Ecofreindly, candle precursor (Paraffin wax) production by Iranian indigenous bacterium to reduce the indoor health risks

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ABSTRACT

The potential indoor air impacts of burning candles have drawn increased attention in recent years. Burning paraffin waxes itself, regardless to wick and the essence, produce some allergens and even carcinogenic compounds. Further biorefining of petroleum paraffin wax with proper bacteria is one of the proposed resolutions to decrease the health threat. Therefore in this study Iranian heavy crude oil sample were screened for the best isolate to reach the target. Manual culture and plate count method and screening were done in MSM medium supplemented with paraffin wax as the sole source of carbon and energy in 40°C and 150rpm. The growth curve of the best isolate was depicted through spectrophotometry during 24 hours. Then the isolate was characterized by biochemical test and molecular identification using 16srRNA. The spore forming, biosurfactant producing bacterium was identified as Bacillus cereus. Gas chromatogaraphs revealed the efficient degradation of long chain paraffin wax with the selected *Bacillus cereus* into 15 to 25 carbon length n- alkanes. The high physicochemical endurance of this bacterium including salinity tolerance from 0.5to 15 percent, pH from 4 to 10 and temperature growth range from 40 to 65°C, makes it a decent choice for industrial and environmental approaches.

Keywords: Paraffin wax; Indoor air pollution; *Bacillus cerus*; Biodegradation

INTRODUCTION

Paraffin wax is a white or colorless soft solid hydrocarbon molecule containing between twenty and forty carbon atoms derived from petroleum, coal or oil shale [1]. Common applications for paraffin wax include lubrication, electrical insulation, and candles and fruit waxing [2]. In chemistry, paraffin is used synonymously with alkane, indicating hydrocarbons with the general formula C_nH_{2n+2}. The name is derived from Latin *parum* ("barely") + *affinis*, meaning "lacking affinity" or "lacking reactivity", referring to paraffin's unreactive nature [3].

Paraffin wax is exploited in different branches of industry like candle-making, Coatings for waxed paper or cloth and Food-grade paraffin wax. When released in the environment, alkanes don't undergo rapid biodegradation, because they have no functional groups (like hydroxyl or carbonyl) that are needed by most organisms in order to metabolize the compound. However, some bacteria can metabolize some alkanes (especially those linear and short), by oxidizing the terminal carbon atom. The product is an alcohol, that could be next oxidized to an aldehyde, and finally to a carboxylic acid. The resulting fatty acid could be metabolized through the fatty acid degradation pathway [4].

One of the main challenges that bacteria face is to overcome the difficulty of complex hydrocarbon uptake. For instance, as bioavailability is the main limitation factor for biodegradation of petroleum hydrocarbons [5, 6] due to their chemical structure [7], bacteria produce surfactants to increase bioavailability of hydrocarbons, desorption and solubility of them in the aqueous phase[7, 8] and consequently enhance the biodegradation rate[9, 10, 11]. Candles Paraffin is the last possible petroleum distillate product. Even asphalt is extracted before paraffin in the refining process. The use of paraffinic compounds in candle-making industry is one of the health concerning issues, as an indoor pollutant material for scientific societies

nowadays. When candles' paraffin wax burns, trace amounts of organic chemicals emit, including acetaldehyde, formaldehyde, acrolein, and cyclic alkanes [12] which are all constituent of public health concern.

