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Comparative Study of Biodegradation and Deoiling Techniques for Isolation of Microcrystalline Waxes

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ABSTRACT

One stage fractional crystallization and biodegradation techniques have been used to separate different microcrystalline waxes grades; with different characteristics; from Alexandria crude petrolatum (waste by-product). The deoiling technique was performed using butyl acetate as solvent at ambient temperature of 20°C, at different dilution solvent ratios (S/F by weight) ranging from 2:1 to 8:1 at constant washing ratio of 2:1 for the first technique. While, Alexandria crude petrolatum waste by-product was subjected to biotreatment using five isolated *Bacillus* species at 30°C and various incubation periods, 7, 14 and 21 days. The results obtained from HPLC for the 15 samples showed that the aromatics contents decreased, especially, for that treated with *Bacillus* sp. MAM-27 which degrade PAHs faster at 1% (w/v) concentration of crude petrolatum (waste by- product) and exhibited high biodegradation ability within 1 week. *Bacillus sp. MAM-27* degraded 98.9% of PAHs, while *Bacillus sp. MAM-24* and *Bacillus sp.MAM- 3* degrades 97.88% and 95.5% respectively of PAHs within 2 weeks and then the degradation ability is slightly increased afterwards. The gas chromatography analysis of the samples before and after treatment with *Bacillus spp.* showed that, the aromatics, naphthenes and iso-alkanes were more degradable than saturated n-paraffins. Treatment by *Bacillus sp.MAM- 27, Bacillus sp.MAM- 24 & Bacillus sp.MAM-3* can be an effective method for biodegradation of Alexandria crude petrolatum (waste by-product) leading to microcrystalline waxes which have a lot of industrial applications. Thus, from economic point of view, the biotechnology technique is more suitable for deoiling of Alex. crude petrolatum than fractional crystallization technique since it a useful and an efficient method for the refining of Suez crude petrolatum since, it saves time, money and not causes damage to the environment because it done in one step without using any solvents while, the traditional methods, deoiling techniques, are done by two processes; fractional crystallization followed by adsorption techniques using expensive materials.

Keywords: Biodegradation, deoiling technique, Alexandria crude petrolatum, *Bacillus Spp.,* microcrystalline wax.

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INTRODUCTION

Microcrystalline waxes are extremely small crystals or microscopic in size and sometimes called amorphous. They are usually derived from heavy petroleum distillates or residues or tank bottoms. They are petroleum waxes containing substantial proportion of hydrocarbons other than normal paraffins. They consist mainly of highly branched chain paraffins, cyclo-paraffins and small amounts of n-paraffins and alkylated aromatics [1-3]. Microcrystalline waxes have been widely used in many applications such as household chemicals as candles and polishes, pharmaceutical, cosmetic as lipsticks, building construction, paper, match, rubber and other industrial purposes [4].

The most predominant deoiling process is the wax re-crystallization (fractional crystallization) which is sometimes called wax fractionation process and can be used to fractionate or deoil all types of waxes [5,6]. Generally Petroleum refiners have been used solvent deoiling technique especially the fractional crystallization to refine the slack wax to produce different grades of petroleum waxes. Our previous study revealed that some different grades of petroleum waxes can be produced by fractional crystallization of light, middle, heavy slack waxes and crude petrolatums at ambient temperature by using different solvents [7-10]. It has been reported that many indigenous microorganism are capable of degrading petroleum hydrocarbons, such as alkanes, paraffinic and aromatics [11, 12]. Bacteria belonging to the genus Bacillus have been extensively related to petroleum biodegradation and have been found in petroleum and polycyclic aromatic hydrocarbons (PAHs) - contaminated environments [13, 14]. PCR results obtained using the *B.pumilus*- specific primer set reinforce the wide distribution of Bacillus spp. in oil fields [12]. *Pseudomonas spp.* reduces wax precipitation in waxy crude oils and so enhances oil recovery. GC analysis shows an increase in concentration of iso-alkanes in range of C_{15}- C_{20} and bioconversion of heavy iso-alkanes in the range C_{21}- C_{22+} [15]. Alkanes are the major component in fossil oil. The release of oil has created serious environment problem, which requires efficient method for the degradation of alkanes, especially the less volatile long-chain n-alkanes. Many microorganisms are known to grew on n-alkanes and thus degrade the hydrocarbons [16]. Biodegradation of long chain n-alkanes has also been considered as a useful tool for enhancing oil recovery in reservoir due to the removal of heavy oil components [17]. *Rhodococcus SP. MOj-3449* was used for fast biodegradation of long chain n-alkanes and crude oil at high concentration, thus, enhanced oil recovery and cleaning [18]. Slop wax waste by-product obtained through the lube oil manufacture was subjected to biotreatment using five isolated Bacillus species at 30 °C and various incubation periods, 7, 14 and 21 days. The results obtained from HPLC for the 15 samples showed that the aromatic contents decreased, especially, for that treated with Bacillus sp. MAM-27 which degrade PAHs faster at 1% (w/v) concentration of slop wax waste by-product and exhibited high biodegradation ability within 1 week. Bacillus sp. MAM-27 degraded 99.9% of PAHs, while Bacillus sp. MAM-24 degrades 99.8% of PAHs within 2 weeks and then the degradation ability is slightly increased afterwards. Treatment by Bacillus sp.MAM-27 and Bacillus sp.MAM-24 can be an effective method for biodegradation of slop wax waste by-product leading to paraffin waxes match with plastic paraffin wax according to USSR 1121284 specifications [19]. Thus, the present study is an attempt to use biodegradation technique to degrade the oily constituents which are mainly aromatics and low melting waxes. Also, this study deals with the differentiation between the fractional crystallization (the most used one) and biodegradation techniques for separation of various grades of microcrystalline waxes of different specifications from Alex. crude petrolatum which is a waste by-product that doesn't find any high quality.

