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Isolation, Cloning and Characterization of Oil Degrading Genes from Microbes and their Bioinformatics Analysis

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Abstract

The isolation of new oil-degrading microbes from water or soil and the evaluation of their capacity to degrade oil were both very essential steps in the process of cleaning up oil-polluted habitats and extracting crude oil. For the quick and effective cloning of enzymatic genes of isolated oil degrading microbes, cloning was a relatively new method. Although the cloning method often relies on partial amino acid sequence in order to generate adequate DNA primers, the cloning process is tied to availability to accurate and sensitive enzyme tests. This method was quick and may potentially be used to characterize extracellular enzymatic genes. In this article, we also detailed the cloning procedures that are employed in the investigation and characterization of the enzymatic genes expression in microbes. In recent years, because to the revolution of microbial families, high-throughput sequence technologies have been created. These technologies, together with their analysis using appropriate bioinformatics methodologies, have played a vital role in the exploration of microbial genes. In conclusion, we covered the mechanisms of oil degradation from microbes, the isolation of oil degrading microbes, and the isolation of several oil degrading genes. categorized them using two distinct ways as follows: traditional physiochemical and 16S rRNA gene sequence analysis, as well as cloning and study of the expression of genes in microbes using bioinformatics.

Keywords: Oil-degrading microbes, enzymatic genes, hydrocarbon, cloning expression, bioinformatics analysis, bacteria, fungi, algae.

Introduction

Throughout industries and everyday life, oil-based products were the primary source of energy [1]. These products, which were produced from crude oil and included hydrocarbon compounds as their primary components, included gasoline, gasoline, kerosene, diesel oil, and lubricating oil, among others [2]. It is thought to have originated from ancient germs like algae and plants that

were discovered in reservoirs underneath the surface of the world. Saturates, aromatics, resins, and asphaltenes are the four categories of products made from oil [3]. Hydrocarbons were released into the environment as a result of human activities, which also contributed to the contamination of water and soil with oil [4]. According to the bioremediation process, microorganisms are utilized to remove pollutants due to the numerous metabolic capabilities that they possess. This approach is an emerging method for the removal and degradation of many pollutants, including by-products of oil industries [5]. The bioremediation process was non-invasive and reasonably inexpensive [6]. The principal and optimal processes for removing oils and other hydrocarbon pollutants from the environment were biodegradation by microorganisms, which was also less expensive in coastal areas than other removal technologies [7, 8]. In this setting, a wide variety of microorganisms, including as bacteria, fungus, and algae, may be found in the soil and aquatic environment. These organisms are equipped with the enzymatic capabilities necessary for the full mineralization of those hydrocarbons[1]

The oxidation process of organic molecules is finished by mineralization, which is included in the degradation process. (b) the transformation of pollutants into intermediates that are smaller and less toxic via biological processes (c) the reduction process, in which compounds with highly electrophilic nitro and halo- groups are transformed to less toxic versions by the transfer of electrons from an electron donor, such as sugar or fatty acid, to the contaminant [9]. Since there are microorganisms present, the process of hydrocarbon biodegradation may be sustained if there is an enough concentration of nutrients and oxygen. The pH ranged from 6 to 9. Physical and chemical properties of oils, as well as their surface area, were significant factors in determining bioremediation. There were primarily two approaches to oil spill bioremediation: (a) bio-augmentation, in which oil degrading bacteria were added to an already existing microbial population; and (b) bio-stimulation, in which the growth of indigenous oil depredation was stimulated by the addition of nutrients or growth-limiting co-substrates.

Where, Biodegradation was a complicated process that was dependent on the environment and the quantity of hydrocarbons that were present in it. Saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) are the four types of hydrocarbons [10]. According to Cooney et al., [11], that deterioration was caused by a number of different reasons. One of the most critical factors was the restricted availability of microorganisms capable of limiting the biodegradation of oil pollutants in the environment. It is difficult for it to be degraded after it has bound with soil oil [12]. The susceptibility of different types of hydrocarbons to microbial assault varies. It might be arranged in the following order: linear alkanes > branched alkanes > tiny aromatics > cyclic alkanes [7, 13]. High molecular weight polycyclic aromatic hydrocarbons (PAHs) are one example of a chemical that cannot be degraded [14]. So, in this review, we discussed the isolation, cloning, and characterisation of oil degrading genes in microbes, as well as their bioinformatics analysis.

Isolation of oil degrading microbes

(i) Hydrocarbon degrading bacteria: In order to efficiently and effectively breakdown distinct percentages of the total petroleum hydrocarbons, Mandal et al. isolated 324 bacteria from 110

different species of oil sands and crude oil sludge [15]. Mahjoubi et al. described isolated bacterial genera "*Pseudomonas, Ochrabactrum, Bacilllus, Agrobacterium, Stenotrophomonas, Brevundimonas, Gordonia, Acinetobacter, Achromobacter, Microbacterium, Sphingobium, Rhodococcus, Luteibacter, Kocuria, and Novosphingobium*" that were contaminated with oil [16]. In a different investigation, Chaillan et al. [17] identified isolated bacterial genera "*Gordonia, Burkholderia, Aeromicrobium, Mycobacterium, Dietzia, and Brevibacterium"* from petroleum contaminated soil. Pseudomonas species, on the other hand, were an important family of microbes that degraded xenobiotic substances [18]. P. aeruginosa DSVP20, which degrades 97% eicosane, 75% pristine, and 47% fluoranthene in the presence of pure biosurfactant after incubation for up to one week, was reported by Sharma and his colleagues[18].

On the other hand, Hamza et al. [19] explain that P. aeruginosa was able to effectively decrease hydrocarbon components ranging from C12 to C30. After 24 hours, the strain was found to breakdown 48% of the total petroleum hydrocarbons, and after 48 hours, it had degraded 77% of them; after incubation, it had degraded 77% of them. During 21 days of degradation caused by Pseudomonas, Linda et al. detected 88.5% of the 2% petroleum [20]. According to Mahjoubi and colleagues, the Acinetobacter species was the most prevalent strain capable of using hydrocarbons in marine sediments [16]. In a separate investigation, Throne-Holst et al. [21] report how Acinetobacter sp. strain DSM 17874 mineralizes alkanes with carbon chain lengths ranging from $C_{10}H_{22}$ to $C_{40}H_{82}$. A significant proportion of hydrocarbon molecules are also mineralized by Enterobacter species. For example, the biosurfactant exopolysaccharide EPS 71A is used by Enterobacter cloacae to break down benzene, hexane, xylene, paraffin, kerosene, wax, and other cooking oils [22]. Enterobacter sp. strains EK3.1 and EK4 utilise a combination of hydrocarbons as their only energy sources. The bacterial strains metabolize a hydrocarbon combination of branched alkane 2,6,10,14-tetramethylpentadecane, toluene, and PAHs acenaphthylene and acenaphthene as carbon sources for growth and energy in mineral salts medium[23].

