liqaa***

# Appendix 1

## Determination of PAH compound by using GC- mass

| Name ( ppm ) | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| **Acenaphthene** | 166.5 | 128.9 | 120.4 | 184.5 | 140.5 | 145.9 | 214.8 |
| **Acenaphthylene** | 181.4 | 147.9 | 138.9 | 197.1 | 156.9 | 163.9 | 201.8 |
| **Anthracene** | 97.9 | 74.8 | 65.9 | 120.6 | 88.4 | 91.4 | 123.9 |
| **Benzo( A ) Anthracene** | 76.9 | 66.2 | 60.6 | 96.5 | 72.4 | 75.8 | 142.6 |
| **Benzo( B) Fluoranthene** | 85.4 | 54.8 | 50.9 | 124.5 | 63.2 | 68.9 | 130.5 |
| **Benzo( K ) Fluoranthene** | 52.1 | 47.9 | 41.8 | 66.9 | 52.4 | 56.9 | 124.8 |
| **Benzo ( G , H ) Perylene** | 61.4 | 52.6 | 47.9 | 81.4 | 59.8 | 62.4 | 97.8 |
| **Benzo ( A ) Pyrenen** | 30.5 | 24.5 | 20.1 | 35.6 | 26.9 | 30.4 | 66.3 |
| **Chrysene** | 89.7 | 79.8 | 67.5 | 105.6 | 85.4 | 88.7 | 117.8 |
| **Dibenzo ( A , H ) Anthracene** | 74.1 | 70.5 | 66.9 | 93.2 | 74.5 | 76.9 | 123.6 |
| **Fluranthene** | 63.9 | 57.4 | 50.3 | 841 | 62.1 | 64.8 | 124.5 |
| **Fluroene** | 55.8 | 48.9 | 41.2 | 68.6 | 51.4 | 55.9 | 98.7 |
| **Indeno** | 43.6 | 36.9 | 33.2 | 58.7 | 39.6 | 41.8 | 88.9 |
| **Naphthalene** | 81.4 | 75.6 | 68.4 | 103.6 | 77.4 | 81.4 | 130.9 |
| **Phenanthrene** | 63.9 | 54.8 | 50.1 | 82.4 | 58.9 | 60.8 | 97.4 |
| **Pyrene** | 44.1 | 40.3 | 35.9 | 63.9 | 43.6 | 45.8 | 87.0 |

| Name ( ppm ) | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|
| **Acenaphthene** | 158.9 | 325.9 | 228.7 | 122.8 | 123.7 | 114.8 | 201.5 | 133.6 |
| **Acenaphthylene** | 169.8 | 259.8 | 195.6 | 114.8 | 129.7 | 95.8 | 185.6 | 152.4 |
| **Anthracene** | 145.6 | 203.9 | 155.7 | 120.8 | 136.9 | 96.8 | 101.8 | 124.8 |
| **Benzo( A ) Anthracene** | 98.7 | 198.7 | 142.5 | 81.4 | 97.9 | 74.1 | 124.8 | 71.4 |
| **Benzo( B) Fluoranthene** | 74.9 | 245.6 | 169.8 | 62.8 | 66.9 | 59.8 | 98.7 | 60.5 |
| **Benzo( K ) Fluoranthene** | 98.4 | 198.9 | 154.8 | 82.1 | 87.1 | 71.5 | 108.7 | 80.1 |
| **Benzo ( G , H ) Perylene** | 75.1 | 157.8 | 133.6 | 70.1 | 84.5 | 60.4 | 80.3 | 63.5 |
| **Benzo ( A ) Pyrenen** | 45.8 | 69.8 | 50.9 | 40.3 | 45.9 | 35.9 | 51.4 | 30.5 |
| **Chrysene** | 78.9 | 185.9 | 148.9 | 69.8 | 75.2 | 58.9 | 84.6 | 70.2 |
| **Dibenzo ( A , H ) Anthracene** | 57.8 | 179.8 | 136.9 | 84.1 | 88.9 | 77.4 | 99.8 | 44.8 |
| **Fluranthene** | 71.8 | 169.8 | 142.9 | 83.6 | 90.4 | 71.4 | 100.5 | 53.9 |
| **Fluroene** | 56.8 | 177.6 | 133.9 | 64.8 | 74.8 | 52.6 | 74.9 | 41.8 |
| **Indeno** | 52.5 | 169.8 | 136.9 | 52.5 | 65.9 | 49.8 | 63.5 | 41.2 |
| **Naphthalene** | 85.4 | 148.9 | 114.8 | 96.8 | 112.6 | 80.5 | 115.9 | 70.4 |
| **Phenanthrene** | 52.5 | 136.9 | 98.9 | 66.4 | 70.5 | 55.4 | 75.9 | 41.8 |
| **Pyrene** | 33.6 | 124.8 | 85.9 | 54.8 | 63.9 | 45.9 | 66.9 | 25.9 |

## Conclusions:

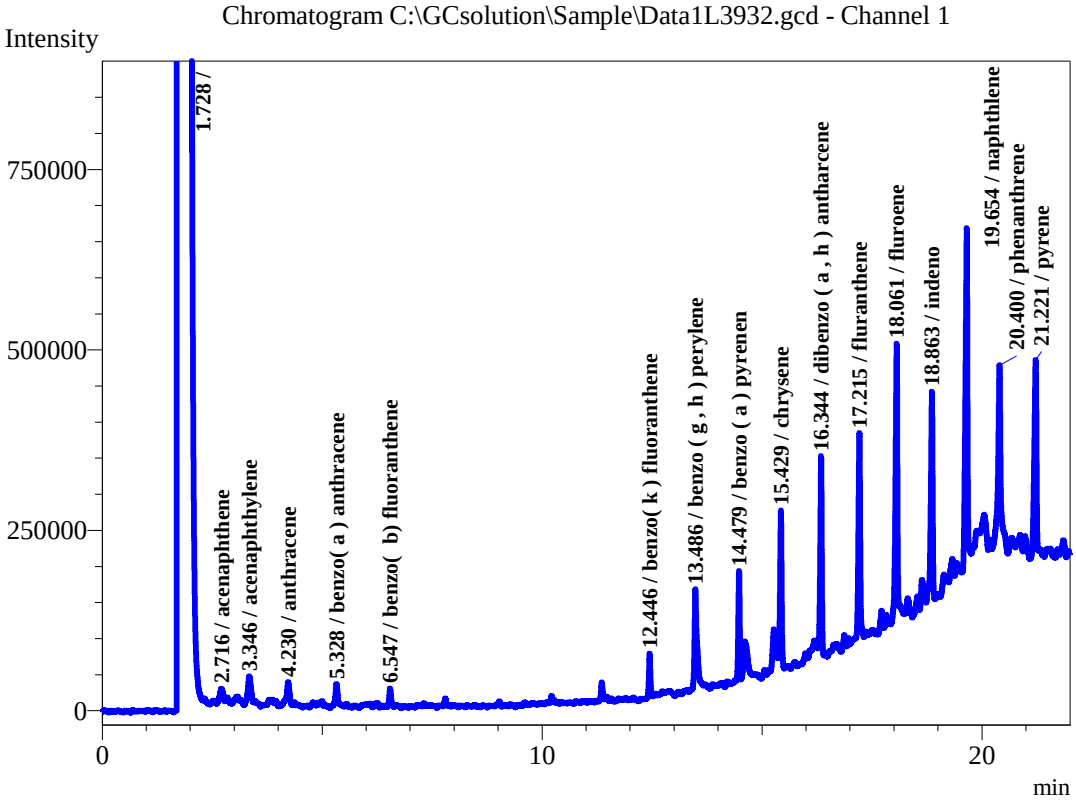
1. These are many bacteria of different species isolated from soil contaminated oil were able to biodegarade of polyaromatic hydrocarabons.
2. All bacterial isolates used in this study were able to produce biosurfactant, and *K.kristinae* and *D.acidovorans* were the most efficient one.
3. Fixing an ideal way for production of biosurfactant from *K.kristinae* and *D.acidovorans* sources in control condition such as best , pH, temperature, carbon sources, nitrogen sources, and incubation period.
4. The chemical composition of biosurfactant revealed that it consists of 71.066 percentage proteins, Lipid and carbohydrataes were not found.
5. Detection of 8 genes for biodegradation, only 6 genes were positive for several isolates while 2 genes not found in all isolates.
6. Microbial consortium were more efficient for degradation when estimate; best PH, O.D, plate count and for screening biosurfactant production compared with single strain cultured.
7. immobilized bacteria (*K.kristinae* OM838480.1. and *D.acidovorans* OM838393.1. ) were used to show their ability to treat soil contaminant oil by using HPLC and estimate reactivity by FTIR.

## Recommenadations:

1. Increase knowledge of the biodegradation of PAH compound may demonsatrate useful approaches towards microbial degradation.
2. Additional studies on gene that is responsible of encoding biosurfactant will provide valuable insights into the role of biodegaradation in studies of bacterial species.
3. Further studies on biosurfactant are needed to explore their values in industrial application such as food and therapeutic purposes.
4. Additional study for removing organic pollutant by using microorganisms .

