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Promising Egyptian soil bacterial isolates for hydrocarbon waste biodegradation: petroleum wax

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Abstract

In terms of the environmentally friendly processing of organic waste, biodegradation is one of the most promising waste management techniques to have recently emerged. One of the byproducts generated by the methods used to refine crude oil that poses a significant issue is light slack wax. Our effort was to isolate microorganisms from nearby soil that had been contaminated by light slack wax. Then, these microbes were elevated to test their ability to break down hydrocarbon waste. The most effective isolates were physically and biologically analyzed. The screening approach revealed that strains H4 and H6 exhibited the best biodegradation efficiencies for light slack wax, with rates of 88.23% and 84.76%, respectively after 14 days of treatments. After the treatment with H4 and H6 for 2 weeks, hydrocarbons like; aromatics, naphthene, and iso-alkanes were degraded rather than saturated n-paraffins according to the gas chromatographic analyses of the samples. The bacterial isolates were further described using 16s rRNA analysis and showed that two strains were from the genus Bacillus. These remarkable results are due to the bacteria's ability to produce biosurfactants and enzymes that facilitate the breakdown of hydrocarbon waste. Further research and development in this area may lead to implementing effective bioremediation methods for managing hydrocarbon waste and lowering pollution issues.

Keywords: Biodegradation; Hydrocarbons; Light slack wax; Petroleum wax; Waste management

1. Introduction

The accidental leakage of oil into the environment as a result of the petroleum refining process and storage is one of the most annoying environmental issues [1]. Oil pollutants, especially petroleum hydrocarbons (PHs), are classified under the family of toxic compounds to all living organisms [2]. Shahzadi reported that about 8.8 million metric tons of PHs are disposed of into the environment annually [3]. The PHs are considered carcinogens and neurotoxic organic pollutants which cause many human health risks [4]. In addition, the PHs accumulation in the soil leads to significant damage to the local system which may cause mutations and death to plants and animals [5] distributing nitrogen cycle in soil and inhibiting beneficial microbial communities in soil [6]. Therefore, it is required to use long-term remediation approaches to clean the environment from hydrocarbon contaminants. There are many approaches for soil remediation such as the physical approach, evaporation, washing, dispersion of soil, and the chemical approach, oxidation-reduction [7]. These technologies, however, are costly and can result in inadequate breakdown of pollutants [8]. Now, the biological approach is considered to be the most promising and effective approach for soil remediation as it is eco-friendly, cheap, and more efficient than other approaches [9], [10]. The biological approach is classified as biodegradation and phytoremediation. Biodegradation is the mechanism of using microorganisms like; bacteria and fungi to use PHs as a carbon energy source [11]. As reported by Magan *et al.*, fungi can consume more time than bacteria and

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have low competitive capabilities [12]. However, bacteria have plenty of catabolic genes which are helping in the purification of PHs in a short period [13]. In the biodegradation process, bacteria have the ability to utilize aliphatic and aromatic hydrocarbons and degrade PHs, aerobically or anaerobically, by using catalytic enzymes and biosurfactants [14], [15]. Under aerobic conditions, an oxidation reaction of aromatic hydrocarbons and n-alkanes takes place using monooxygenases and dioxygenases enzymes to induce primary alcohols [16]. Depending on the nature and the composition of PHs, many enzymes play an important role in the oxidation process for example, alkane 1-monooxygenase and alcohol dehydrogenase are suitable for alkanes, while naphthalene 1,2-dioxygenase is involved in naphthalene biodegradation [17], [18]. After the attachment of bacteria to PHs substrates, the production of biosurfactants takes place. Biosurfactants play an important role in the bioremediation process by increasing the surface area of oil, allowing bacteria to use and remove pollutants. [19]. It has been reported that microbial oxidation of hydrocarbons involves a series of catalytic events that result in transitory metabolic products including ketones, alcohols, fatty acids, aldehydes, and carboxylic acids, which are oxidised to carbon dioxide [20], [21]. Many bacterial species displayed potential in the PHs degradation. For n-alkanes, *Actinobacteria* sp. utilizes chain length C10-C40 [22]. Moreover, *Pseudomonads* bacteria species tend to degrade a wide range of PHs by producing glycolipids biosurfactants. Furthermore, *Pseudomonas* and *Rhodococcus* species were investigated for biodegradation of PAHs [23]. In addition, *Thermus brockie* and *Bacillus* sp. can metabolize pyrene [24], [25]. According to Adlan *et al.*, *Geobacillus kaustophilus*, *Geobacillus jurassicus*, and *Geobacillus stearothermophilus* have been reported by their ability to degrade more than 70% of crude oil [26]. Al-Wasify & Hamed discovered five types of bacteria that showed 88.5% of Egyptian crude oil degradation [27]. Abdulla *et al.*, proved that *Bacillus cereus* has a high potential to treat and degrade polyaromatic hydrocarbons (PHs) in contaminated soil [28].