Materials and Methods

Materials

One appropriate crude petrolatum (petroleum wax by product) obtained from heavy residue from Alexandria Petroleum Company (mixed crudes from Belayim and west desert) used in this study for isolation of some grades of microcrystalline waxes via solvent deoiling and biodegradation techniques.

Fractional Crystallization Technique

Alexandria crude petrolatum was subjected practically to one stage fractional crystallization using butyl acetate solvent at ambient temperature of 20°C and at different solvent feed ratios (S/F by weight) ranging from 4:1 to 8:1 at fixed washing solvent ratio to produce microcrystalline waxes [20-22].

A known weight of Suez crude petrolatum wax was dissolved in the corresponding amount of solvent in a beaker and heated till the mixture becomes homogenous. Then the mixture was cooled gradually at room temperature. The beaker and the buchner funnel were transferred to a controlled temperature unit and gradually cooled to the desired temperature. The beaker contents were transferred to the funnel and filtered through a Whatman filter paper No. 43 by using gentle suction. The wax cake was washed with additional solvent at the same temperature and added at small increments. Solvents were removed from the wax cake by distillation.

Microorganisms and culture condition

Five *Bacillus* spp. (*Bacillus sp. MAM-3, Bacillus sp. MAM-24, Bacillus sp. MAM-27, Bacillus sp. MAM-29* and *Bacillus sp. MAM-33) were* isolated from depository of petroleum field onto chronic soil of Cairo Refining Petroleum Company, Mostorod, El-kaluobia, Egypt. In all tests performed, isolates and P.Sajor-Caju were inoculated in flasks containing basal salt medium (BSM) [19, 23]

Effect of different microorganisms on Alexanderia crude petrolatum degradation

The five bacterial strains were inoculated individually in 100ml BSM containing 1 %(w/v) Alexandria crude petroltum. The bacterial isolated strains were grown in L.B medium composed of (g/l distilled water) 10.0 Tryptone; 5.0 yeast extract and 5.0 Nacl [24]. The bacterial isolates inoculated in L.B medium were incubated at 30°C for 48 hours and then centrifuged at 10.000rpm for 10 minutes. The pellets were washed three times with strile BSM. After that the washed pelletes were resuspended in BSM and used to inoculate (10% v/v) the BSM containing Alexandria crude petrolatum as a sole carbon and energy source. Three replicates were used for each strain. The inoculated flasks were incubated at 30°C in shaking incubator for 7, 14 and 21 days. Three replicates were used for each period. Samples for analysis were withdrown at the end of each incubation period.