## Sample Information

Sample Name = PAHs ( 10 ppm )
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa




Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.728 | 775386323 | 98.8787 | 92004540 | |
| 2 | 2.716 | 90132 | 0.0115 | 18243 | acenaphthene |
| 3 | 3.346 | 169756 | 0.0216 | 35803 | acenaphthylene |
| 4 | 4.230 | 104925 | 0.0134 | 29012 | anthracene |
| 5 | 5.328 | 77623 | 0.0099 | 26482 | benzo( a ) anthracen |
| 6 | 6.547 | 71802 | 0.0092 | 24122 | benzo( b) fluoranthe |
| 7 | 12.446 | 141604 | 0.0181 | 59704 | benzo( k ) fluoranthe |
| 8 | 13.486 | 506686 | 0.0646 | 134304 | benzo ( g , h ) peryle |
| 9 | 14.479 | 362686 | 0.0463 | 150691 | benzo ( a ) pyrenen |
| 10 | 15.429 | 530525 | 0.0677 | 208978 | chrysene |
| 11 | 16.344 | 689413 | 0.0879 | 268790 | dibenzo ( a , h ) anth |
| 12 | 17.215 | 844607 | 0.1077 | 282342 | fluranthene |
| 13 | 18.061 | 1096844 | 0.1399 | 366932 | fluroene |
| 14 | 18.863 | 825341 | 0.1052 | 279814 | indeno |
| 15 | 19.654 | 1482182 | 0.1890 | 458168 | naphthlene |
| 16 | 20.400 | 837097 | 0.1067 | 233776 | phenanthrene |
| 17 | 21.221 | 961431 | 0.1226 | 266698 | pyrene |
| Total | | 784178977 | 100.0000 | 94848399 | |

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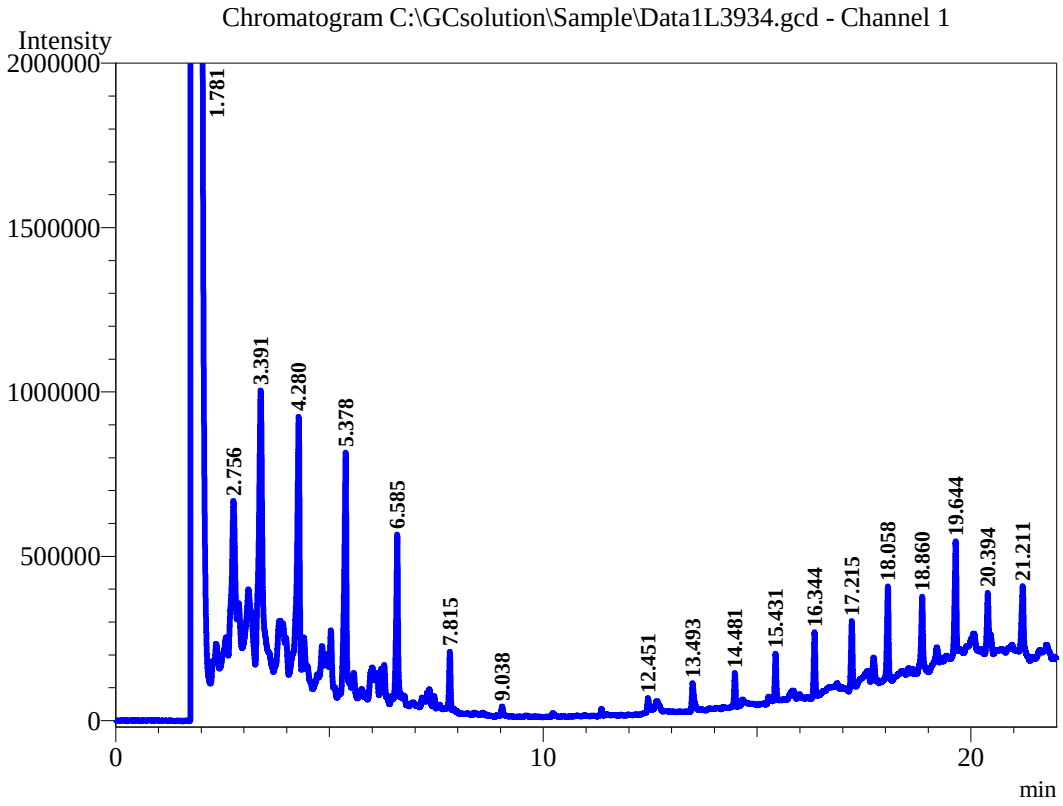
## Sample Information

Sample Name = 1
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

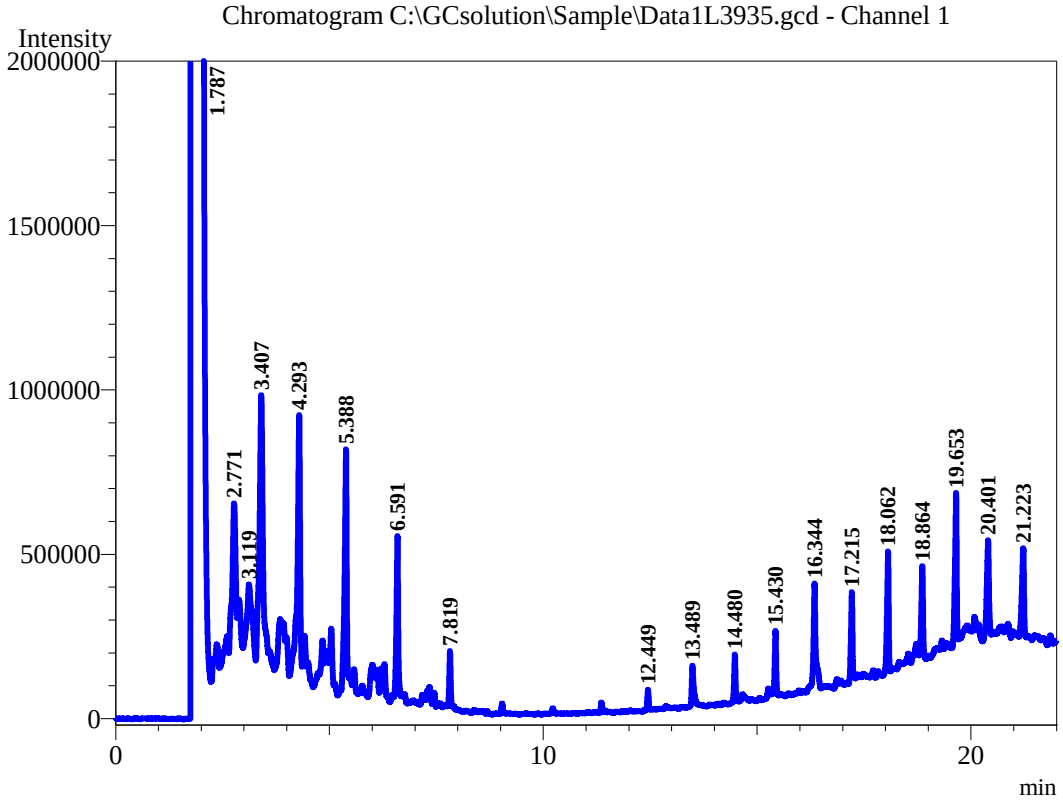



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.781 | 638207501 | 97.6601 | 82261495 | |
| 2 | 2.756 | 1152389 | 0.1763 | 310647 | |
| 3 | 3.391 | 2583428 | 0.3953 | 654702 | |
| 4 | 4.280 | 2117014 | 0.3240 | 668711 | |
| 5 | 5.378 | 1930918 | 0.2955 | 653480 | |
| 6 | 6.585 | 1261320 | 0.1930 | 464712 | |
| 7 | 7.815 | 429650 | 0.0657 | 167506 | |
| 8 | 9.038 | 71722 | 0.0110 | 27963 | |
| 9 | 12.451 | 93794 | 0.0144 | 39507 | |
| 10 | 13.493 | 299752 | 0.0459 | 84080 | |
| 11 | 14.481 | 239002 | 0.0366 | 102547 | |
| 12 | 15.431 | 337825 | 0.0517 | 142734 | |
| 13 | 16.344 | 462743 | 0.0708 | 193678 | |
| 14 | 17.215 | 523553 | 0.0801 | 199300 | |
| 15 | 18.058 | 800268 | 0.1225 | 284834 | |
| 16 | 18.860 | 719359 | 0.1101 | 226410 | |
| 17 | 19.644 | 811840 | 0.1242 | 314795 | |
| 18 | 20.394 | 724438 | 0.1109 | 183863 | |
| 19 | 21.211 | 731885 | 0.1120 | 205602 | |
| Total | | 653498401 | 100.0000 | 87186566 | |