The ability of bacteria to degrade PHs depends on many factors that affect the growth of bacteria. The composition and concentration of PHs affect the utilization in which bacteria can simply degrade n-alkanes rather than asphaltenes as a result of lacking some enzymes required for asphaltenes degradation. In addition, bacteria require many components in the biodegradation process such as nitrogen, sulphur, and several trace elements besides hydrogen and carbon in hydrocarbons [29]. Temperature, pH value, viscosity of oil, and oxygen are the most important physical factors which play an important role in bacterial biodegradation [30].

PHs-waxes are defied as by-products from the dewaxing process of lubricating oil. They are a mixture of straight and branched hydrocarbons between 18 and 70 carbon atoms [31], [32]. Slack wax is the refinery product from the dewaxing process of crude oil [33]. The demand of using petroleum waxes increases in many industries such as paper, cosmetics, cleansers, ink, rubber, and energy storage industries [31]. On the other hand, wax is still considered a waste material in the petroleum field which should be used and treated with non-costly techniques.

Although there is no highly useful application of slack wax, it could be degraded by chemical or biological methods and used in many applications such as phase change material (PCM) in thermal energy storage systems [34-36]. In addition, degraded wax could be used to generate bio-hydrogen gas which is used in generating electricity with high efficiency and good quality [37], [38].

Accordingly, waxes are considered as PHs wastes which should be treated with the aid of biological treatment (biodegradation). The aim of this study was directed to isolate local bacterial isolates from contaminated soil in order to evaluate their efficiency in the biodegradation process of purifying light slack wax. After bacterial isolation and purification, screening was applied to select the most potent microbial isolates depending on the biodegradation efficiency. The biodegradation processes of purifying light slack wax were optimized to increase their efficiency and gas chromatographic analysis was performed before and after degradation. The selected most potent isolates were finally identified using 16S rRNA.

2. Experimental

2.1 Hydrocarbon waste collection

Light slack wax was obtained from El-America Refining Company, Alexandria, Egypt.

2.2 Isolation of hydrocarbons degrading bacteria from soil

The soil rhizosphere of the basil plant (10.0 cm depth) was obtained from an agricultural site located in north Cairo, Egypt. Briefly, 100 g of soil was supplemented with 1% of light slack wax. The supplemented soil with wax was kept for three months with regular watering in triplicate. After 3 months, soil samples were collected with sterilized tools and dried in a shade at room temperature for 3 days. The isolation of bacteria was done by using soil enrichment method by incubating the two-soil in 200 ml of mineral salt media M9 (0.2 g/l $MgSO_4$, 1 g/l KH_2PO_4, 1 g/l $(NH_4)_2SO_4$, 0.02 g/l $CaCl_2$, 0.05 $FeCl_2$) with (v/v) 1% of soil and 1% of hydrocarbon (light slack wax) [39]. Each soil was incubated in triplicates and kept in a horizontal shaking incubator at 37 °C and 150 rpm for 10 days. Tenfold dilution was done for each soil suspension using sterile saline water up to 10^{-5} dilution. A 100 µl from the last three dilutions was cultured by spreading technique onto M9 agar plates supplemented with 1% (v/v) of wax as a sole carbon source then incubated at 37°C for 3 days. Different morphological bacterial isolates were sub-cultured on the same media to get purified single colonies. Out of thirty different isolates, six bacterial isolates were selected for further analysis based on their abilities for growth. Selected isolates (H1, H2, H3, H4, H5, and H6) were subjected to test the suitable time for degradation. The isolates were cultured on mineral salt media M9 supplemented with 1% wax, respectively. The culturing was performed in triplicates and incubated at 37 °C for 7, 14, and 21 days. After that, Light slack wax was extracted from the culture by using a solvent and kept for 48 hours for solvent evaporation to have pure wax.