Comparing the most three potent *Bacillus Spp*. in degrading slop wax

The most promising *Bacillus spp., Bacillus sp. MAM-24*, *Bacillus sp. MAM-27 and Bacillus sp. MAM-3* were selected to perform the previous step in large scale to get a sufficient quantity of the remainder of Alex. crude petrolatum after biodegradation for chemical and physical analysis. The two isolated strains were inoculated (10% v/v) into 2000 ml BSM in 10.000 ml conical flask for each strain, which containing (1% w/v) crude wax as sole carbon and energy source. The inoculated culture media were incubated at 30°C for 14 days in shaking incubator (150 rpm) [19].

Physical property analysis

Alexandria crude microcrystalline wax and the isolated waxes were physically characterized according to American Society for Testing and Materials (ASTM) standard methods [25]. The type of the isolated waxes was specified according to Technical Association of the Pulp and Paper Industry TAPPI-ASTM equation **[**26**].**

The total aromatic content of Suez crude petrolatum and separated waxes were determined using liquid solid column chromatography technique [27]. A 1.3 cm diameter and height of 130 cm column packed with activated (60-200 mesh) silica gel was used. The column was then moistened with 100 ml of n-hexane to dissipate the heat of adsorption. A 10 g sample of the sample dissolved in few milliliters of n-hexane was transferred to the column. The column was then eluted with 300 ml of n-hexane followed by 200 ml benzene and finally 150 ml of a 1:1 mixture of absolute methanol and benzene. Fractions of 25 ml were taken from the column, the solvent distilled off and the refractive index of each fraction was determined. According to the refractive index data at 20°C, eluates were combined into saturates mono-, di- and poly-aromatics. The saturate hydrocarbons have refractive indices not more than 1.48. The mono-cyclic, bi-cyclic and poly-cyclic aromatics have refractive indices from 1.48 to 1.53, 1.53 to 1.59 and higher than 1.59, respectively [28].

Chemical property analysis

Polycyclic Aromatic Hydrocarbon Analysis

PAHs identification and quantification were performed using HPLC technique. The apparatus used was model Waters HPLC 600E, equipped with dual UV absorbance detector Waters 2487 and auto sampler Waters 717 plus attached to computerized system with Millennium 3.2 software. PAHs standards were obtained from Supelco. The conditions of separation [29] are as follow:

Column: Supelcosil. LC-PAH, 5µm particles, 15cm length and 4.6mm ID, Mobile phase: gradient acetonitrile: water 60 to 100 % acetonitrile (v/v) over 45 minutes. Flow rate: 0-2 min. 0.2 ml/min., 2-45 min. 1.0 ml/min. Detector: set at 254 nm.

Gas chromatographic analysis

The analysis of n-alkane and iso-alkane before and after microbial growth was done using a GC technique. The GC apparatus used was PerkinElemer (Clarus 500), equipped with a hydrogen flame ionization detector and fused silica capillary column (60 m length × 0.32 mm i.d), packed with poly (dimethyl siloxane) HP-1 (non-polar packing)of 0.5µm film thickness. In the chromatograph, the injector was heated at350°C. the column temperature was programmed from 100 to 300°C at a fixed rate of 3°C/min, and nitrogen (oxygen-free) was used as a carrier gas with flow rate of 2 ml/min. the detector was heated at 350°C, and operated with a hydrogen flow rate adjusted to optimize the detector sensitivity. The sample was melted and 0.1µl of it was introduced into the injector. A mixture of pure n-paraffins was used as standard. The peak area of each resolved component (consisting of either n- and iso-paraffin) is determined individually. However, the unresolved complex mixtures (humps); composed of non n-paraffins presumably mainly cyclo-paraffins and aromatics with long side chains; were determined only as a total.

RESULTS AND DISCUSSION

Characterization of Crude Wax

The general features of Alexandria crude microcrystalline wax are listed in Table 1. It is clear that, Alexandria crude wax is characterized by its higher refractive index, kinematic viscosity, oil content and needle penetration due to its high aromatics content. The higher oil content is an indication for low quality wax. These aromatics constitutes are mono-aromatics and di-aromatics components. Data of sulfur content and color are parallel to the previous results. It is obvious, from molecular composition data that, Alexandria crude wax is characterized by its high iso- and cycloparaffins content (54.65 wt.%), higher aromatic content (34.85 wt.%) and consequently low n-paraffin content (10.50 wt.%).

Fractional Crystallization Technique

Alexandria crude petrolatum has been subjected to one stage fractional crystallization process using butyl acetate solvent at ambient fractionating temperature of 20°C [30].