## Sample Information

Sample Name = 2
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

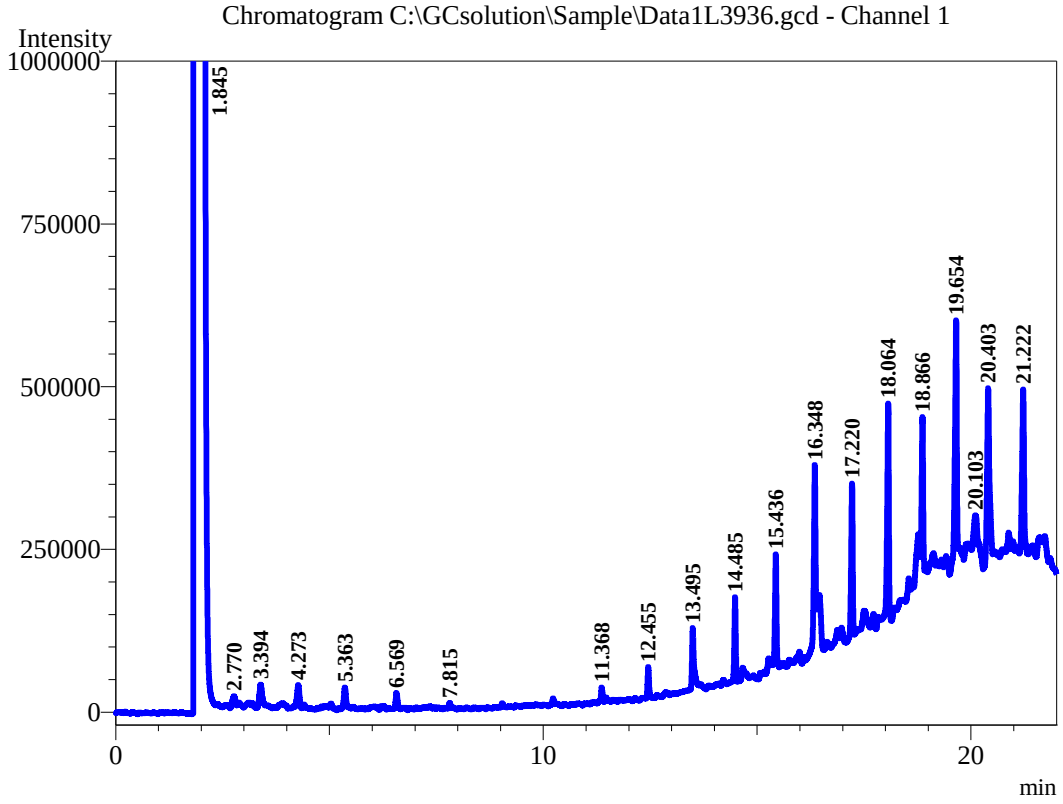



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.787 | 741935792 | 96.7979 | 85690501 | |
| 2 | 2.771 | 2062427 | 0.2691 | 392785 | |
| 3 | 3.119 | 1756296 | 0.2291 | 210617 | |
| 4 | 3.407 | 4533334 | 0.5914 | 795894 | |
| 5 | 4.293 | 3421744 | 0.4464 | 759066 | |
| 6 | 5.388 | 2425237 | 0.3164 | 705080 | |
| 7 | 6.591 | 1458570 | 0.1903 | 485466 | |
| 8 | 7.819 | 404464 | 0.0528 | 168105 | |
| 9 | 12.449 | 140673 | 0.0184 | 61089 | |
| 10 | 13.489 | 392116 | 0.0512 | 120535 | |
| 11 | 14.480 | 339160 | 0.0442 | 142972 | |
| 12 | 15.430 | 473935 | 0.0618 | 192163 | |
| 13 | 16.344 | 1166973 | 0.1523 | 309484 | |
| 14 | 17.215 | 726904 | 0.0948 | 267713 | |
| 15 | 18.062 | 1058868 | 0.1381 | 365602 | |
| 16 | 18.864 | 806075 | 0.1052 | 273091 | |
| 17 | 19.653 | 1359694 | 0.1774 | 450935 | |
| 18 | 20.401 | 1056379 | 0.1378 | 289470 | |
| 19 | 21.223 | 960284 | 0.1253 | 269490 | |
| Total | | 766478925 | 100.0000 | 91950058 | |

## Sample Information

Sample Name = 3
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

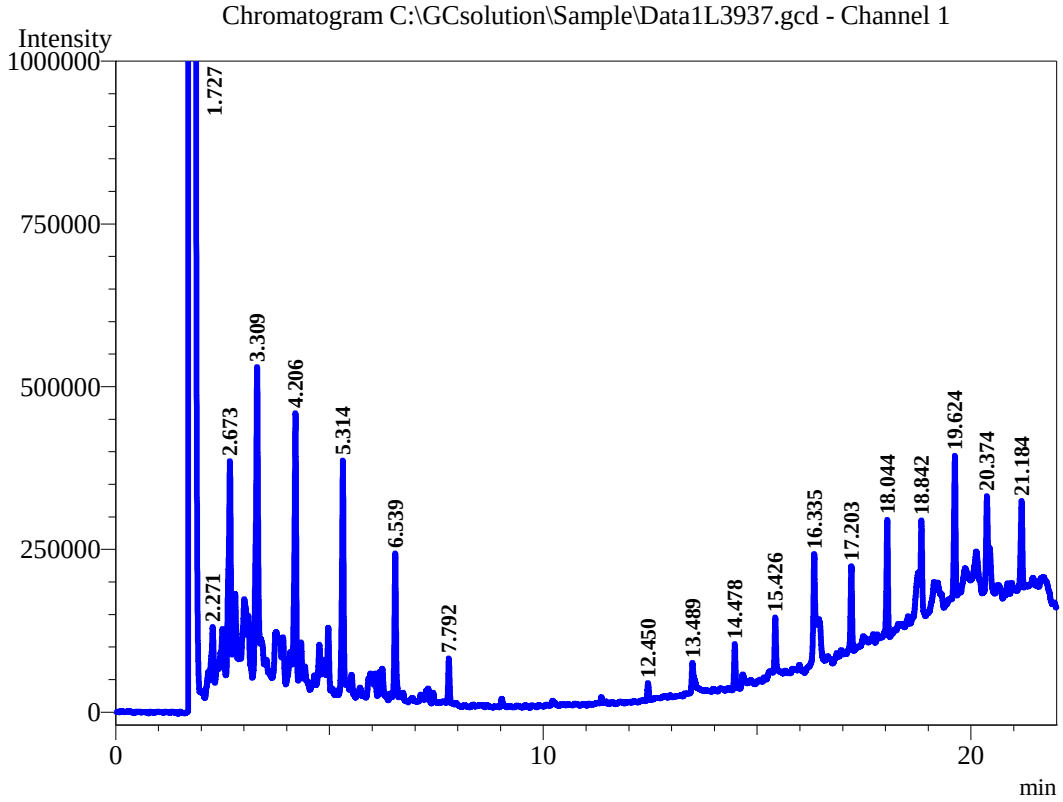



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.845 | 2652929513 | 99.7140 | 30198508 | |
| 2 | 2.770 | 52756 | 0.0020 | 12606 | |
| 3 | 3.394 | 132548 | 0.0050 | 32029 | |
| 4 | 4.273 | 126731 | 0.0048 | 33661 | |
| 5 | 5.363 | 94035 | 0.0035 | 30182 | |
| 6 | 6.569 | 63949 | 0.0024 | 22741 | |
| 7 | 7.815 | 24906 | 0.0009 | 8083 | |
| 8 | 11.368 | 58721 | 0.0022 | 21938 | |
| 9 | 12.455 | 115375 | 0.0043 | 46898 | |
| 10 | 13.495 | 281189 | 0.0106 | 91589 | |
| 11 | 14.485 | 296341 | 0.0111 | 128431 | |
| 12 | 15.436 | 430588 | 0.0162 | 170865 | |
| 13 | 16.348 | 621733 | 0.0234 | 243784 | |
| 14 | 17.220 | 618246 | 0.0232 | 233064 | |
| 15 | 18.064 | 916379 | 0.0344 | 326948 | |
| 16 | 18.866 | 529408 | 0.0199 | 215387 | |
| 17 | 19.654 | 1075412 | 0.0404 | 357659 | |
| 18 | 20.103 | 244055 | 0.0092 | 49587 | |
| 19 | 20.403 | 1089945 | 0.0410 | 265234 | |
| 20 | 21.222 | 837976 | 0.0315 | 251366 | |
| Total | | 2660539806 | 100.0000 | 32740560 | |