2.3 Physical analysis of light slack wax

The light slack wax samples were physically characterized before and after microbial development using American Society for Testing and Materials (ASTM) standard techniques [40]. Congealing point (ASTM D-938), kinematic viscosity (ASTM D-445), and refractive index (ASTM D-1747) are the main procedures for analysis. The refractive index of the light slack wax samples after and before the treatment was determined according to ASTM D-1218. The Yield (%) of wax was calculated according to the following equation:

$$\text{Yield (\%)} = \frac{\text{Amount of oil produced}}{\text{Amount of oil used}} * 100$$

2.4 Characterization of the most promising bacteria

Most The ability of the most promising isolates (H4 and H6) to utilize wax as the carbon source was examined. The isolates were cultured in triplicates on M9 broth medium supplemented with 1% wax in a shaking incubator at 150 rpm at 37°C for 14 days. Then, bacterial isolates (H4, H6) were initially characterized morphologically on Luria Broth (LB; Laboratories Conda SA, Madrid, Spain) agar plates for colony characteristics and investigated under a microscope for cell morphology. Gram stain, spore formation, and biochemical were evaluated based on Bergey's Manual of Determinative Bacteriology [40].

2.5 Gas chromatographic analysis

The most powerful bacterial isolates (H4 and H6) were utilized to determine the light slack wax concentrations before and after microbial breakdown. In this experiment, the PerkinElmer (Clarus 500) GC equipment was used that has a hydrogen flame ionization detector and a fused silica capillary column (60 m length; 0.32 mm i.d.) packed with poly (dimethyl siloxane) HP-1 (non-polar packing) of 0.5 m. The carrier gas was nitrogen (oxygen-free) at a flow rate of 2 ml/min, and the column temperature was planned to increase from 100 to 300 °C at a fixed rate of 3 °C/min. While the detector was heated to 350 °C, the hydrogen flow rate was optimized to maximize detector sensitivity. Wax was heated and 0.1 was added into the injector. Pure wax was used as a standard. Each resolved component, whether n- or iso-paraffin, has a unique peak region. Amplification of 16S rDNA of the most degrading bacterial isolates.

2.6 Amplification of 16s rDNA of the most degrading bacterial isolates

LB medium was used to cultivate the most potent pure colonies of bacterial isolates. Then, centrifugation at 10,000 rpm was used for harvesting the bacterial cells for 2 min which then were washed by phosphate-buffered saline (PBS). First to extract DNA, the genomic DNA isolation kit (QIAGEN, Germany) was used as directed by the manufacturer's instructions. The amplification of 16S rRNA of the selected isolates was performed by PCR (GenAmp 9700 thermal cycler; Applied Biosystems, USA) using the universal PCR forward primer 27 F (5'-

AGAGTTTGATCMTGGCTCAG-3') and the reverse primer of 1492 R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR reactions were established by mixing 25 µl of 2X Dream Taq master mix, and 1 µl from each primer (200 nM) with 2 µl of bacterial DNA template (50 ng), and the volume was completed to 50 µl by sterile distilled water. According to the PCR program, the reaction started with initial denaturation (94°C for 3 min), then 30 cycles of denaturation (94°C for 30 sec), after that annealing at (60°C for 30 sec), and finally extension (72°C for 30 sec). The PCR amplicon from each bacterial isolate was separated in 0.8% (w/v) agarose gels by electrophoresis, purified by PCR purification kit (Bio-basic, Canada) and then sequenced using Macrogen Services (Daejeon, Korea) in both directions using the primers 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'CCGTCAATTCMTTTRAGTTT-3'). The resulting forward and reversed sequences of each bacterial isolate were assembled into a contig sequence, approximately 1.5 kb in size, using DNA Star Laser gene software (V.7). The contig sequence was aligned for a similarity search in the National Center for Biotechnology Information (NCBI) database using the BLAST 2 Sequence to get phylogenetic relationships. The obtained bacterial contig sequence was compared with those in the 16s rRNA database in the NCBI GenBank and the alignment was checked. The MEGAX software was used to draw the phylogenetic relationship with the four bacterial isolates by the maximum likelihood method and the Kimura 2-parameter model and the evaluation was based on 1000 bootstrap.