(ii) Hydrocarbon degrading fungi: Fungal genera such as "*Amorphoteca, Fusarium, Graphium, Aspergillus, TalaromycesNeosartorya, Paecilomyces, and Penicillium"* were isolated from petroleum contaminated soil [17]. Eight of the twelve fungal species that were recovered from oil contaminated soils and crude oil biodegradation were isolated by Uzoamaka et al. [24]. These eight species demonstrated the greatest and quickest biodegradation compared to Aspergillus versicolor and Aspergillus niger. Yet, in both aquatic and terrestrial environments, algae and protozoa were the most significant members of the microbial family. Walker and his colleagues isolated an alga called Protothecazopfi that was able to use crude oil and mixed oil substrate and shown substantial degradation of n-alkanes, iso-alkanes, and aromatic hydrocarbons [25]. Nine cyanobacteria, five green algae, one red alga, one brown alga, and two diatoms were shown to be able to oxidize naphthalene, according to Cerniglia et al. The use of hydrocarbons was not shown by Protozoa, however[26].

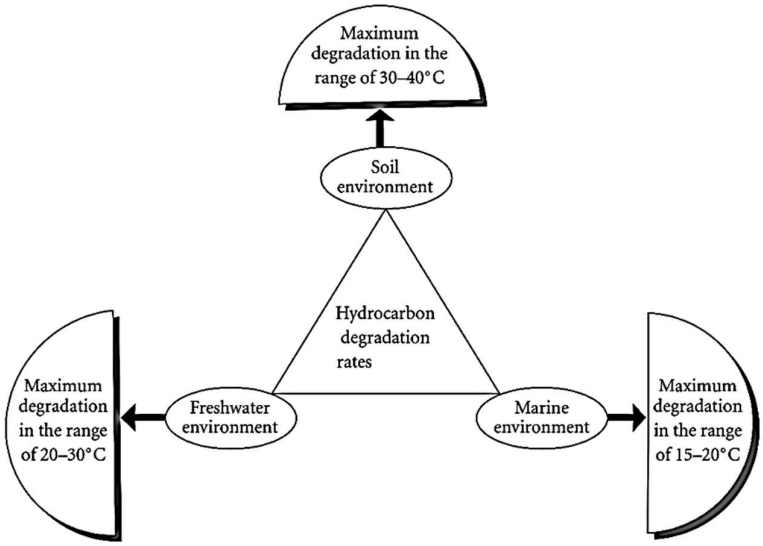

Figure 1: Hydrocarbon degradation rates in soil, fresh water and marine water[27, 28].

Distribution of pheN, alkB, CYP153A, and almA genes

For the assessment of phenol degraded strains of bacteria, phenol hydroxylase, which catalyzed the first stage of the phenol catabolic pathway, was often utilized [29-31]. Around 60 isolates were found to have the pheN gene, 35 of which contained six different phylotypes and represented 10.7% of the total number of isolates cultured at ambient temperature. The remaining 25 isolates contained eight different phylotypes and represented 9.6% of the total number of isolates cultured at constant temperature. On the other hand, several strains harboring the pheN gene that were isolated by the direct-plating method were included in 34 isolates, 11 phylotypes, or 13.6% of the total strains. compared to the enrichment method of isolation, where 26 isolates, 4 strains, or 7.7% of the total strains. PheN genes were divided into two groups based on phylogenetic distance (Figure 2). Cluster I comprises of 52 sequences (4 genotypes) from 52 isolates (10 phylotypes) that belonged to Marinobacter, Pseudomonas, Achromobacter, and Halomonas. Others belonged to Gammaproteobacteria, whereas Achromobacter was connected to Betaproteobacteria. Pseudomonas strains pheN and the gene sequences in Cluster I are closely linked. Cluster II was made up of 8 sequences (3 genotypes) from 4 phylotypes (8 isolates) that belonged to Pseudomonas, Paracoccus, Porphyrobacter, and Brevundimonas. As a result, Cluster II pheN genes were acquired in constant temperature cultures, whereas some strains were produced from ambient temperature cultures and a few strains were obtained from constant temperature cultures and contained Cluster I pheN genes. Nevertheless, the pheN gene was found in the strains Marinobacter sp. SLG510A10-4, Paracoccus sp. SL013B 8A1, Porphyrobacter sp. SL014B-17A3, and Halomonas sp. SL014B-63A1-2 and SL013A-18A, although phenol degrading abilities were not found in these strains (Figure 2). In bacteria, the reaction is carried out by a variety of enzymes and is encoded by the genesalmA, CYP153A, and alkB/alkM. All of those unique CYP153A sequences were acquired from 91 bacterial isolates that were distributed across 13 genera; 24 unique alkB/alkMsequences were derived from 93 isolats in nine genera; and 14 almA sequences were collected from 34 isolates that were distributed across two genera [32-34].

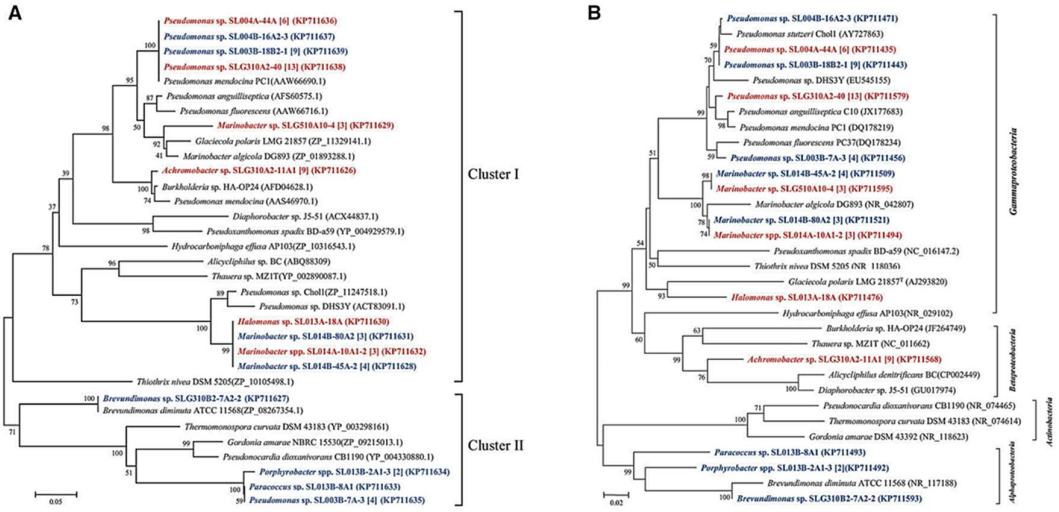

Figure 2: Phylogenetic diagram which related to partial amino sequences of (A) phenol hydroxylase and (B) 16S rRNA gene sequence of bacterial strains which obtained fromoil-contaminated soils[35].