## Sample Information

Sample Name = 4
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

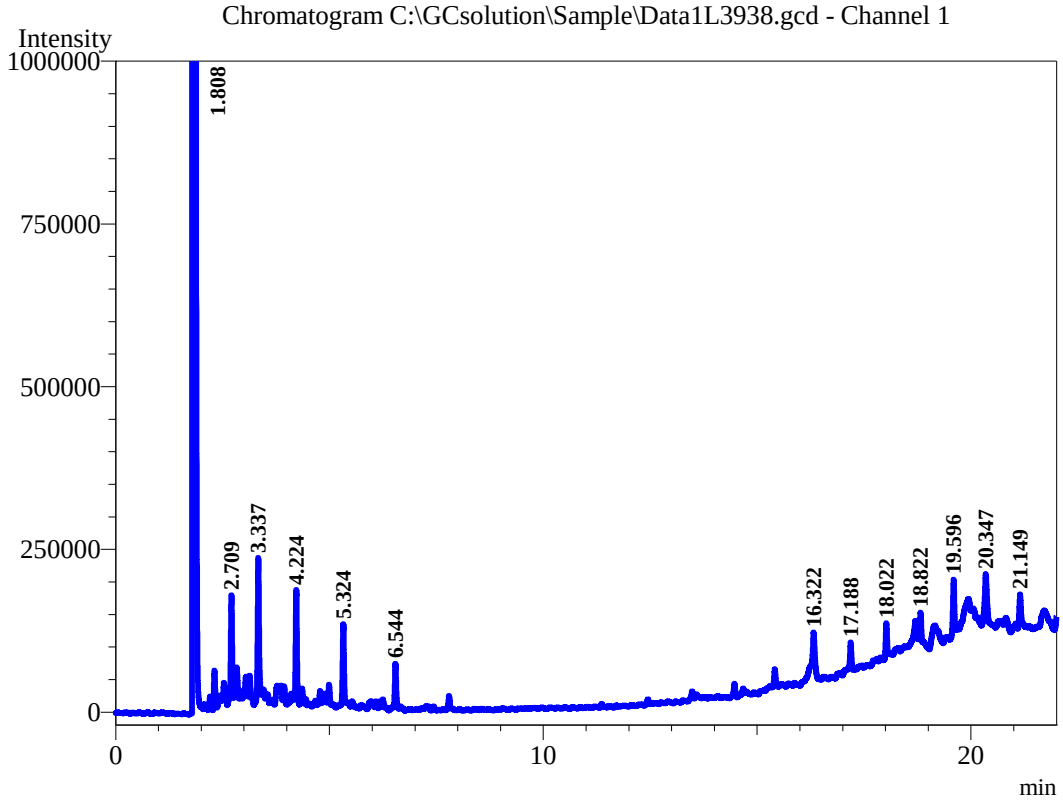



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.727 | 374011932 | 97.6465 | 71814477 | |
| 2 | 2.271 | 279008 | 0.0728 | 84086 | |
| 3 | 2.673 | 1123618 | 0.2934 | 308946 | |
| 4 | 3.309 | 1573525 | 0.4108 | 438747 | |
| 5 | 4.206 | 1222255 | 0.3191 | 382724 | |
| 6 | 5.314 | 989306 | 0.2583 | 350065 | |
| 7 | 6.539 | 579777 | 0.1514 | 218027 | |
| 8 | 7.792 | 159549 | 0.0417 | 68280 | |
| 9 | 12.450 | 62846 | 0.0164 | 26151 | |
| 10 | 13.489 | 92592 | 0.0242 | 36858 | |
| 11 | 14.478 | 163503 | 0.0427 | 69057 | |
| 12 | 15.426 | 186151 | 0.0486 | 82467 | |
| 13 | 16.335 | 353376 | 0.0923 | 136767 | |
| 14 | 17.203 | 320431 | 0.0837 | 128887 | |
| 15 | 18.044 | 433407 | 0.1132 | 176442 | |
| 16 | 18.842 | 245330 | 0.0641 | 117096 | |
| 17 | 19.624 | 571193 | 0.1491 | 214524 | |
| 18 | 20.374 | 276928 | 0.0723 | 109302 | |
| 19 | 21.184 | 381920 | 0.0997 | 133459 | |
| **Total** | | 383026647 | 100.0000 | 74896362 | |

## Sample Information

Sample Name = 5
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

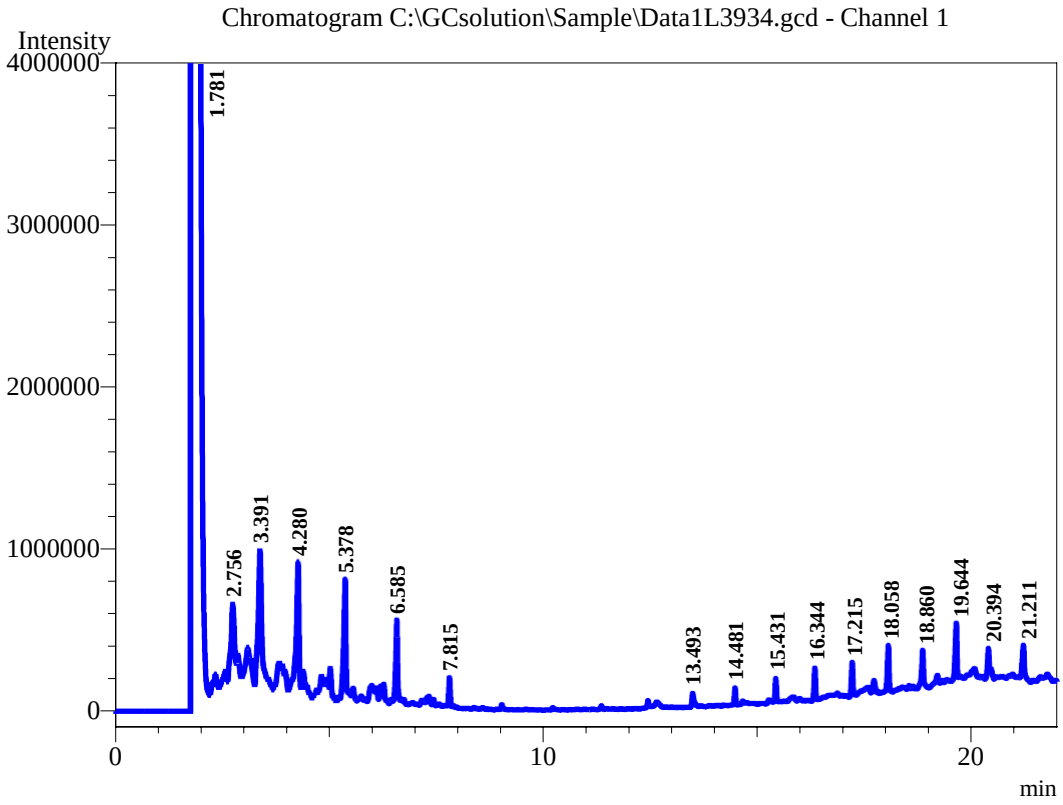



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.808 | 112596284 | 97.5876 | 39985092 | |
| 2 | 2.709 | 334815 | 0.2902 | 157686 | |
| 3 | 3.337 | 519590 | 0.4503 | 212814 | |
| 4 | 4.224 | 382441 | 0.3315 | 169360 | |
| 5 | 5.324 | 279260 | 0.2420 | 121985 | |
| 6 | 6.544 | 162194 | 0.1406 | 67069 | |
| 7 | 16.322 | 180329 | 0.1563 | 57640 | |
| 8 | 17.188 | 93308 | 0.0809 | 40217 | |
| 9 | 18.022 | 118935 | 0.1031 | 51666 | |
| 10 | 18.822 | 97311 | 0.0843 | 42483 | |
| 11 | 19.596 | 204972 | 0.1776 | 82083 | |
| 12 | 20.347 | 273577 | 0.2371 | 73479 | |
| 13 | 21.149 | 136680 | 0.1185 | 49469 | |
| Total | | 115379696 | 100.0000 | 41111043 | |