Effect of Dilution Solvent Ratio

The dilution and the amount of solvent used in fractional crystallization have an obvious and great effect upon the yield and quality of isolated waxes from Alex. crude petrolatum using butyl acetate solvent at ambient fractionating temperature of 20°C and at fixed washing solvent ratio of 2:1 by weight is represented in table1.

It can be noticed from the data that by increasing the dilution solvent ratio from 4:1 to 8:1, the wax yield decreases sharply with increasing of dilution ratios. It may be attributed to the increase of the solvent power for oil inherent to the wax crystals. This facilitates the removal of entrained oil and is in line with the data of congealing point of the isolated waxes, which give higher values on increasing dilution solvent ratio. It is interest to note that the decrease in the oil content is accompanied with decrease of refractive index,

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viscosity needle penetration and sulfur content (table 1). This may be attributed to the type of the inherent oil which may be of higher molecular naphthenes or alkylated benzene.

Isolated Wax Type

Examining the isolated wax type in Table 1; on the basis of TAPPI-ASTM equation; it can be noticed that all the waxes isolated from Alex. crude petrolatum; at all dilution solvent ratios; lie in the category of microcrystalline waxes as they characterized by refractive indices higher than that given by the equation and by viscosities at 98.9°C more than 10 centistokes. Meanwhile, according to petroleum wax and U.S. Pharmacopoeia and National Formulary Specifications [5,31] congealing, refractive index, viscosity and oil content of the isolated wax fractions separated at all dilution ratios are in limits of microcrystalline wax group.

Biodegradation Technique

The physical characteristics and the molecular type composition for Alex. Crude petrolatum are represented in table (1). Data indicate that crude petrolatum high viscosity and high oil content due to its high isoparaffin and naphthenic compound beside to aromatics content. Thus, in order to produce useful petroleum products from crude petrolatum (waste by-product), the aromatics, isoparaffin and naphthenic compounds must be reduced by subjected waste wax to biodegradation process using *Bacillus spp.*

In this study five isolated *Bacillus spp.* were used to evaluate their potential for degradation of the aromatics, isoparaffin and naphthenic ones. The physical result show a sharp increase in melting point and a decrease in refractive index of Alexandria crude petrolatum after growth especially for samples treated with *Bacillus sp. MAM-24 27*& *Bacillus sp. MAM-3* at different incubation period compared with that before *Bacillus spp*. treatment as indicated in Figs.(1 & 2). This may be attributed to the increase of n-paraffins content.

Characteristics	Alex. crude petrolatum	Wax fractions isolated at different solvent feed		
		4:1	6:1	8:1
Yield on crude, wt.%	100	60.20	42.50	38.45
Congealing point ,°C	70	74	76	78
Kinematic viscosity, 98.9°C, mm/^{2}s	12.75	11.90	11.00	10.8
Refractive index, 98.9°C	1.4544	1.4480	1.4439	1.4407
Mean molecular weight	623	712	719	725
Oil content, wt%·	20.20	5.89	2.64	1.50
Needle penetration, 25°C	42	22	18	16
Sulfur content, wt %	1.67	0.4	0.25	0.19
Color (ASTM-D 1500)	2.0	0.6	0.5	0.5
Refractive index by TAPPI-ASTM equation	-----	1.4315	1.4325	1.4331
Type of wax	-----	Micro-crystalline		
Molecular Type Composition				
Total saturates, wt. %	65.15	81.20	85.37	89.94
n-Paraffin content, wt. %	10.50	19.82	22.50	24.82
Iso- and cyclo-paraffins content, wt. %	54.65	74.38	62.87	64.67
Total aromatics, wt. %	34.85	18.80	14.63	10.06
Mono-aromatics, wt.	21.90	17.30	14.63	10.06
Di-aromatic content, wt %	12.95	1.50	0.0	0.0

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Table 1: Effect of dilution solvent ratio on the physical characteristics, molecular type composition and type of the isolated waxes by one stage fractional crystallization of Alexandria crude microcrystalline wax using butyl acetate solvent at fractionating temperature= 20 ^{0}C and S\F for washing of 2:1.