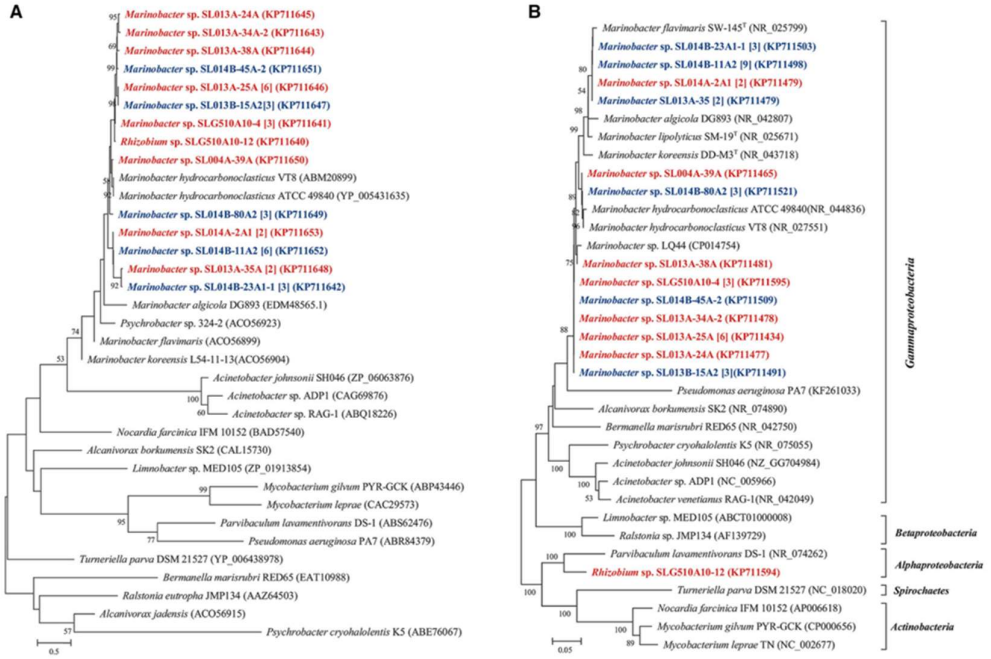

Figure 3: Phylogenetic diagram which formed based on partial amino sequence of (A) alkane mono-oxygenase (AlmA) and (B) the 16S rRNA gene sequences of bacterial strains obtained from the oil-contaminated soils. The scale bar represents 0.5 and 0.05 substitutions per site[35].

Metabolism of long-chain n-alkanes was mediated by an additional monooxygenase of the flavin-binding family AlmA. Among 36 isolates from two genera, Marinobacter and Rhizobium, all 12 distinct almA sequences were found. Those genes, despite their diverse taxonomic positions, are closely linked to strains of Marinobacter. As a consequence of this, 5.5% (19 isolates) of ambient temperature isolates possessed almA genes, whereas 6.1% (17 isolates) of

constant temperature isolates retained the same genes (Figure 3). Several alkane hydroxylase genes were expressed by different Marinobacter strains. In addition, three distinct types of alkane mono-oxygenase genes, such as alkB, CYP153A, alma, and phenol hydroxylase genes, were found to be present concurrently in "Marinobacter spp. SLG510A10-4, SL014B-80A2, and SL014B- 45A-2. Marinobactergudaonensis SL014B-11A2T, Marinobacter spp. SL013A-35A, SL004A-39A, SL013A-34A-2, SL013A-38A, SL013A-24A, SL013A-25A, and SL013B-15A2 expressed alkB, CYP153A, and almA genes" [35]. The CYP153A gene was present in two copies in strain SL013A-34A-2. Both the alkB and CYP153A genes were found in Marinobacter sp. SLG210B1-9A1, and the CYP153A and almA genes were found in Marinobacter sp. SL014B-23A1-1. In addition to this, the CYP153A gene was found to be present in Marinobacter sp. SL013-15A2 in a total of three copies. Yet the majority of these strains were unable to thrive in MSM that had been treated with hexadecane[35].

Mechanism of oil degradation

In aerobic circumstances, organic pollutants degrade the quickest and most completely. Figure 4 depicted the method by which hydrocarbons degrade aerobically[36].

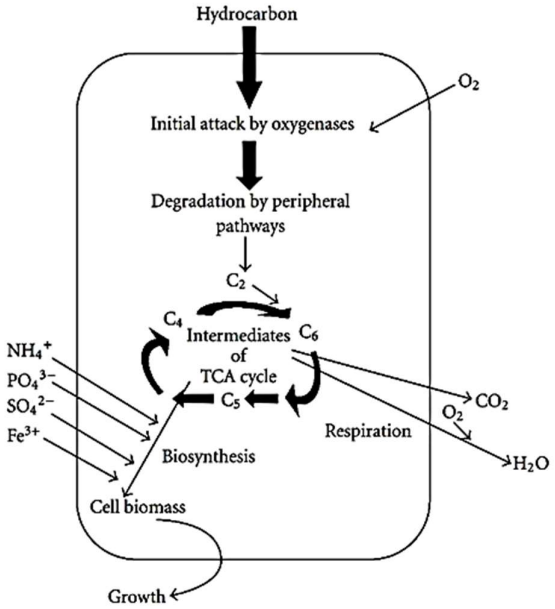

Figure 4: Principle of hydrocarbon degradation via microbes [36].