## Sample Information

Sample Name = 6
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

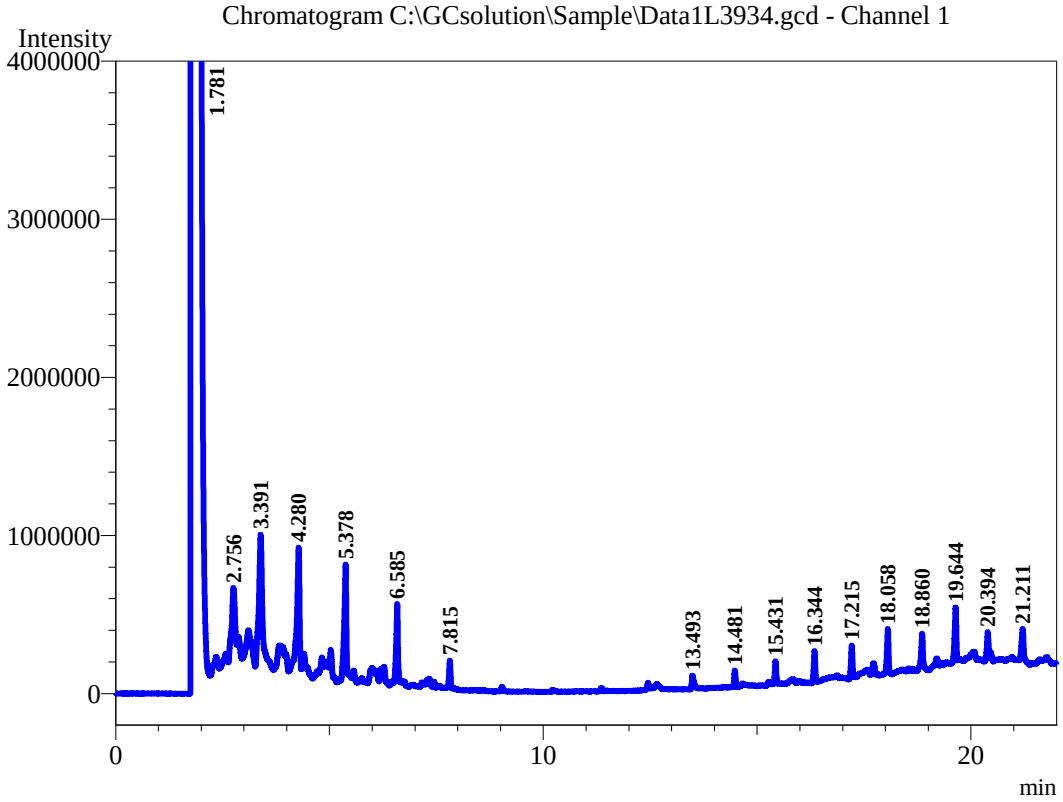



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.781 | 55777107 | 98.4455 | 8082038 | |
| 2 | 2.756 | 192984 | 0.1633 | 527077 | |
| 3 | 3.391 | 1239996 | 0.2462 | 843926 | |
| 4 | 4.280 | 1461646 | 0.2850 | 958864 | |
| 5 | 5.378 | 5693366 | 0.1647 | 247426 | |
| 6 | 6.585 | 287965 | 0.1550 | 739938 | |
| 7 | 7.815 | 815377 | 0.0267 | 29360 | |
| 8 | 13.493 | 89767 | 0.0171 | 35011 | |
| 9 | 14.481 | 21124 | 0.0030 | 81117 | |
| 10 | 15.431 | 53189 | 0.0069 | 22710 | |
| 11 | 16.344 | 348018 | 0.0683 | 187467 | |
| 12 | 17.215 | 25580 | 0.0504 | 124357 | |
| 13 | 18.058 | 525968 | 0.0919 | 283554 | |
| 14 | 18.860 | 292514 | 0.0529 | 124453 | |
| 15 | 19.644 | 648728 | 0.1143 | 297129 | |
| 16 | 20.394 | 290715 | 0.0513 | 134284 | |
| 17 | 21.211 | 325732 | 0.0575 | 144182 | |
| Total | | 566577776 | 100.0000 | 84423093 | |

## Sample Information

Sample Name = 7
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

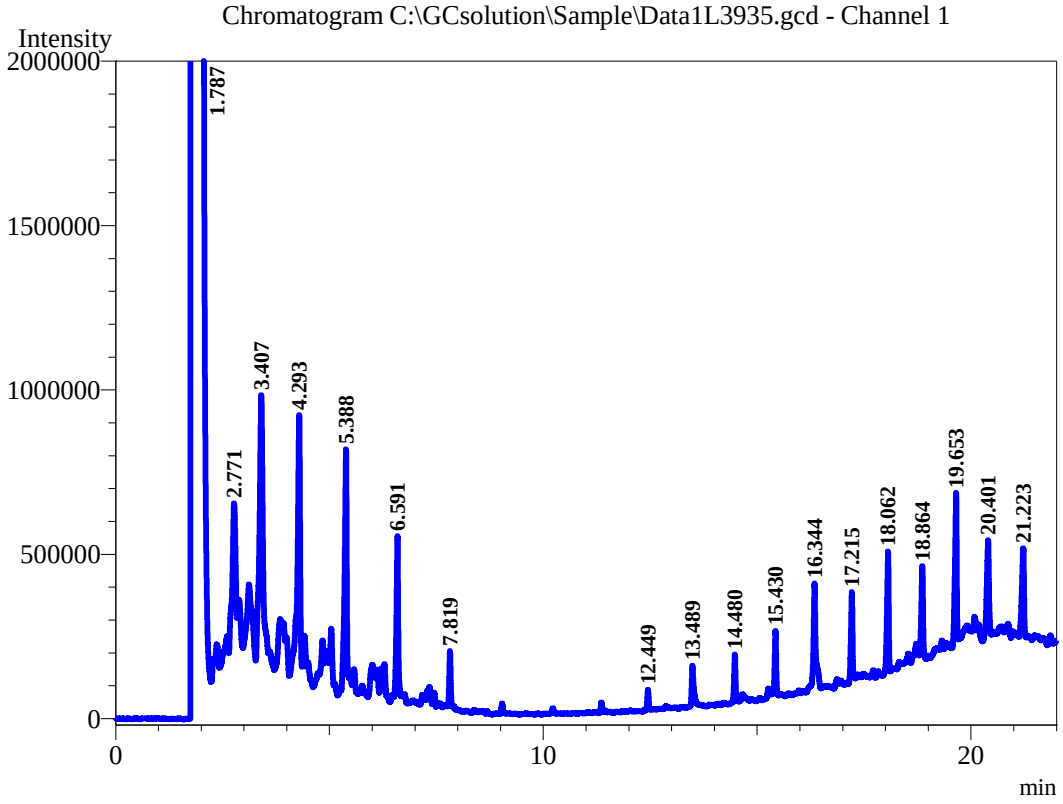



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.781 | 627914809 | 98.4186 | 82148423 | |
| 2 | 2.756 | 924984 | 0.1450 | 270377 | |
| 3 | 3.391 | 1811698 | 0.2840 | 540134 | |
| 4 | 4.280 | 1614646 | 0.2531 | 588064 | |
| 5 | 5.378 | 1352535 | 0.2120 | 566217 | |
| 6 | 6.585 | 455648 | 0.0714 | 303263 | |
| 7 | 7.815 | 225732 | 0.0354 | 120476 | |
| 8 | 13.493 | 108589 | 0.0170 | 51915 | |
| 9 | 14.481 | 114165 | 0.0179 | 64512 | |
| 10 | 15.431 | 39744 | 0.0062 | 26803 | |
| 11 | 16.344 | 305209 | 0.0478 | 155913 | |
| 12 | 17.215 | 523553 | 0.0821 | 199300 | |
| 13 | 18.058 | 520968 | 0.0817 | 235654 | |
| 14 | 18.860 | 517863 | 0.0812 | 204343 | |
| 15 | 19.644 | 647728 | 0.1015 | 271529 | |
| 16 | 20.394 | 438991 | 0.0688 | 147036 | |
| 17 | 21.211 | 487152 | 0.0764 | 175797 | |
| Total | | 638004014 | 100.0000 | 86069756 | |

## Sample Information

Sample Name = 8
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

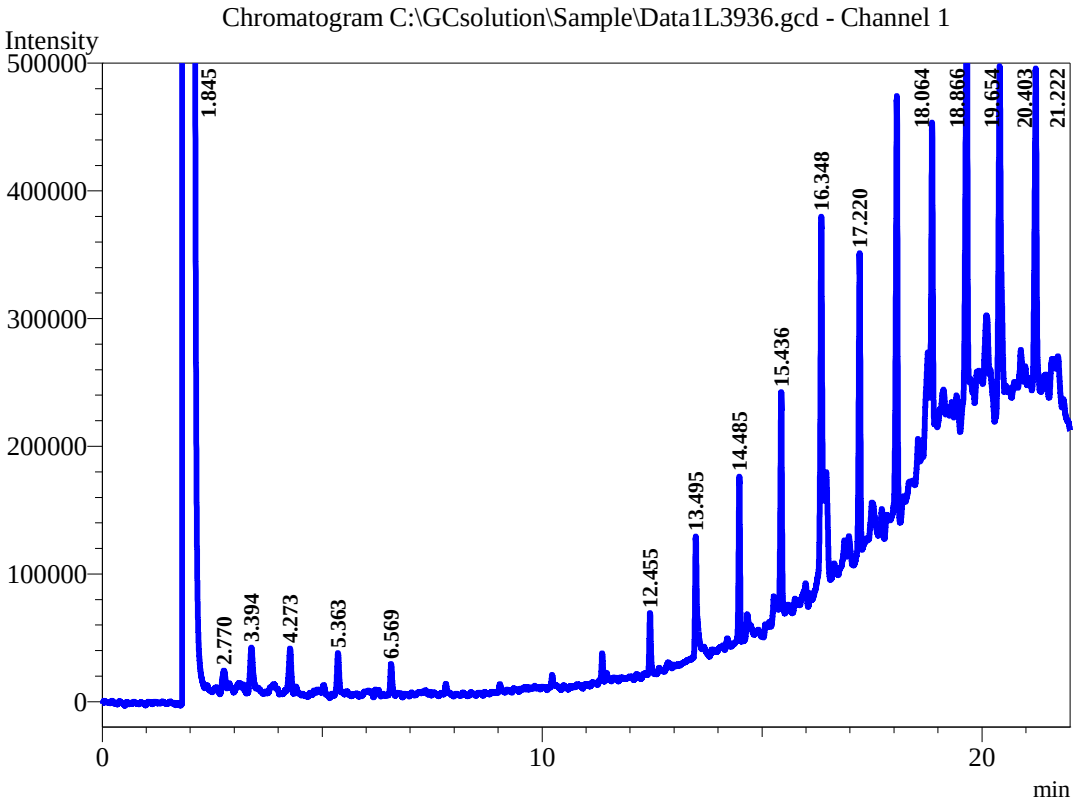



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.787 | 731022634 | 98.2370 | 85550201 | |
| 2 | 2.771 | 1833849 | 0.2464 | 373885 | |
| 3 | 3.407 | 1369461 | 0.1840 | 450903 | |
| 4 | 4.293 | 2197173 | 0.2953 | 682938 | |
| 5 | 5.388 | 1668474 | 0.2242 | 597509 | |
| 6 | 6.591 | 1077515 | 0.1448 | 419316 | |
| 7 | 7.819 | 322636 | 0.0434 | 150085 | |
| 8 | 12.449 | 130544 | 0.0175 | 59622 | |
| 9 | 13.489 | 223435 | 0.0300 | 99232 | |
| 10 | 14.480 | 25518 | 0.0034 | 40973 | |
| 11 | 15.430 | 218318 | 0.0293 | 127691 | |
| 12 | 16.344 | 438304 | 0.0589 | 213935 | |
| 13 | 17.215 | 367311 | 0.0494 | 204413 | |
| 14 | 18.062 | 825179 | 0.1109 | 332116 | |
| 15 | 18.864 | 598200 | 0.0804 | 240939 | |
| 16 | 19.653 | 747786 | 0.1005 | 352879 | |
| 17 | 20.401 | 598628 | 0.0804 | 213554 | |
| 18 | 21.223 | 476749 | 0.0641 | 189031 | |
| Total | | 744141714 | 100.0000 | 90299222 | |