3. Results and discussion

TABLE 1 depicts the physical properties and molecular type composition of El-Ameria Refining Company's light slack wax. According to the data, light slack wax has a low congealing point, kinematic viscosity, and refractive index as a result of the low boiling point range and/or mean molecular weight of light slack wax. According to the molecular type composition data, the light slack wax has a high saturate content (97.63 wt.%) and a low aromatic content (2.37 wt.%) which are mono-aromatic aromatic compounds. The sulphur content and color measurements are consistent with the previous observations.

The Gas chromatography was used to assess the normal paraffin concentration of light slack wax. The statistics show that the light slack wax has a high normal paraffin content (78.35 wt.%) and a low iso- and cyclo-paraffin percentage (19.28 wt.%) (Table 1). To obtain valuable products utilized as fuel from waste byproducts, aromatics and low melting point components (isoparaffinic and naphthenic compounds) must be reduced by biodegrading light slack wax using bacterial isolates.

In this study, thirty isolates were morphologically identified and it was noticed that about 75% of the bacterial isolates, can cultivate on M9 media supplemented with 1 % wax, which needed incubation time (14 days) to grow and degrade the wax. Only six isolates were selected namely (H1 to H6) for further evaluation. Yield, refractive index, and melting point were measured for isolates.

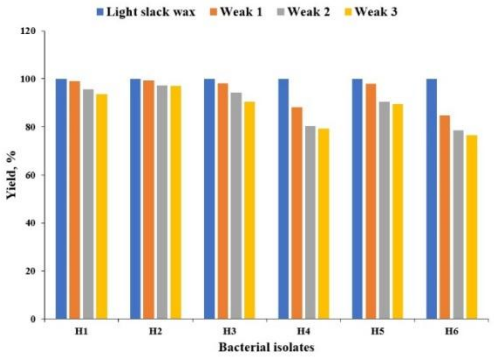

Figure 1: Yield (%) of the light slack wax after the treatment with six different bacterial isolates (H1, H2, H3, H4, H5, and H6) compared with the original wax at different incubation periods, after week 1, week 2, and week 3.

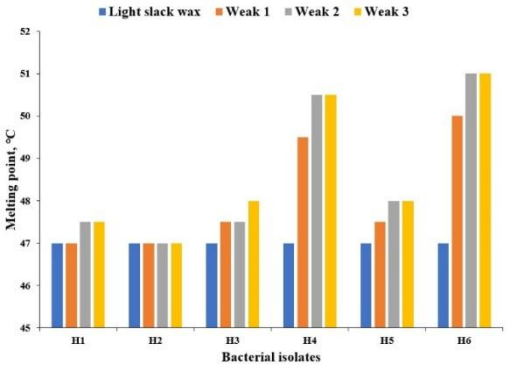

Figure 2: Melting point of the light slack wax after the treatment with six different bacterial isolates (H1, H2, H3, H4, H5, and H6) compared with original wax at different incubation periods, after week 1, week 2, and week 3.

Table 1: Molecular type composition and physical characteristics of the original light slack wax and the isolated after treatment with H4 and H6

Characteristic	Light slack wax	Isolated wax using H4	Isolated wax using H6
Yield, wt.%	100	80.34	78.5
Congealing point, □	47	0.0	0.0
Kinematic viscosity at 100 □ ,mm^2/s	3.04	3.12	3.10
Oil content, wt.%	5.52	1.21	0.92
Penetration at 20 □	43	24	22
Melting point, □	47	50.5	51
Refractive index at 83 □	1.4200	1.4170	1.4161
Molecular type composition total saturates content, wt, %	97.63	100	100
n-paraffins content, wt.%	78.35	86.22	91.25
Iso- and cyclo-paraffins content, wt.%	19.28	13.78	8.75
Total aromatic content, wt.%	2.37	0.0	0.0