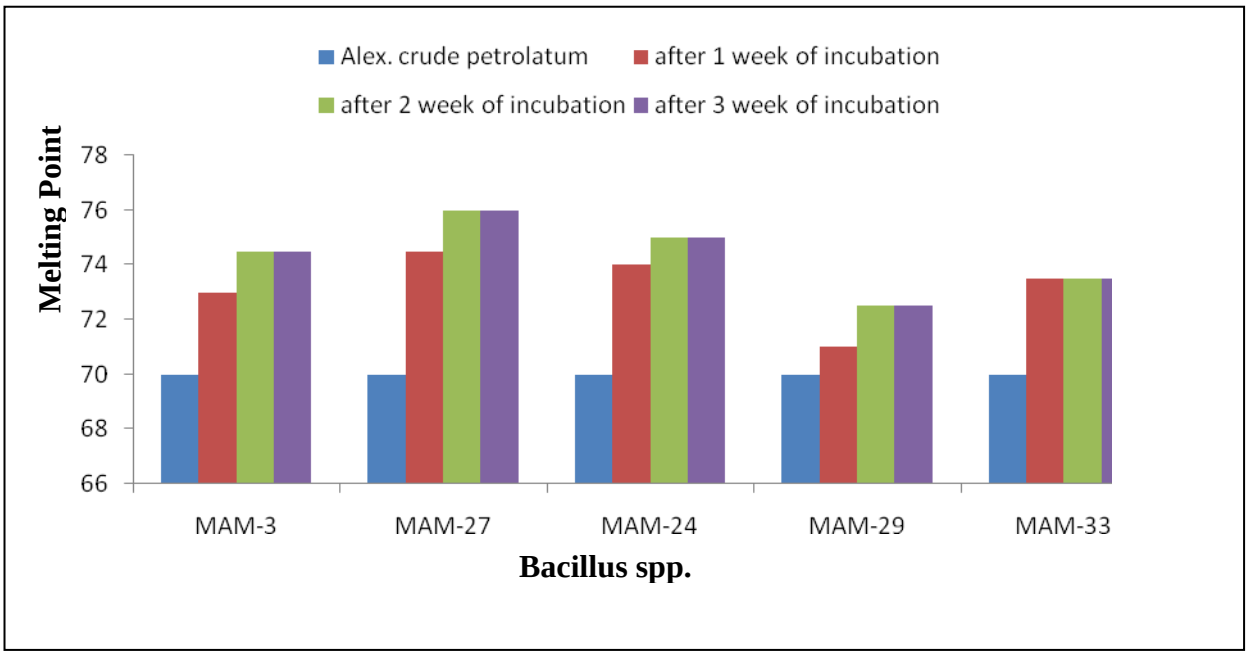

Figure 1: Melting point of Alex. crude petrolatum before and after treated with Bacillus spp. at different incubation period