## Sample Information

Sample Name = 9
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

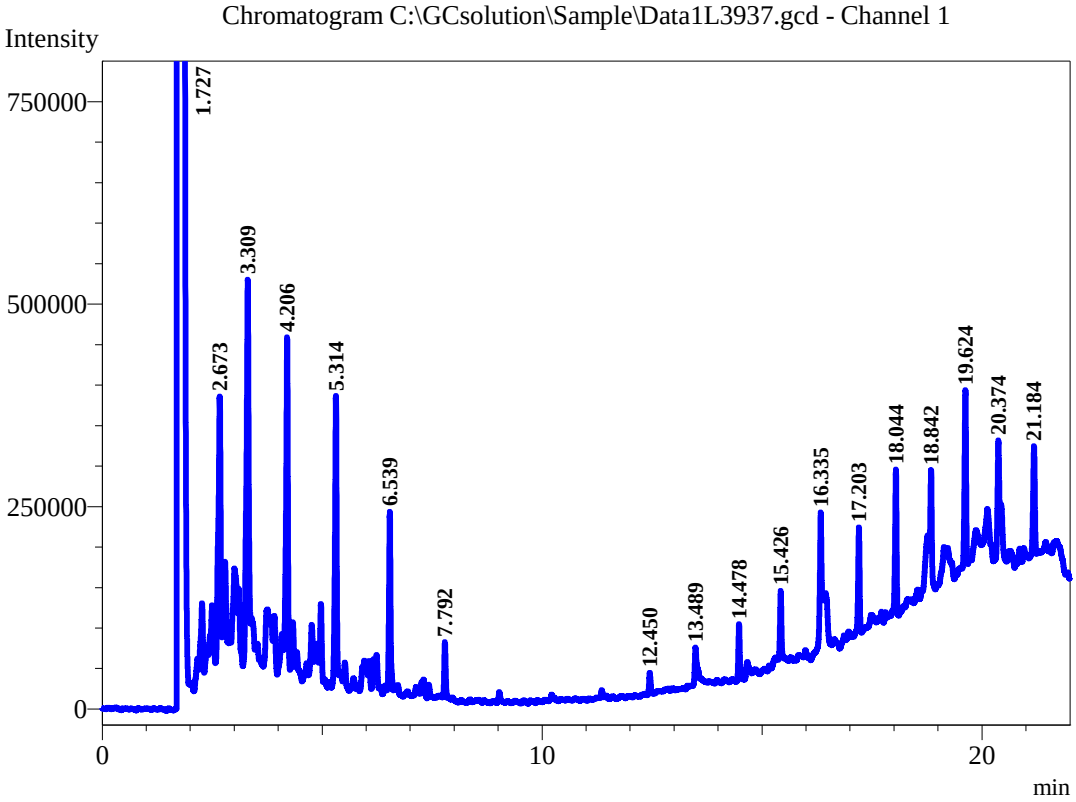



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.845 | 2652553340 | 99.7681 | 30194690 | |
| 2 | 2.770 | 19298 | 0.0007 | 6975 | |
| 3 | 3.394 | 60746 | 0.0023 | 21191 | |
| 4 | 4.273 | 68578 | 0.0026 | 23921 | |
| 5 | 5.363 | 86783 | 0.0033 | 29083 | |
| 6 | 6.569 | 24768 | 0.0009 | 12441 | |
| 7 | 12.455 | 65766 | 0.0025 | 33557 | |
| 8 | 13.495 | 182899 | 0.0069 | 77424 | |
| 9 | 14.485 | 190307 | 0.0072 | 103957 | |
| 10 | 15.436 | 310482 | 0.0117 | 139927 | |
| 11 | 16.348 | 1074267 | 0.0404 | 260286 | |
| 12 | 17.220 | 439125 | 0.0165 | 195683 | |
| 13 | 18.064 | 537295 | 0.0202 | 255430 | |
| 14 | 18.866 | 516229 | 0.0194 | 212970 | |
| 15 | 19.654 | 684211 | 0.0257 | 294026 | |
| 16 | 20.403 | 1067244 | 0.0401 | 262302 | |
| 17 | 21.222 | 837976 | 0.0315 | 251366 | |
| Total | | 2658719314 | 100.0000 | 32375229 | |

## Sample Information

Sample Name = 10
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

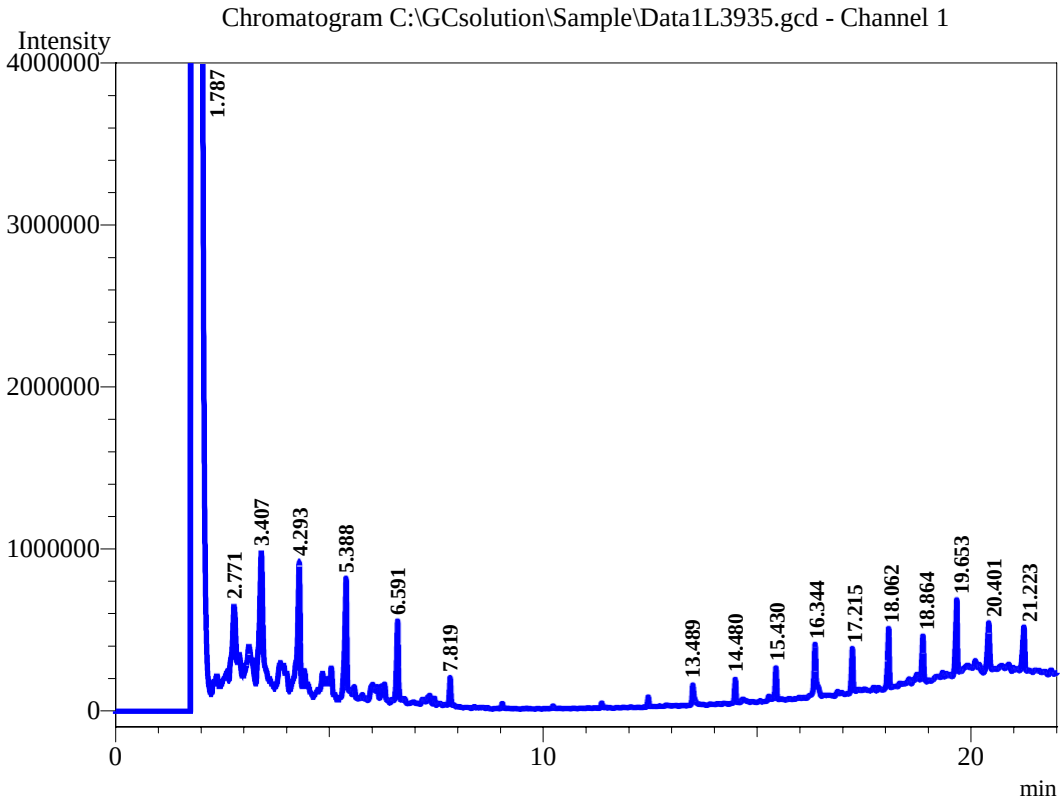



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.727 | 337943575 | 98.1149 | 67301389 | |
| 2 | 2.673 | 610740 | 0.1773 | 237350 | |
| 3 | 3.309 | 875096 | 0.2541 | 329901 | |
| 4 | 4.206 | 1019551 | 0.2960 | 374147 | |
| 5 | 5.314 | 864945 | 0.2511 | 333513 | |
| 6 | 6.539 | 506355 | 0.1470 | 206993 | |
| 7 | 7.792 | 51784 | 0.0150 | 35400 | |
| 8 | 12.450 | 57777 | 0.0168 | 24556 | |
| 9 | 13.489 | 74712 | 0.0217 | 33361 | |
| 10 | 14.478 | 80294 | 0.0233 | 47587 | |
| 11 | 15.426 | 134835 | 0.0391 | 69153 | |
| 12 | 16.335 | 488923 | 0.1419 | 133478 | |
| 13 | 17.203 | 259590 | 0.0754 | 117268 | |
| 14 | 18.044 | 399550 | 0.1160 | 170750 | |
| 15 | 18.842 | 434309 | 0.1261 | 129939 | |
| 16 | 19.624 | 322384 | 0.0936 | 163160 | |
| 17 | 20.374 | 192260 | 0.0558 | 89489 | |
| 18 | 21.184 | 119951 | 0.0348 | 71175 | |
| Total | | 344436631 | 100.0000 | 69868609 | |