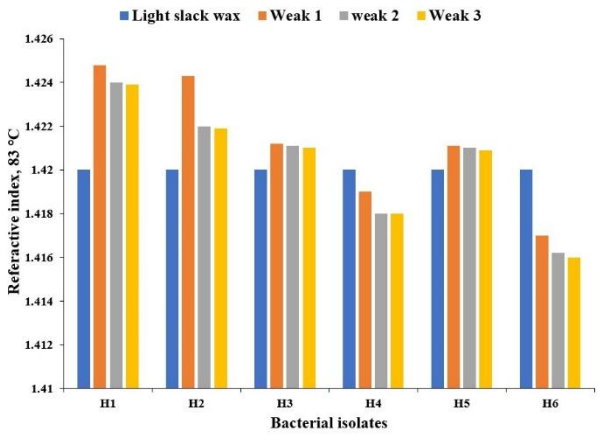

Figure 3: Refractive index of the light slack wax after the treatment with six different bacterial isolates (H1, H2, H3, H4, H5, and H6) compared with original wax at different incubation periods, after week 1, week 2, and week 3.

Data in (FIG.1, FIG.2, and FIG.3) showed a sharp increase in melting point and a decrease in both yield and refractive index of isolated waxes after growth especially for samples treated with isolates H4 & H6 at different incubation periods compared with the light slack wax before biodegradation. H1, H2, H3, and H5 bacteria isolates had no significant effect on the yield, melting point, and refractive index during different incubation periods. This may be attributed to an increase in n-paraffins content and a decrease in aromatic, iso-paraffin, and naphthenic compounds.

From such findings, the most promising bacteria isolates were H4 & H6 selected for biodegradation of light slack wax by-products with an incubation period of 2 weeks. The optimum degradation of aromatic and low melting waxes was found for isolates H4 & H6 after 2 weeks incubation and remains constant for 3 weeks incubation.

By lowering the oil content of the wax, needle penetration decreases, and congealing point increases. Furthermore, the viscosities and melting point of the isolated waxes increase which are separated from light slack wax by biodegradation utilizing bacterial isolates. This is due to the oil's composition, which consists mainly of iso-paraffins with lower refractive indices and viscosities. In addition, the data of molecular type composition confirms the aforementioned conclusions as the iso- and cyclo-paraffins concentrations that were separated from light slack wax are decreased.

The selected most promising isolates for biodegrading of the light slack wax (H4 & H6) were subjected to biochemical and morphological analysis.

TABLE 2 displayed the physical examination of the isolates (H4 and H6). Moreover, the biochemical analysis of selected isolates (H4 and H6) was reported in TABLE 3. Gram staining and microscopic examination showed that all colonies are G+ve and rods shape. Further biochemical analysis demonstrated that three strain (H6) is catalase positive, VP (Voges-proskauer) positive, and motile. The Isolates can grow on NaCl concentration of (6.5 %) and cannot grow at 55°C. In addition, the isolates can utilize glycerol and citrate and can hydrolyze casein and starch. On the other hand, the H4 strain was starch and citrate negative. It grows at up to 55 °C and up to 14% NaCl (w/v). All analysis demonstrates that the isolates (H4 and H6) may be attributed to *Bacillus* sps

Table 2: The physical examinations of the most potent bacterial isolates (H4 and H6)

Physical examination					
Bacterial isolates	**Shape**	**Motility**	**Spore formation**	**Maximum Temperature (°C)**	**Maximum NaCl resistant (%)**
H4	Rods	Motile	+	55	14
H6	Rods	Motile	+	55	6.5

Table 2: Biochemical examinations of the most potent bacterial isolates (H4 and H6)

Biochemical Tests							
Bacterial isolates	**Gram Staining**	**Catalase Activity**	**Voges-proskauer**	**Glycerol utilization**	**Citrate utilization**	**Casein hydrolysis**	**Starch hydrolysis**
H4	+	_	+	+	_	+	+
H6	+	+	+	+	+	+	+