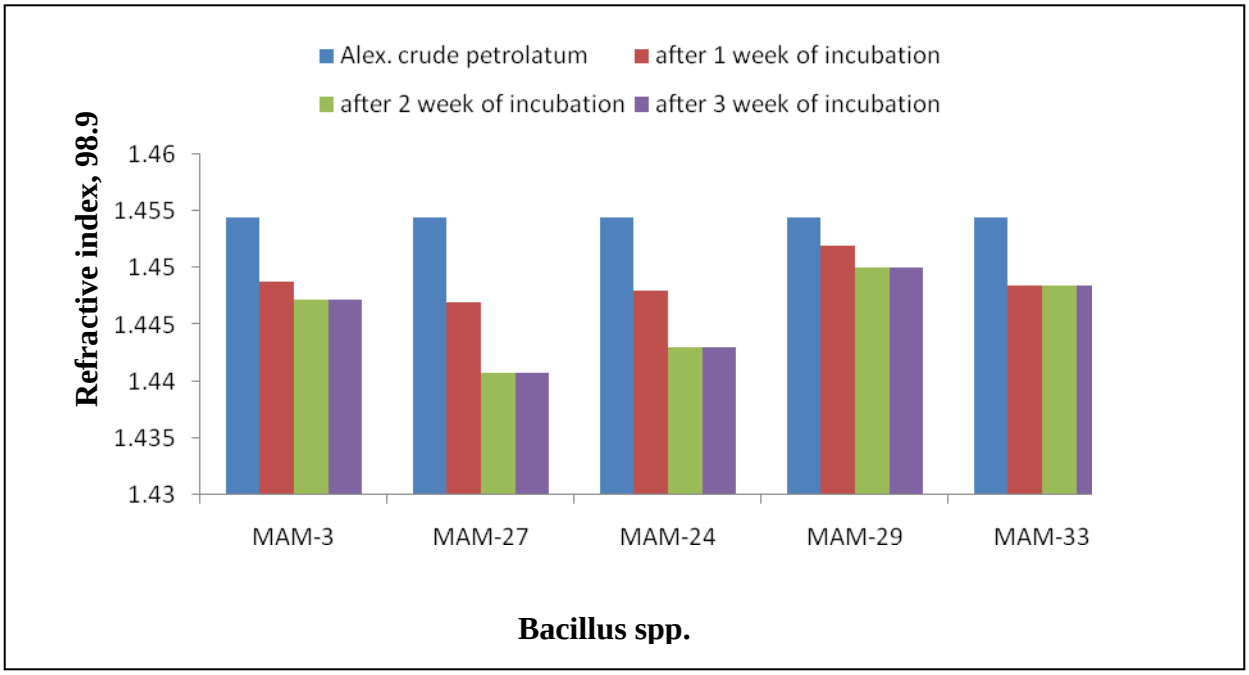

Figure 2: Refractive index of Alex. crude petrolatum before and after treated with Bacillus spp. at different incubation period

and decrease of aromatics, isoparaffin and naphthenic compounds [32]. These results were with agreement with GC and HPLC analysis. Gas Chromatographic (GC) was carried out on untreated Alexandria crude

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petrolatum and treated with *Bacillus spp*. at different incubation period. Figure (3) shows GC analysis of n-paraffin's before and after different incubation periods of microbial treatment. At first weak, there was noticeable increase in n-paraffins content especially for samples treated with *Bacillus sp. MAM-24 & MAM-27*, which increases more rapidly especially after first week for both *Bacillus sp. MAM-27* and the same for sample treated with *Bacillus sp. MAM-3* after 2 weeks. This could be attributed to the nature of microbes especially *Bacillus sp. MAM-27*, *Bacillus sp. MAM-24 and Bacillus sp. MAM-3* are capable to degrade isoparaffins and naphthenic compounds rather than paraffinic fraction. From Figure (3), it is obvious that the optimum degradation of iso-alkane and naphthenic compounds was found for *Bacillus sp. MAM-27* after 14 days incubation and remains constant. The same was obtained for *Bacillus sp. MAM-24 and Bacillus sp. MAM-3* after 14 days and remains constant. From GC results, all the five *Bacillus spp*. are useful for degradation of isoparaffins and naphthenic compounds especially *Bacillus sp. MAM-24*, *MAM- 27* and *Bacillus sp. MAM-3* The above results were in agreement with that of *Hao et.al* (2004). They found that the microbial growth of the microbial growth of the thermophilic bacterium on crude oil resulted in losses of aromatic hydrocarbons, resins and asphaltenes and bioconversion of crude oil leads to enrichment in lighter hydrocarbons and overall redistribution of these hydrocarbons [33].

Biodegradation of polycyclic aromatic hydrocarbons Alexandria crude petrolatum waste by-product were determined by High Perfomce Liquid Chromatography HPLC before and after different incubation periods as indicated in Figure (4).The results obtain from HPLC were in agreement with that of physical properties and GC analysis. The *Bacillus spp*. were efficiently capable of removing PAHs, *Bacillus sp. MAM-27* was capable of removing almost PAHs about 98.9% after 2 weak followed by *Bacillus*