Some of the early investigations revealed excessive amounts of hazardous byproducts of paraffin burning and arise lots of scientific debates and concerns. It has been proved that acetaldehyde levels for 30 candles burned in an enclosed room for 3 hours were modeled at excess cancer risk level. Formaldehyde levels were measured at 0.190g/m and 17g/m [12] which were above the EPA's risk level. Maximum concentrations of acrolein and Formaldehyde also excess the cancer level. A cigarette burned in a similar environment produces that much acrolein [13]. Fine et al. (1999) found that the majority of emissions from candles consisted of organic compounds including alkanes, wax esters, alkanoic and alkenoic acids, and alkenes [14]. Some of the compounds found were thermally altered products of the unburned wax, while others were unaltered in the volatilization process. Due to those approaches, some studies have been conducted to decrease the health risk of using candle in enclosed indoors. The longer the paraffinic wax chain is, the more hazardous by-products are produced thanks to increase in possibility of cyclic hydrocarbon production. Therefore one of the biotechnological approaches is producing paraffin waxes with shorter hydrocarbon chains. Produce long chain alkanes not longer than 25 carbons in the chain and not less than 15 which cause it to become liquid, is one of the purposes of industrial studies. In practice to have an approximately exact cut in hydrocarbon chain could only be achieved biologically through enzymatic processes [15]. Petrochemical processes not only cannot accurately reach this propose but also itself produce lots of hazardous by products. Although this approach appears costly it is the sole less petrochemically severe and more beneficial resolution for this process. Thus, the purpose of this study is to isolate Iranian indigenous bacteria able to use paraffin as the sole source of carbon and energy, to cut down the large cultivation expenses. One of the best environments in which paraffin degrading bacteria are more likely to live is in heavy crude oil were the amount of accessible paraffin wax is considerable. So to address the best bacterial choice an Iranian crude oil sample was used.

MATARIALS AND METHODS

A sample of heavy Iranian crude oil was continuously enriched for a month with paraffin wax crystals as the sole source of carbon and energy [16, 17] in a minimal salt medium (MSM) consisting of K_2HPO_4 ,0.5(g); NH_4Cl,1(g); Na_2SO_4 ,2(g); KNO_3 ,2(g); $CaCl_2$.$6H_2O$,0.001(g); $MgSO_4$.$7H_2O$,1(g); $Feso_4$. $7H_2O$, 0.001(g) in 1 liter distilled water pH 7.0 [18]. It was kept under constant shaking at 150 rpm in 40°C ,that was the ambient temperature at the sampling site, for a month with the objective of enriching those microorganisms which can exploit the heavy structure of paraffin wax as their sole source of carbon and energy [19].The sample was enriched because indigenous bacteria in the soil can degrade a wide range of target constituents of the petroleum, but their population and efficiency are affected when any toxic contaminant is present at high concentrations [20, 21]. This adapted crude oil indigenous bacterial consortium with solid paraffin, consisted of several culturable bacteria, isolated by standard plate count method. Among the isolates the best grown bacteria on paraffin was chosen for further identification.

The growth and long chain alkane-degrading ability of the bacterium studied in minimal salt medium as described, supplementing with 5% paraffin as the sole source carbon and energy [22]. The bacterial growth curve was depicted measuring turbidity change hourly for 24 hours. Before each inoculation a primary preculture was prepared as following: 5 ml of the bacterial suspension with optical density equal 1(using T80 UV/VIS spectrometer) enriched in 100 ml nutrient broth; after 48 hours incubation at 30°C, that was inoculated in MSM supplemented with 5% Parrafin, spread on the flask wall, and 0.1% glucose as a growth stimulator and agitated for 48 hours in 150 rpm at 40°C, then this preculture was used as inoculum in different experiments [23,24].

Biosurfactant production was proved by Blood hemolysis [24], drop collapse [8, 25, 26] and oil spreading techniques [27]. Degradation of

paraffin-wax by the selected isolate was measured in 250 ml Erlenmeyer flasks (in triplicate) containing 100 ml MSM with 0.1% (w/v) of paraffinic wax as the sole carbon source embedded on the flask wall. Samples were incubated on a rotary shaker (150 rpm) at 40°C for 7 days. The isolates were grown overnight in Luria Bertani broth to a cell density of 10CFU ml^{-1} and 5% (v/v) inoculum was used to inoculate MSM with paraffin wax as the sole carbon source. Uninoculated controls were considered to measure loss of paraffin-wax after bacterial treatment. The residual paraffin-wax was extracted thrice with equal volumes of hexane. For quantitative analysis, 1 ml of the paraffinic wax (dissolved in 10 ml hexane) was analysed by GC (Hewlett Packard 5890 Series II fitted with flame ionization detectors (FID) on a DB 2887 column [21]. The chromatograph of the paraffinic fraction, extracted from inoculated flasks, was compared with that obtained from uninoculated control flask [28]. Each assay was performed three times and the average is reported.