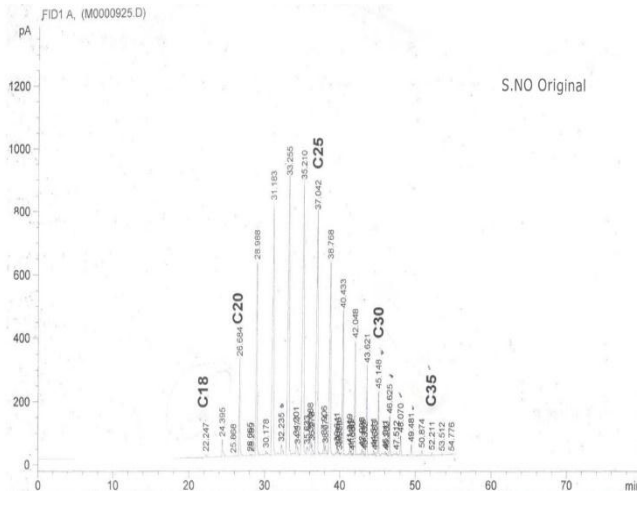

Figure 4: Shows GC analysis of the original light slack wax before treatment with bacteria.

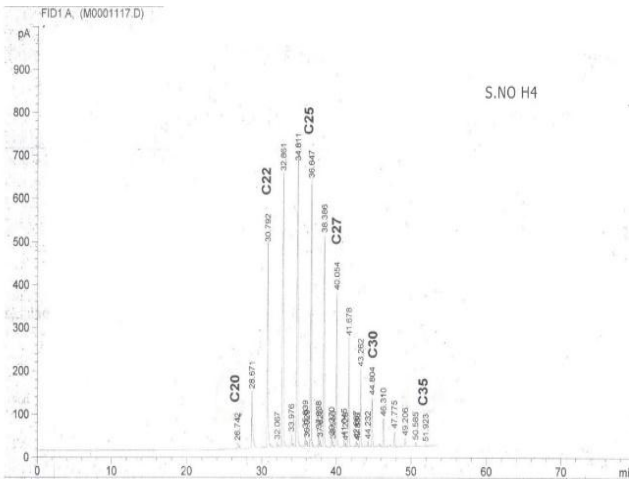

Figure 5: Shows GC analysis of light slack wax after degradation with H4 isolates after 14 days of treatment.

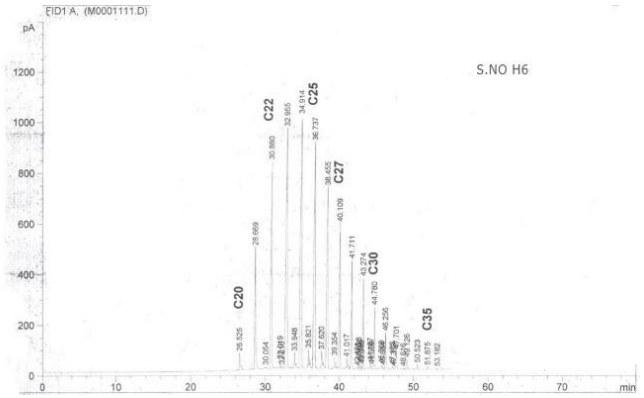

Figure 6: Shows GC analysis of light slack wax after degradation with H6 isolates after 14 days of treatment.

Gas chromatography (GC) was performed on untreated and treated light slack wax with isolates H4 & H6 after a 2-week incubation period. FIGS. 4, 5, and 6 depict GC analysis of n-paraffins before and after a 2-week microbial incubation period. When compared to the original feed (78.35 wt. %), there is a significant increase in n-paraffin content, particularly for isolate H6 (86.22 wt.%) and H4 (91.25 wt.%). This is based on the tendency of bacterial isolates to degrade iso-paraffin and naphthenic chemicals rather than paraffin fractions. These findings were consistent with [42]. They discovered that the thermophilic bacterium's growth in crude oil resulted in losses of aromatic hydrocarbons, asphaltenes, and resins. Also, the bioconversion of crude oil leads to an enrichment in lighter hydrocarbons and overall redistribution of these hydrocarbons [43].

Solid-liquid chromatography was used to measure aromatic compounds in the light slack wax waste by-product of the biodegradation before and after a 2-week incubation period, as shown in Table 3. The results of solid-liquid chromatography agreed with those of physical properties and GC analysis. After two weeks of incubation, the isolates H4 and H6 were able to remove nearly 100% of mono-aromatic compounds. These conclusions are supported by sulfur and color data. According to Zhengzhi *et al*., a constructed microbial consortium composed of seven bacteria related to bacterial isolates isolated from oil-contaminated soils was more efficient in crude oil degradation (approximately 87.5% of total petroleum hydrocarbons (TPHs)) than individual strains (approximately 10-64%) of TPHs [44]. Because of synergistic effects, microbial consortiums can breakdown hydrocarbons from oil-contaminated regions more efficiently than pure isolated microorganisms [45].