The aerobic degradation of hydrocarbons was shown in Figure 4. Intracellular assault of organic pollutants was first an oxidative process, and the incorporation of oxygen was initially an enzymatic catalysis reaction carried out by oxygenase and peroxidases. Intermediates for intermediary metabolism, such as the tricarboxylic acid cycle, are produced through the peripheral degradation pathway. Biosynthesis of cell biomass on metabolites such as acetyl-CoA, succinate, and pyruvate. Many bio syntheses required sugars, and gluconeogenesis was another name for cell development.

Via a particular enzyme system, hydrocarbon degradation might be followed. According to Figure 5, which displayed the early assault on xenobiotics known as oxygenases [36]. Additional

mechanisms included the following: (1) the attachment of microbial cells to substrates; and (2) the synthesis of biosurfactants[37].

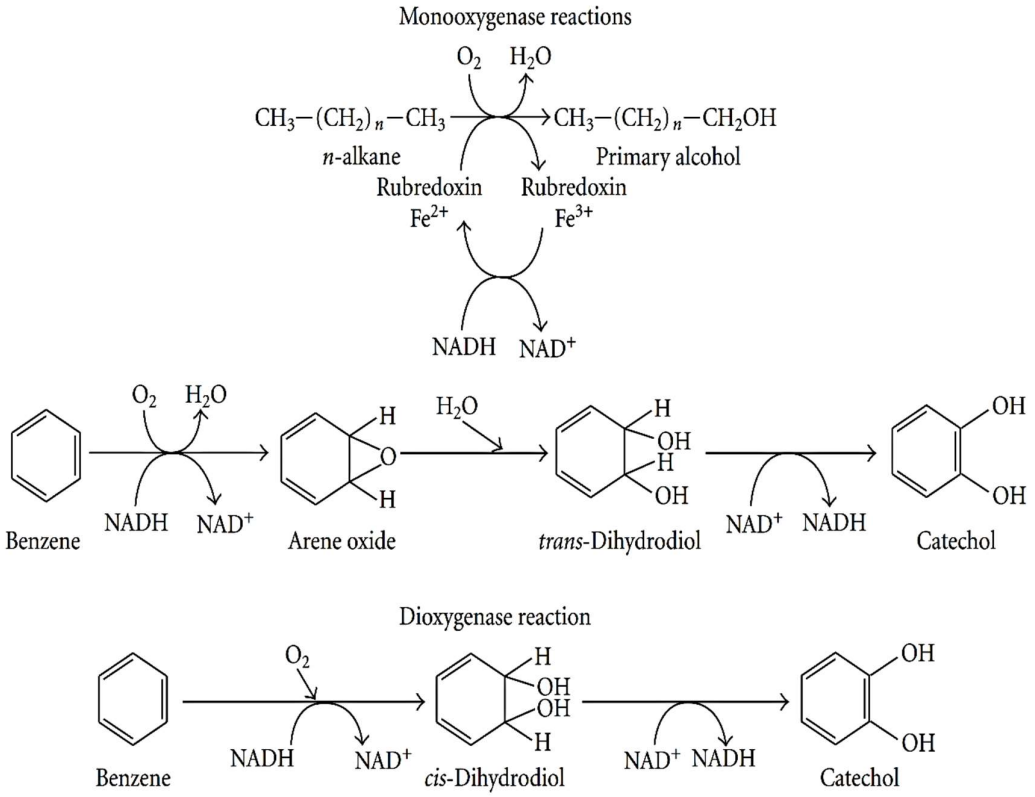

Figure 5: "Enzymatic reactions enhanced the hydrocarbons degradation process"[36, 37].

Participation of enzymes in hydrocarbon degradation

Cytochrome P450 alkane hydroxylases were members of a family of widely distributed heme-thiolate mono-oxygenases that were crucial in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and other substances [38]. It is dependent on the length of the chain, the enzyme systems, and the oxygen content of the substrate in order to commence biodegradation (Table 1). Higher eukaryotes, which comprised a wide variety of P450 families, each of which contributes an isoform to an ensemble that is involved in the metabolic conversion of substrate. In microorganisms like P450, which only exist in a select few species [39]. The biodegradation of petroleum hydrocarbons was aided by cytochrome P450 enzyme systems (Table 1). Many species of yeast utilise n-alkanes and other aliphatic hydrocarbons as a source of carbon and energy, which is mediated by the presence of various microsomal forms of cytochrome P450. Several cytochrome P450 enzymes were isolated from yeast species such as Candida maltosa, Candida tropicalis, and Candida apicola [40]. Several alkane oxygenases, including Cytochrome P450 enzymes, integral membrane di-iron alkane hydroxylases (such as AlkB), soluble di-iron methane mono, and membrane bound copper-contained methane oxygenases mono, are present in prokaryotes and eukaryotes and actively participate in alkane degradation under aerobic conditions[41].

Table 1: Enzymes involved in biodegradation

Enzyme	Substrates	Microbes	Reference
Soluble methane mono oxygenases	C_1–C_8 alkanes alkenes andcycloalkanes	*Methylococcus Methylosinus Methylocystis Methylomonas Methylocella*	[42]
Particulate methane monooxygenases	C_1–C_5 (halogenated) alkanes andcycloalkanes	*Methylobacter Methylococcus, Methylocystis*	[42]
AlkB related alkane hydroxylases	C_5–C_{16} alkanes, fatty acids, alkyl benzenes, cycloalkanes and so forth	*Pseudomonas Burkholderia Rhodococcus, Mycobacterium*	[43]
Eukaryotic P450	C_{10}–C_{16} alkanes, fatty acids	*Candida maltosa Candida tropicalis Yarrowialipolytica*	[44]
Bacterial P450 oxygenase system	C_5–C_{16} alkanes, cycloalkanes	*Acinetobacter Caulobacter Mycobacterium*	[45]
Dioxygenases	C_{10}–C_{30} alkanes	*Acinetobacter sp.*	[46]

Factors affecting rate of biodegradation

Physical condition, chemical composition, and hydrocarbons' concentration all have a role in the rate of biodegradation [1]. The primary distinctions in the biodegradation rate in soil and aquatic eco systems after an oil spill [47]. The primary variations affect the physicochemical makeup of oil. Terrestrial oil spills connected with vertical penetration rather than horizontal transport of oil into soil which avoid evaporative losses of volatile hydrocarbons which are toxic to microorganisms [8].

The physico-chemical makeup of oil in soil is also affected by a number of primary components. It is possible that oil components have a mild toxic impact through the absorption and adsorption process of hydrocarbons to humid substances, which may lead to the production of persistent oil film and silks [48]. The size and intensity of activity of degrading bacteria can affect the biodegradability of pollutants [49].