The selected bacterium was characterized by standard morphological, Gram-reaction, physiological and biochemical techniques [44] and preliminarily identified as a *Bacillus sp.* The selected *Bacillus* was identified molecularly through 16SrRNA sequencing. fD1 and rD1 primers (Sinagene/Tehran/Iran) were used for PCR:

fD1*:5'ccgaattcgtcgacaacagagtttgatcctggctcag3',

rD1*:5'cccgggatccaagcttaaggaggtgatccagcc3'.

Then the PCR product [29] has been cloned in *P Bleuescript Sk* plasmid. White colonies had been chosen and after confirmation of the presence of 16SrRNA sequence in that, the fragment has been sequenced using fD1 and rD1 primers.

Physicochemical Endurance of the isolate was studied. Effect of temperature on the isolate was studied using 100ml mineral salt medium with 5ml VR and 1ml preculture at pH 6.8 on shaker at 150 rpm for 48 hrs. Then the pH and salinity endurance of the bacteria in MSM broth at 150 rpm for 48 hrs was respectively studied [30].

All experiments were done in triplicate at the adaptation temperature (40°C) and in a short period of 20 days (for biodegradation). To analyze the biodegradation an un-inoculated flasks with VR were served as control under each experiment condition.

RESULTS

Bacterial growth in minimal medium supplemented with paraffin wax was evident from the significant increase in the population as compared to control by measuring cell density at 600nm in 20 days of incubation period [31].

The bacterium which could more efficiently transform the rough appearance of paraffin wax into a white foamy structure in shorter time and could tolerate the toxicity of it with better growth rate in growth media was chosen for further identification.

The selected isolate grew continuously and the turbidity increased (Figure 1). Values represent the average of three experiments.