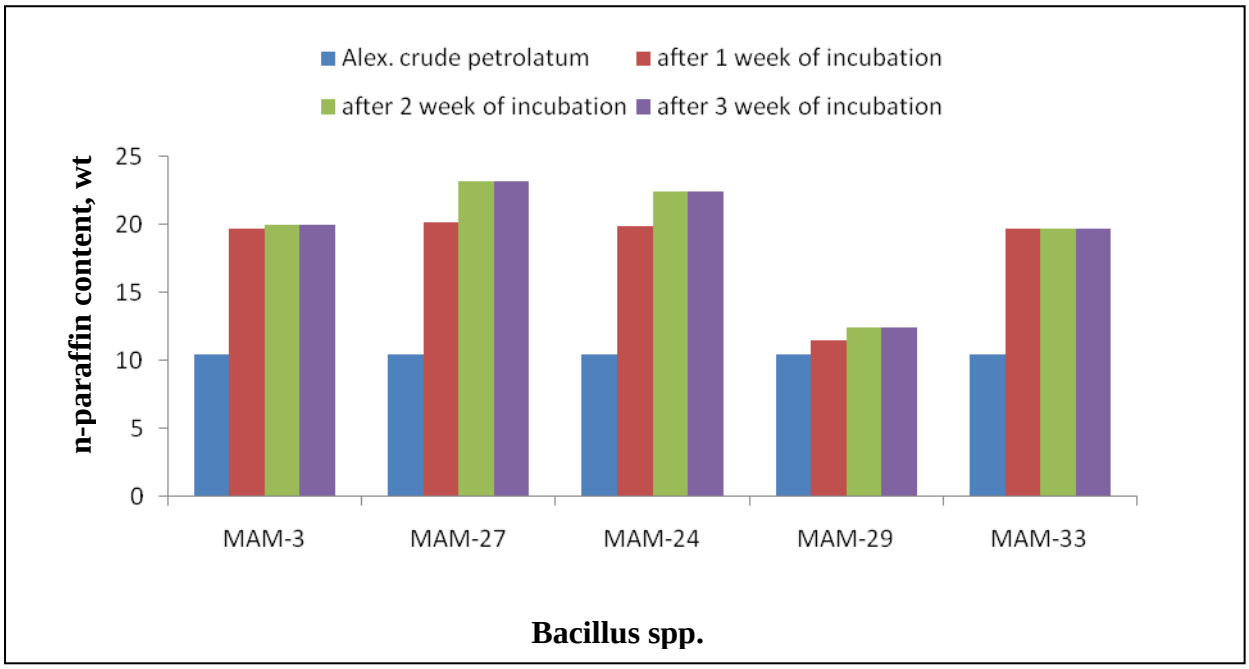

Figure 3: GC analysis of Alex. crude petrolatum before and after treated with Bacillus ssp. at different incubation period

sp. MAM-24 after 2 weeks which 97.88% and *MAM-3* after 2 weeks which is 95.50 % and the least aromatic removing was *Bacillus sp. MAM-29* removed about 35.42%, after 3 weeks. That is to say *Bacillus spp.MAM-27*, *MAM-24* and *MAM-3* were the most efficient in removing total PAHs of Alexandria crude petrolatum after 2 weeks incubation periods. Other *Bacillus spp*. removed the highest percentage of total PHAs in all microbial treatment used in crude petrolatum at 7, 15 and 21 days respectively. There is increasing interest in the biodegradation of toxic aromatic hydrocarbon organic wastes, including polycyclic aromatics hydrocarbons (PAHs), because many PAHs and their epoxides are highly toxic, mutagenic and/ or carcinogenic to microorganisms as well as to higher systems including humans [34,35]. The U.S. Environmental Protection Agency (USEPA) had classified 16 PAHs as priority pollutants whose remediation is considered indispensable for environment clean up and human health [36].

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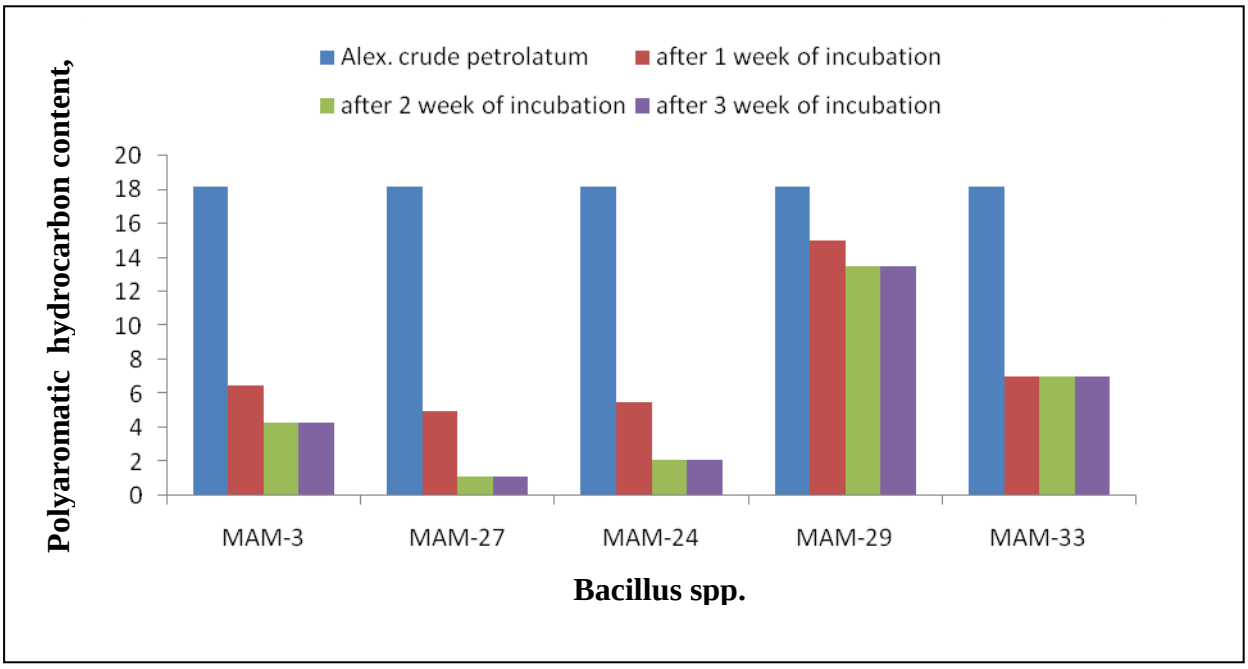