The rate of hydrocarbon degradation was also shown to be affected by temperature, oxygen concentration, and nutritional concentration [8]. Salinity and pressure degradation affect hydrocarbons in various aquatic habitats. On the other hand, moisture and pH affect the degradation process in soils [49]. The kind and degree of microbial hydrocarbon degradation were under control, as well as the temperature [8]. It directly influences the physico-chemical properties of hydrocarbons, such as diffusion, viscosity, and volatization, which modify the hydrocarbon content and bioavailability, resulting in a net impact on the rate of biodegradation

[50]. High rate of degradation was observed at high temperature. As a result, solubility also improved. Furthermore, voletization also increases membrane toxicity [50] and slows the rate of toxic degradation process [8]. For example, biodegradation of hydrocarbons was discovered to take place throughout a broad temperature spectrum. Whyte and colleagues documented the degradation of n-alkanes and diesel oil by Rhodococcus sp. strain Q15 at optimum temperatures of 0 and 5 degrees Celsius [50]. On the other hand, Holmes and colleagues discovered biodegradation at a high temperature of 85 °C for the hyperthermophilic bacteria Ferroglobusplacidus [51].

The concentration of oxygen in soil and groundwater also impacts the biodegradation of petroleum [8]. The rate of microbial oxygen consumption and the presence of oxygen in substrates that deplete the oxygen in the soil [52]. The concentration of carbon, nitrogen, and phosphorus also affect the rate of microbial biodegradation. The amount of these nutrients was decided by the biochemical oxygen and the demand for the contaminated location. The effects of adding phosphate and nitrogen were reported by Manilal and Alexander. The biodegradation process was slowed down by the addition of nitrate, but it was sped up by the addition of phosphorus [53]. The combination of the two minerals increased the mineralization rate at a rate that was lower than what was seen when phosphate was introduced in it separately. Nitrogen and Phosphorus are restricted in soils; but, by adding urea-phosphate, N-P-K fertilizers, and ammonium and phosphate salts, the rate of biodegradation may be increased [54].

Many heterotrophic related bacteria and fungi rely on a neutral pH, with fungi being more tolerant to acidic environments [9]. The capacity of microbes to digest hydrocarbons was negatively impacted by an excessive amount of pH in particular soils. By using Sphingomonaspaucimobilis BA 2 in soil with a pH of 5.2, Kastner et al. observed reduced pyrene biodegradation. Yet, a 10-fold increase in acidity caused the pH to rise to 7. The pH of the soil had no effect on the degradation of pyrene by another strain, BP 9[55].

Characterization

Using classical physiochemical analysis and 16S rRNA gene sequence analysis, all microbes, including bacteria degrading crude oil, were described.

(i) Classical physiochemical analysis: The Identification Manual of Systematic Bacteriology released in China [57] and the standard Bergey's Manual of Systematic Bacteriology [56] were used to describe crude oil degrading bacteria. The identification of microbes such as bacteria, algae, and fungi was done using their morphological characteristics, growth characteristics (pH and temperature), and enzyme activity (oxidase and catalase).

(ii) 16S rRNA gene sequence analysis: Total DNA of crude-oil-degrading microbes like bacteria was extracted via SDS method for 16S rRNA gene sequence analysis [58]. The 16S rRNA region was amplified by PCR used the universalforward primer 50-AGAGTTTGATCCTGGCTCAG-30 andreverse primer 50-TACGGCTACCTTGTT ACGACTT-30. The amplificationreaction was performed in a “total volume of 25 ml containing15.5 ml sterile deionized water, 2.0 ml Mg^{2+} (25 mM), 2.0 ml dNTP(2.5 mM), 1.0 ml of each forward and reverse primer (25 mM), 0.5 mlDNA template, 0.5 ml *Taq*DNA

polymerase (5 u ml^{-1}), and 2.5 ml10 X buffer solution (20 mM). Amplification for 35 cycles was done in GeneAmp@ PCR System 9700, while the temperatureprofile for PCR was 95 °C for 5 min pre-denaturation (1 cycle), 94 °Cfor 30 s denaturation, 53 °C for 1 min annealing and 72 °C for 2 minextension (35 cycles), 72 °C for 10 min extension after the finalcycle. PCR amplicons were running 1.0% agarose gelelectrophoresis, purified and then cloned into pMD18Tvectorfor sequencing"[59].

Cloning

Principle of cloning expression

The sensitive cloning method was based on the isolation of enzymatic genes from microbes such as fungi by combining the capacity of S. cerevisiae to heterologous genes with sensitive and accurate enzyme tests. The principle of cloning expression was shown in figures 6 and 7.