## Sample Information

Sample Name = 11
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

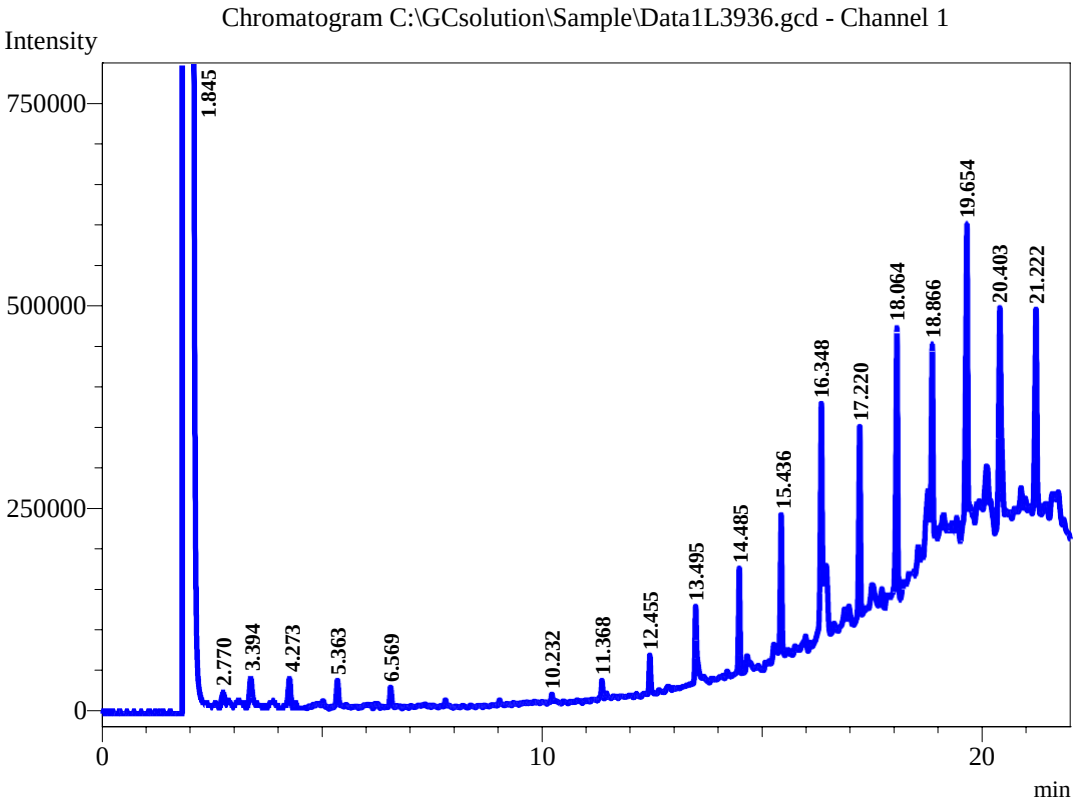



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.787 | 734235077 | 98.5700 | 85596309 | |
| 2 | 2.771 | 5113459 | 0.1522 | 530325 | |
| 3 | 3.407 | 9136961 | 0.1838 | 945003 | |
| 4 | 4.293 | 8142514 | 0.1914 | 852298 | |
| 5 | 5.388 | 7148980 | 0.2000 | 657854 | |
| 6 | 6.591 | 697958 | 0.1316 | 441541 | |
| 7 | 7.819 | 231395 | 0.0421 | 514889 | |
| 8 | 13.489 | 417081 | 0.0229 | 28552 | |
| 9 | 14.480 | 517338 | 0.0232 | 19158 | |
| 10 | 15.430 | 67655 | 0.0103 | 54372 | |
| 11 | 16.344 | 683962 | 0.0859 | 255616 | |
| 12 | 17.215 | 129612 | 0.0264 | 124053 | |
| 13 | 18.062 | 582454 | 0.0704 | 286901 | |
| 14 | 18.864 | 524243 | 0.0729 | 222733 | |
| 15 | 19.653 | 784786 | 0.1004 | 385279 | |
| 16 | 20.401 | 227772 | 0.0373 | 121337 | |
| 17 | 21.223 | 5891294 | 0.0794 | 280570 | |
| Total | | 744886841 | 100.0000 | 89801390 | |

## Sample Information

Sample Name = 12
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

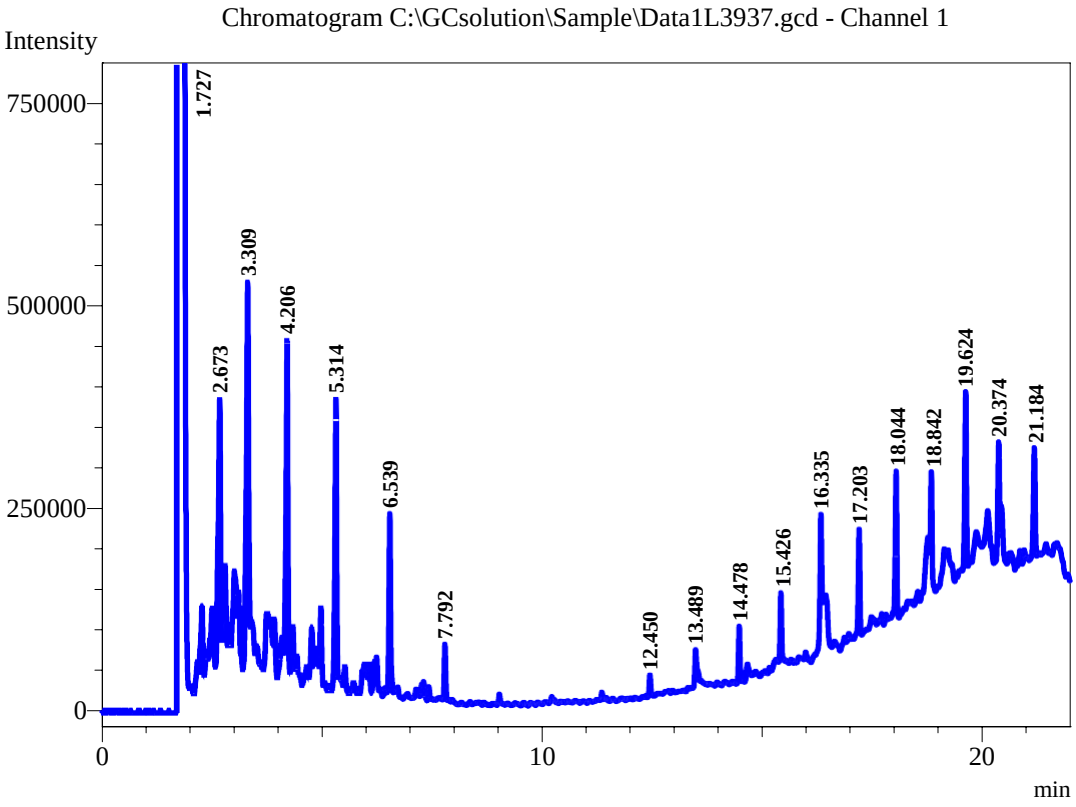



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.845 | 264628381 | 99.8232 | 3013337 | |
| 2 | 2.770 | 48142 | 0.0016 | 51195 | |
| 3 | 3.394 | 48464 | 0.0017 | 81766 | |
| 4 | 4.273 | 109218 | 0.0039 | 63060 | |
| 5 | 5.363 | 47313 | 0.0016 | 41946 | |
| 6 | 6.569 | 25880 | 0.0011 | 51488 | |
| 7 | 10.232 | 17947 | 0.0007 | 8872 | |
| 8 | 11.368 | 32491 | 0.0013 | 12756 | |
| 9 | 12.455 | 67576 | 0.0025 | 38357 | |
| 10 | 13.495 | 112761 | 0.0044 | 62093 | |
| 11 | 14.485 | 108283 | 0.0039 | 68104 | |
| 12 | 15.436 | 312042 | 0.0117 | 136927 | |
| 13 | 16.348 | 707533 | 0.0266 | 244417 | |
| 14 | 17.220 | 432965 | 0.0166 | 202070 | |
| 15 | 18.064 | 2972082 | 0.0110 | 175875 | |
| 16 | 18.866 | 5172629 | 0.0195 | 212270 | |
| 17 | 19.654 | 686421 | 0.0258 | 298426 | |
| 18 | 20.403 | 596745 | 0.0225 | 206365 | |
| 19 | 21.222 | 544202 | 0.0204 | 209034 | |
| Total | | 2650921845 | 100.0000 | 32081958 | |