Figure 4: Poly aromatic hydrocarbons measurement for Alex. crude petrolatum before and after treated with Bacillus spp. at different incubation period

Feitkenhauer et.al., (2003) reported that *Bacillus spp.* and *Thermus sp.* were used to degrade PAH compounds and mixtures [37]. Abo-State and Moustafa (2005) found that *B. megaterium* degrade chrysene and dibenzo (a, h) anthracene completely from crude oil [38]. Surprisingly, *Bacillus sp.2* showed biodegradation percentages > 90% for dihydrophenanthrene and phytane, reveling hydrocarbon degradation percentages from petroleum samples not found in the literature [12].

From the above findings, the most promising are *Bacillus sp.MAM-24, MAM-27 and MAM-3* were selected for biodegradation of Alexandria crude petrolatum waste by-product. Table (2) indicated the physical properties of the isolated waxes after treating by *Bacillus sp.MAM-27, MAM-24* and *MAM-3.* By examining the physical properties of the isolated waxes after treating by *Bacillus sp.MAM-24, MAM-27& MAM-3*, it was lie in the category of microcrystalline waxes on the basis of petroleum wax and U.S. Pharmacopoeia and National Formulary Specifications [5, 31] congealing, refractive index, viscosity and oil content of the isolated wax fractions separated are in limits of microcrystalline wax group. Microcrystalline waxes are widely used for various applications such as laminated foils and papers for the food

Characteristics	Isolated Wax using *Bacillus sp. MAM-27*	Isolated Wax using *Bacillus sp. MAM-24*	Isolated Wax using *Bacillus sp. MAM-3*
Congealing point , ºC	76	75	74.5
Kinematic viscosity at 100 ºC , mm^2/s	10.20	10.95	11.10
Oil content , wt %	1.50	2.89	4.20
Molecular type Composition			
Total saturates content,wt.%	89.98	88.80	81.70
n-paraffins content, wt.%	23.20	22.50	20.20
Iso-and cyclo-paraffins content,wt.%	66.78	66.30	61.50
Total aromatic content, wt.%	10.02	11.20	18.30

Table (2): Physical characteristics of the isolated waxes after treating with*Bacillus sp. MAM-27, Bacillus sp. MAM-24 and Bacillus sp. MAM-3*.

industry, manufacturing of candles, polishes, coating, household chemicals, pharmaceutical, cosmetic and other industrial purposes [4].

CONCLUSIONS

In this study, we achieved double goals, the first one is to use local isolates from chronic contamined soil to degrade slop wax waste by-product. *Bacillus spp. MAM-27, MAM-24* and *MAM-3* were the most efficient in removing total PAHs of Alexandria crude petrolatum after 2 week incubation periods. The second is utilization of Alex. crude petrolatum which is a waste by-product that does not find any high quality application to obtained microcrystalline waxes which have a lot of industrial applications . Thus, biodegradation process can be a useful and an efficient method for the refining of Alex. crude petrolatum instead of other refining processes which cause damage to the environment. Also, from economic point of view, biotechnology technique is more preferable since the yield of the isolated waxes from fractional crystallization technique (the most used one) is very low if compared with that isolated from biotechnology technique.

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