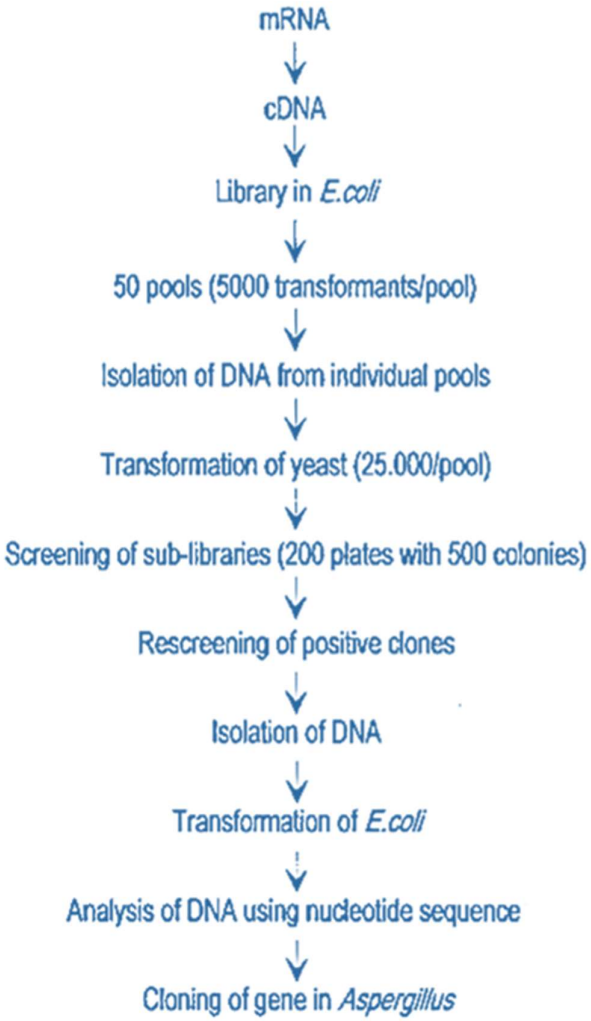

Figure 6: Flow diagram of cloning expression

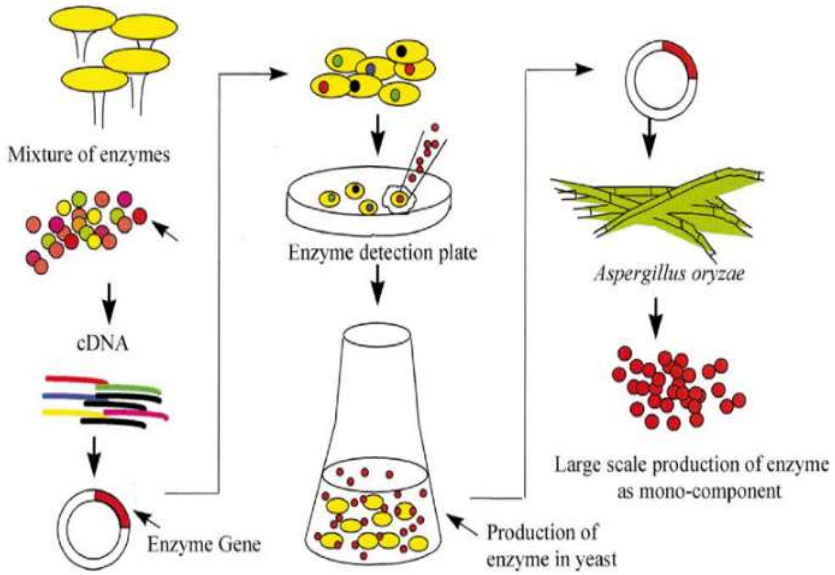

Figure 7: Principle of cloning expression.

The donor strain was propagated in complex media that promoted enzyme activity. Mycelium was obtained and utilized for mRNA isolation at the exact moment of maximum enzyme synthesis. Subsequently, using a shuttle vector that can replicate in both S. cerevisiael and E. coli, cDNA was produced. The cDNA was cloned into the vector in a selectively directed fashion between the strong yeast promotor and terminator. As a result, it will be possible to control the expression of fungal genes in yeast cells. Due to the comparatively low frequency of transformation in yeast. E. coli was used to create the cDNA library. DNA plasmid was isolated from sub-pools of an E. coli library and converted into S. cerevisiae. The yeast transformants were reproduced onto fresh sets of agar plates before being tested on enzyme substrates, which are used to determine whether or not an enzyme is active. On enzyme indicator plates, yeast colonies were then limited by streaking. For the first characterization of the enzyme, small scale cultures of yeast transformants may be propagated. The organism's poor synthesis of enzymes prevented S. cerevisiae from becoming an ideal host. Hence, it was crucial to separate the DNA plasmid from the yeast and insert the segment of cDNA into an appropriate vector expression in another host such as A. oryzae [60]. In order to separate the clones that were not enzymatically active, the 5' end of the cDNA sequence was determined in parallel. Nevertheless, the DNA also showed similarities in other enzyme species. As compared to the traditional cloning of cDNA, the process of cloning expression offered several benefits. It was independent of the enzyme that needed to be cloned and required a lengthy amount of time for purification and characterization before cloning could be performed. By creating several replicas of yeast on agar plates and using diverse screening assays, it performed continuous screening for a wide range of enzymes.

Factors affecting cloning expression: There were few factors which used for establishment of cloning expression in yeastas follows:

(i) cDNA synthesis: The presence of a signal peptide encoded by a heterologous cDNA gene is required for cloning expression. It was necessary for the cloning expression outcome to have full length cDNA that at least covered the gene from the AUG start codon to the stop codon.

Nonetheless, there are a variety of kits available, and the majority of them comprise reagents for cDNA cloning and expression. High-quality mRNA was necessary for the effective synthesis of cDNA [61]. The synthesis of cDNA may be connected to a modification of the Gubler Hoffman method [62], which employed mung bean nuclease as a replacement for SI nuclease. In plasmid-based system, the improved cDNA system had an average library size of > 107 clones. mRNA isolated for optimal generation of extracellular enzyme activity. Compared to cDNA housekeeping genes, the cDNA enzyme is represented. For the isolation of a low abundance cDNA enzyme, it is thus required to plate a portion of the E. coli cDNA library.

(ii) Enzyme assays: Around 5000 clones make up the E. coli library pool (Figure 5). It had an acceptable likelihood for screening a variety of clones, which is analogous to a library in yeast. It was about five times higher. There were between 300 and 500 clones on the screening agar plates, which were numbered from 50 to 100. These big number sets have a few requirements for the assay systems. It was simple to manage, dependable, and sensitive. There were numerous assay principles that could be used in liquid assays, and clones that had been established after being propagated in tiny cultures could be screened.

(iii) Yeast expression system: It was a crucial expression system for cloning that linked to S. cerevisiae as the host, episomal E. coli, and S. cerevisiae shuttle expression. Therefore, the substantial activity seen on screening plates. When yeast clones were propagated on liquid medium, only a little quantity of enzyme was generated [63]. The filamentous fungus A. oryzae has been shown to be quite beneficial for the expression rate of most fungal enzymatic genes. Because to the vast size of the colonies and the fact that they seldom duplicate, it might be transformed on plates. Following that, transformed DNA incorporated in chromosomes, making it harder to rescue, and the frequency of transformation was lower than that of S. cerevisiae.