## Sample Information

Sample Name = 13
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

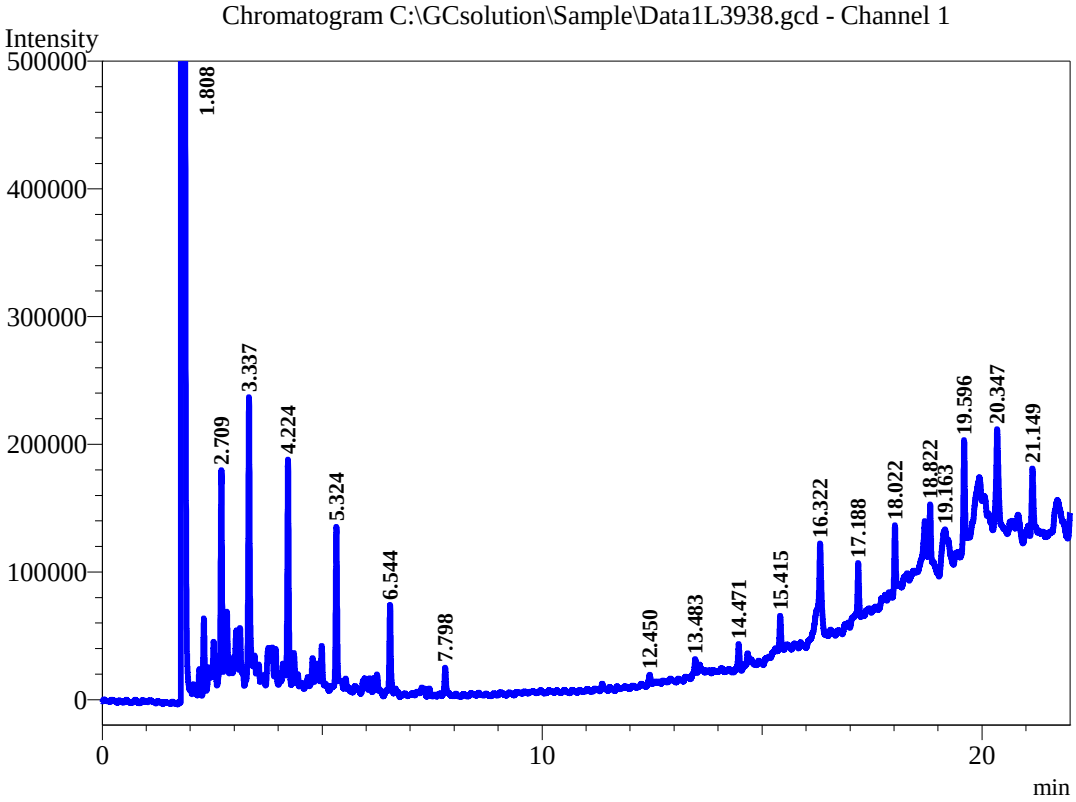



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.727 | 372937715 | 98.2612 | 71593609 | |
| 2 | 2.673 | 842979 | 0.1132 | 160022 | |
| 3 | 3.309 | 1916555 | 0.3070 | 388086 | |
| 4 | 4.206 | 568998 | 0.1816 | 306258 | |
| 5 | 5.314 | 877067 | 0.2030 | 313250 | |
| 6 | 6.539 | 250423 | 0.1328 | 206648 | |
| 7 | 7.792 | 411192 | 0.0295 | 57542 | |
| 8 | 12.450 | 61648 | 0.0045 | 11538 | |
| 9 | 13.489 | 29940 | 0.0261 | 36508 | |
| 10 | 14.478 | 86246 | 0.0164 | 41506 | |
| 11 | 15.426 | 123435 | 0.0355 | 69153 | |
| 12 | 16.335 | 581418 | 0.1356 | 147791 | |
| 13 | 17.203 | 228594 | 0.0751 | 122837 | |
| 14 | 18.044 | 3829950 | 0.1053 | 170750 | |
| 15 | 18.842 | 535974 | 0.1474 | 132909 | |
| 16 | 19.624 | 413332 | 0.1143 | 188389 | |
| 17 | 20.374 | 129260 | 0.0507 | 89489 | |
| 18 | 21.184 | 203070 | 0.0609 | 98971 | |
| Total | | 379537196 | 100.0000 | 74135256 | |

## Sample Information

Sample Name = 14
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa

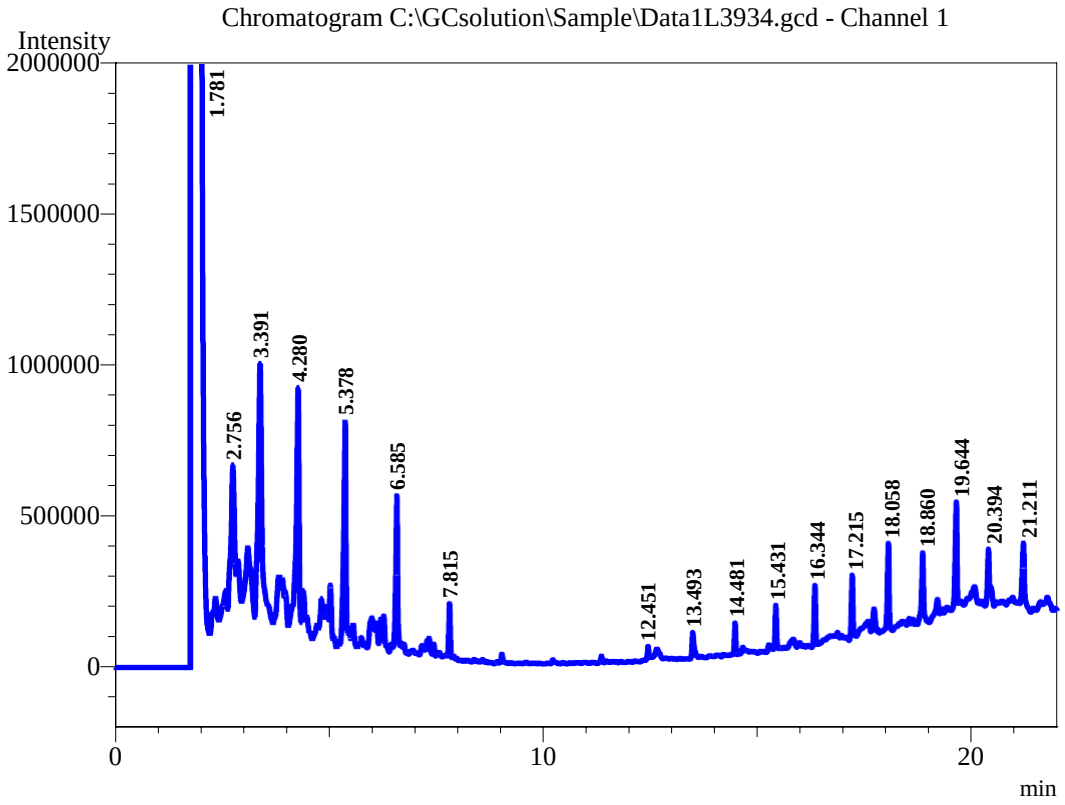



Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.808 | 111534520 | 97.9809 | 39914082 | |
| 2 | 2.709 | 101772 | 0.0894 | 88667 | |
| 3 | 3.337 | 366748 | 0.3222 | 183638 | |
| 4 | 4.224 | 212804 | 0.1869 | 126548 | |
| 5 | 5.324 | 258347 | 0.2270 | 114928 | |
| 6 | 6.544 | 140561 | 0.1235 | 64015 | |
| 7 | 7.798 | 39083 | 0.0343 | 18429 | |
| 8 | 12.450 | 24795 | 0.0218 | 7851 | |
| 9 | 13.483 | 13597 | 0.0119 | 7618 | |
| 10 | 14.471 | 33735 | 0.0296 | 16579 | |
| 11 | 15.415 | 8955 | 0.0079 | 6560 | |
| 12 | 16.322 | 288226 | 0.2532 | 64922 | |
| 13 | 17.188 | 91223 | 0.0801 | 39830 | |
| 14 | 18.022 | 117186 | 0.1029 | 50568 | |
| 15 | 18.822 | 68046 | 0.0598 | 37098 | |
| 16 | 19.163 | 101624 | 0.0893 | 16649 | |
| 17 | 19.596 | 158207 | 0.1390 | 72431 | |
| 18 | 20.347 | 143598 | 0.1261 | 53118 | |
| 19 | 21.149 | 129840 | 0.1141 | 47370 | |
| Total | | 113832867 | 100.0000 | 40930901 | |

## Sample Information

Sample Name = 15
Injection Volume = 1 uL
Tem Injector = 280 C
Tem Detector ( FID ) = 330 C
Column Oven ( Z B - 5 ) = 100 ( hold 1 min ) - 300 C ( hold 2 min ) ( 10 c / min )
pressure= 100kpa




Peak Table - Channel 1

| Peak# | Ret.Time | Area | Area% | Height | Name |
|---|---|---|---|---|---|
| 1 | 1.781 | 634081647 | 98.0642 | 8222092 | |
| 2 | 2.756 | 1998291 | 0.3066 | 239475 | |
| 3 | 3.391 | 2762360 | 0.4212 | 465988 | |
| 4 | 4.280 | 2054496 | 0.3162 | 664569 | |
| 5 | 5.378 | 1521297 | 0.2340 | 854337 | |
| 6 | 6.585 | 1137738 | 0.1821 | 944424 | |
| 7 | 7.815 | 200096 | 0.0310 | 71075 | |
| 8 | 12.451 | 48653 | 0.0134 | 53801 | |
| 9 | 13.493 | 150841 | 0.0167 | 25494 | |
| 10 | 14.481 | 165499 | 0.0240 | 148261 | |
| 11 | 15.431 | 278100 | 0.0436 | 163141 | |
| 12 | 16.344 | 380509 | 0.0472 | 185513 | |
| 13 | 17.215 | 58825 | 0.0136 | 585380 | |
| 14 | 18.058 | 522068 | 0.0806 | 223554 | |
| 15 | 18.860 | 443874 | 0.0678 | 12990 | |
| 16 | 19.644 | 264685 | 0.0381 | 178159 | |
| 17 | 20.394 | 388492 | 0.0594 | 155235 | |
| 18 | 21.211 | 226164 | 0.0404 | 122570 | |
| Total | | 6465432335 | 100.0000 | 86355658 | |