Based on the *TAPPI-ASTM* equation, all of the waxes isolated from light slack wax after treatment by isolates H4 & H6 fall into the category of macro-crystalline waxes, as they have refractive indices lower than those obtained by the equation and viscosities at 98.9 C lower than 7.4 centistokes [46],[41] (TABLE1).

The most promising bacterial strains were identified by 16S rRNA and the phylogenetic trees for wax degraders were displayed (FIG.7). According to the phylogenetic tree for the selected wax degrading bacteria, H4 belonged to *Bacillus siamensis*; H6 belonged to *Bacillus cereus*. All the sequences were deposited into the GenBank database under the accession numbers ON908492 and ON908493 for isolates H4 and H6.

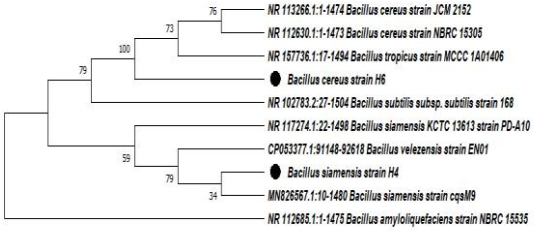

Figure 3: Phylogenetic tree of the most promising WCO biodegrading bacteria constructed by the maximum likelihood method using MEGAX software for the 16S rDNA sequences of Bacillus strains H4 and H6. The numbers at nodes represent the percentage values given by 1000 bootstrap analysis.

Many studies approved the role of soil and water-isolated bacteria as a biological treatment for many types of hydrocarbons [42], [43]. Bacterial degradation of hydrocarbons depends on the ability of bacteria to produce biosurfactants which reduce the surface tension of hydrocarbons and enables bacteria to use carbons in organic wastes as a carbon source [44], [45]. Moreover, in the presence of oxygen, bacteria can produce oxygenase that degrades hydrocarbons to utilize carbons [30]. In this research, the isolated strain (H4, *Bacillus siamensis*) can utilize the light slack wax due to the production of biosurfactants with different types of hydrocarbons [46], [47]. In addition, the isolated strain (H6, *Bacillus cereus*) demonstrated the highest biodegradation of the light slack wax which was approved in many researches [48], [49]. Many investigators approved the role of bacterial isolates *Klebsiella quasi variicola* [50] and *Bacillus cereus* [51].

The isolated paraffin waxes are widely used for various applications, they are a big source for latent heat storage as phase change materials but they suffer from the weakness in their thermal conductivity so different types of additives are needed to enhance their thermal conductivity. According to the differential scanning calorimeter paraffin wax is better in phase change material than microcrystalline waxes because it has two phase

changes solid-solid and solid-liquid, α Nano Alumina were added to the wax with different loading ratios by ultrasonic and the homogeneity was observed by polarized optical microscope. α Nano Alumina Succeeded in improving the thermal conductivity, the more the loading ratio of the α Nano Alumina in the wax composites the more enhancement in the thermal conductivity and the latent heat, PW /2% α Nano Alumina has the highest effect at 323 K [52]. So, the bioconversion of light slack wax waste by-product into fuel was done using isolates H4 & H6 for an incubation period of 2 weeks.

4. Conclusions

It can be concluded that the isolation of local bacterial strains from soil to use them in the bioremediation of hydrocarbon wastes is potential. This research presented evidence that bacterial isolates of *Bacillus cereus*, and *Bacillus siamensis* were the most promising bacterial biodegradation candidates for hydrocarbon wastes such as light slack wax after 14 days of treatments. Additionally, using indigenous bacterial strains for bioremediation not only works well but also offers an environmentally responsible method of handling biowaste. By utilizing these bacterial isolates' inherent skills, the biodegradation process can be carried out without the use of hazardous chemicals, potentially reducing the harm caused by conventional treatment procedures. This study emphasizes the potential of utilizing native bacterial strains to address problems associated with hydrocarbon waste and advance a greener, more sustainable future.

5. Conflicts of interest

There are no conflicts to declare.

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