Bioinformatics analysis of oil degrading microbes

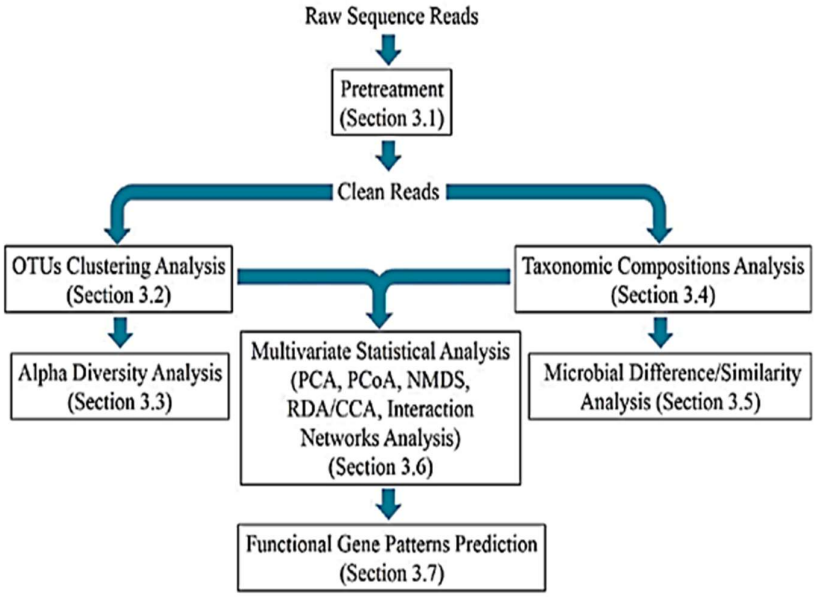

Figure 8: Schematic view of bioinformatics analysis of genes of microbes

According to Figure 8, it demonstrated the use of bioinformatics to the analysis of oil degrading genes in microbes. It also included a number of different aspects, such as pre-treatment of raw sequences, OTUs clustering analysis, alpha diversity analysis, taxonomic compositions analysis, difference / similarity analysis of microbes, multivariate statistical analysis (PCA, PCoA, NMDS, RDA/CCA), and functional gene pattern prediction. There are certain platforms and tools required for each analysis. Ju and Zhang also detailed the platforms and software tools used for bioinformatics analysis and demonstrated a variety of data processing operations such as pre-treatment, assembly, binning, and annotation [64]. In addition, Kumar and Chowdary [65] provided different software tools that incorporated functionalities and online linkages for bioinformatics data analysis.

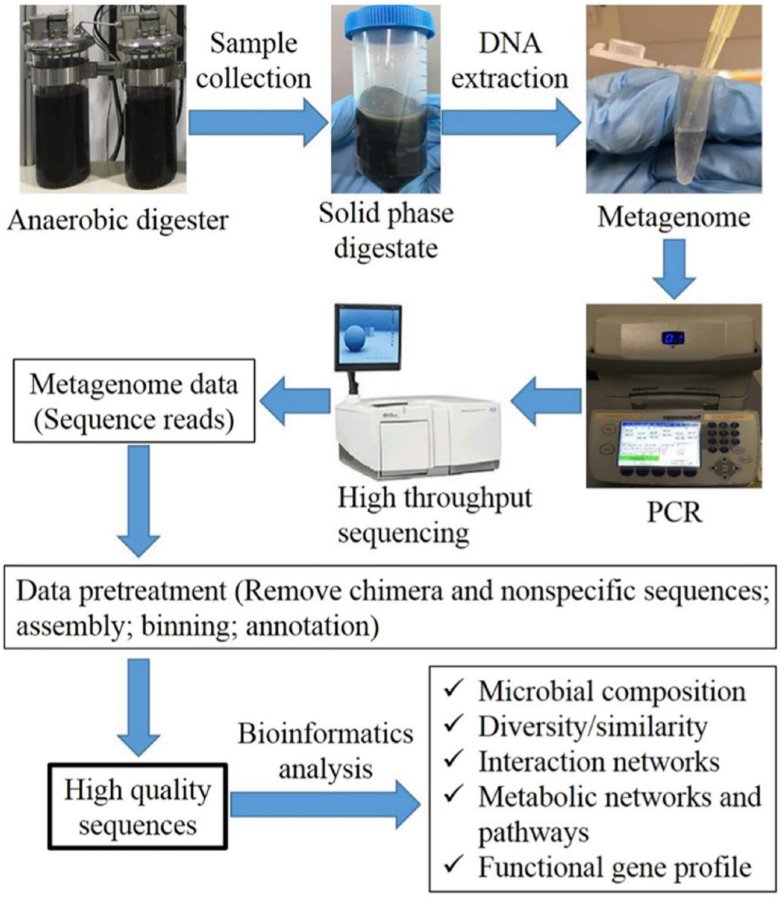

Figure 9: Schematic view of data sequences analysis via bioinformatics method[66]

A technical explanation of the metagenomic data of microbes was supplied using the bioinformatics method (Figure 9)[66].

(i) Pre-treatment of raw sequences

The pre-treatment of raw sequences was an extremely important step that had to be completed after the gathering of the metagenome data in order to provide high-quality reads for the subsequent analysis. In the past, several bioinformatics software tools (Table 2) were created for it. These software tools include Trimmomatic [67], ACE Pyrotag Pipeline (APP) [68], HMMER

[69], MG-RAST [70], Chimera Slayer [71], RDP tools [72], and UCHIME [73-75]. The sequence pre-treatment typically consisted of I the removal of adapters and linkers; (ii) the exclusion of replication; and (iii) the demultiplexing of barcoded samples and quality control. Nevertheless, MOTHUR and QIIME were the two tools that were used the most often to denoise the data. UCHIME was a very essential tool for checking and removing chimeras from the raw sequences. After the main processing, the sequencing was organized into groups, then connected to the individual barcodes, and finally eliminated. Quality control was required for further analysis. The quality control method that was carried out using the online tools provided by the RDP Pyro-sequencing Pipeline disqualified the following sequences: I have less than 150 base pairs (bp), (ii) have more than one unknown nucleotide, (iii) have primer mismatches, and (iv) have poor quality base scores (Phred quality scores of less than 25) before further analysis [72]. To combine the sequences with a 1 bp difference, a "pre-cluster" algorithm was often used.

Table 2: Softwares used in bioinformatic analysis as follows:

Software	Programme	Function	Reference
HMMER	Hmm-search program	Reads trimming and alignment	[69]
MUSCLE	Multiple sequence alignment	Aligned sequences	[76]
ARB rRNA database	BLAST	Detected sequence reads	[76]
CLC Genomics workbench	*de novo* assembly	Genome assembler	[67]
Ion Reporter	NMS ordination	Abundance-based difference visualization	[77]
UCLUST	RDP classifier	Clustering OTUs	[78]
STAMP	Two-sided Welch's *t*-test	Pairwise statistical comparison of taxonomy between two samples	[79]

(ii) OTUs clustering analysis

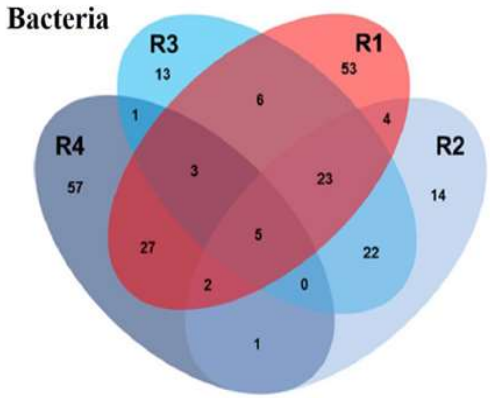

Figure 10: Venn diagram of total number of OTUs (0.03 distance) and shared OTUs among four samples of bacteria [80]

The various sequences were aligned using aligners such as MOTHUR [81], MUSCLE [76], INFERNAL aligner [72], Py-NAST [82], and ClustalW [83], which were combined using bacterial and archaeal data bases such as SILVA [75]. The aligned sequences were then clustered into operational taxonomic units (OTUs) using an average clustering algorithm via the Usearch software [84], and many sequences were classified as having 97% sequence similarity using RDP Bayesian Classifier [72], UCLUST-RDP classifier [78], and MEGA/MEGA5 [75, 83, 85]. Yun and his colleagues produced a Venn diagram using the R-Venn-Diagram program to explain the microbial dynamics that occurred during the mitigation of ammonia inhibition caused by internal dilution in high-rate anaerobic digestion of food waste leachate [80]. The Venn diagram made it simple to determine both the total number of OTUs and the number of OTUs that were shared by all four samples (R1, R2, R3, and R4). Just five OTUs from R1 to R4 that were regularly shared by bacteria made up 2% of the total of 231 OTUs. It shown that a changing parameter was combined via several bacterial dominances.

3. Analysis of alpha diversity

The OTUs-based alpha diversity analysis in terms of Chao1 richness estimator (Chao1), abundance coverage-based species estimator (ACE), Shannon-Weaver diversity index (Shannon), and Simpson diversity index (Simpson) may be performed using the MOTHUR package [86], R software package with VEGAN library [87], and the RDP Pipeline [72]. Several indices included simple calculations based on provided equations, making it possible to quickly and accurately evaluate the biological diversity of microbial groups [88]. Yet, diversified indices such as the Simpson index and the Shannon index integrate rich and even components into a simple index via a single measure, which leads to a loss of relative roles of other possible economic and social value of particular species, etc[89].

4. Taxonomic composition analysis

One easy method to employ significant bioinformatics analysis was taxonomic compositions. In most cases, it may be finished in two stages: I comparison and filtering, and (ii) taxonomic categorization of sequences. Initially, it was run via a BLAST search against sequence databases such as the SILVA database [90], the EzTaxon-e database [69], the GenBank NT/NR database [91], and the RDP database [92], using a threshold that was somewhere between 80 and 90%. As a consequence of this, those sequences were phylogenetically allocated by taxonomic classifications using RDP's naïve Bayesian classifier and Bergey's taxonomy at a distance of 0.03 from the taxonomy[93, 94].

5. Similarities and differences between microbial taxonomic compositions

According to statistical analysis plot and ternary plot, similarities and differences in microbial taxonomic makeup occurred in two different ways. STAMP [95] used a two-sided Welch's t-test with an alpha level of 0.05 to conduct the statistical comparison of the taxonomies of the two samples. The relative abundance of microbial groups was revealed by Yu and colleagues [79]. In general, it was defined as the number of sequences that linked to taxon divided by the total number of sequences per sample (%).

6. Multivariate statistical analysis

The multivariate analysis included principal component analysis (PCA) [68, 96, 97], principal coordinate analysis (PCoA) [98-100], non-metric multi-dimensional scaling (NMDS) [101-103], redundancy analysis (RDA) [83, 104], and canonical correspondence analysis (CCA) [69, 105, 106]. An ordination analysis was performed using the multivariate analysis. Their primary objective was to represent comparable objects close to one another and disparate objects further away [107]. In general, such approaches may be broken down into two categories: unconstrained ordination analysis (PCA, PCoA, and NMDS) and constrained ordination analysis (RDA and CCA), which are based on computational algorithms (linear versus unimodal).

7. Annotation and application of gene functional

Functionalized, annotation of data used for bioinformatics and their tools such BLAST against databases like as SWISSPROT [108], COG [109], KEGG [109], MG-RAST (SEED) [67], GenDB [110], and IMG/M [111]. BLAST is often used for functional annotation, although the GenDB genome annotation system was primarily responsible for functional annotation of long assembled contigs [110]. The MG-RAST server was a SEED-based environment that enabled the uploading of metagenomes for the purpose of performing automated analysis [84]. IMG/M offered a genome annotation pipeline and comparison analysis with microbial genomes, 3D genes, and functions using a variety of tools, including KEGG [127], InterPro [128], Pfam [129], and Gene Ontology [130]. Campanaro and co-workers presented the use of bioinformatics tools such as KEGG, COG, and SEED, for functional relationships between microbial species participating in the AD process and to discover critical microbial genomes encoding enzymes implicated in certain metabolic pathways [67]. On the other hand, MEGAN may also be used for functional analysis of numerous metagenomes. This analysis is based on the SEED hierarchy and the KEGG pathways[112].

Conclusion

Because to oil extraction, refining, shipping, and storage, as well as accidents, oil contamination in eco systems was a worldwide environmental hazard. Here, numerous strategies are discussed for isolating native oil-degrading microbes such bacteria in contaminated sediment and saltwater in the Mediterranean and northern side seas. Bioluminescence inhibition assay analysis of samples showed that samples exhibited an acute toxicity due to toxicity in sediments. There was a substantial correlation between acute toxicity and the level of contamination with hydrocarbons [113]. The inherent biodegradability of hydrocarbons and the widespread presence of competent degrading microbes in the environment contributed to the bioremediation process. In the current research, the capacity of microbes to thrive in mineral media containing hydrocarbons as the like sole source of carbon and energy was used to determine which microbes had the capability to exploit oil hydrocarbons. These oil-degrading species belonged to several genera that produced hydrocarbon-degrading strains. We presented the isolation, identification, and cloning of oil degrading genes in microbes, as well as their bioinformatics analysis. Many novel uses of bioinformatics technology have arisen over the last several decades, and these applications have high levels of efficiency and sustainability in the oil business.

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