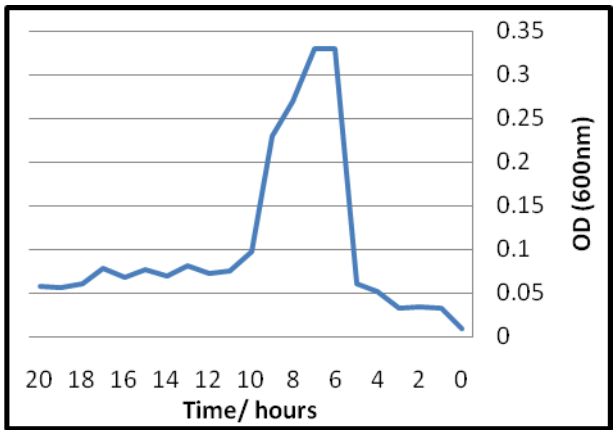

Figure 1. Bacterial Growth in MSM broth

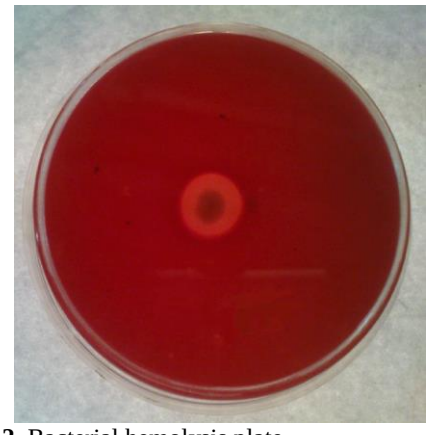

Figure 2. Bacterial hemolysis plate

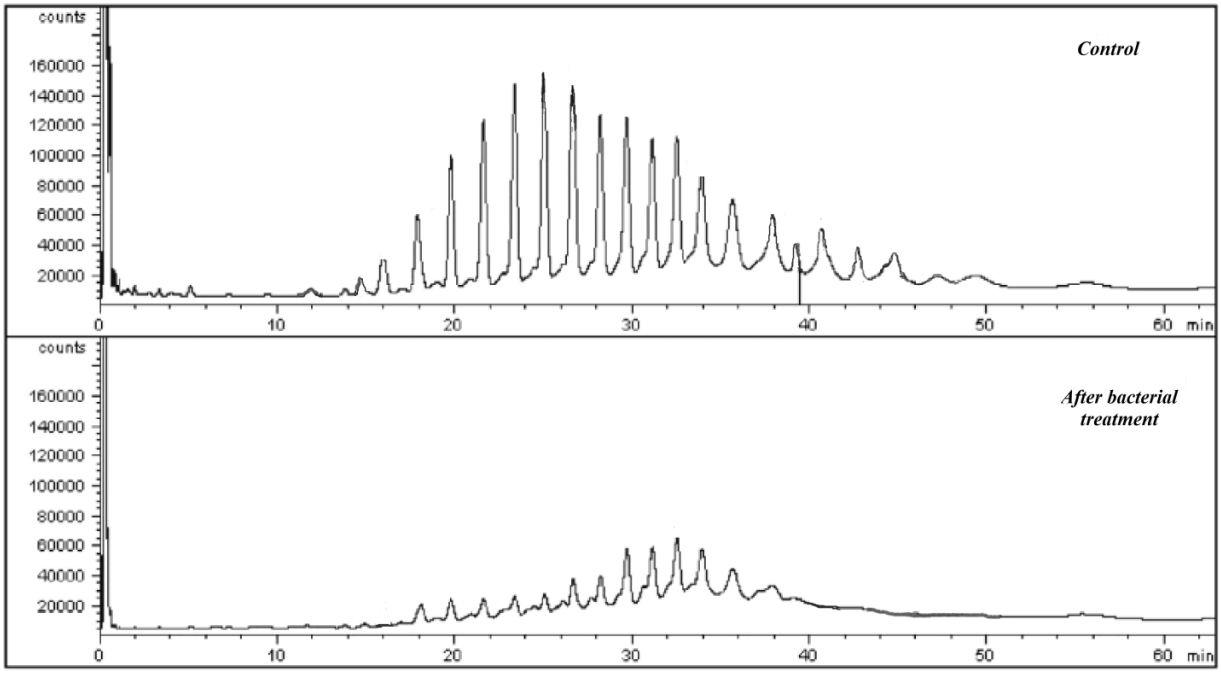

Figure 3. Chromatograph of paraffin wax before and after bacterial treatment

Table 1. Biochemical tests of selected bacterium

TESTS	SELECTED BACTERIUM
Gram staining	+
Spore staining	+
Catalase	+
Citrate	_
SIM	S(-) , Movement (+)
Starch hydrolysis	+
lecithinase	+

Table 2: Physicochemical tolerance of the bacterium

Bacillus cereus	growth
pH	4-10
Salinity	0.5- 15%
Temperature	40-65°C

The drop collapse test, oil spreading technique with 50 mm diameter clear zone and RBC hemolysis with over 75mm diameter clear zone in 24 hours confirmed the biosurfactant production [32]. The result of paraffin chemical analysis for the percentage of alkanes, after 20 days treatment with the bacterium in different, is illustrated in figure.3. The selected bacterium was able to grow in a wide range of pH from 5.5 to 8, salinity up to 3% and temperature from 20°C to 55°C. Biochemical and Molecular analysis (Tables 1 and 2) defined the selected bacterium as a strain of Bacillus cereus.

DISCUSSION

This study indicated that there are capable bacteria to biodegrade long chain alkane, paraffin wax, and utilize it as the only carbon and energy source. This finding could be an applicable approach in environmental biotechnology weather solving environmental issues like bioremediation and bioaugmentation or preventing the environmental pollution like in candle-producing industry.

Several studies have already proved the ability of Bacilli in degradation of various structures of hydrocarbons [23, 31, 33, 34] but there are rare information of paraffin wax degradation in specific length in Iran. Molecular analysis of the selected, spore forming, biosurfactant producing bacterium describes it as *Bacillus cereus*. As this bacterium was isolated from crude oil, It has high environmental endurance that makes it a promising choice in industry.

As paraffin content analysis after bacterial treatment illustrates, degradation in long chain saturate alkanes after treatment with the selected bacterium in 40°C in 150 rpm shaking for 3 weeks in MSM degraded from 12 to 50 carbon lengths into 18 to 38 carbon length, which is the ideal alkane size for candle industry.

CONCLUSION

The high physicochemical endurance of *Bacillus cereus* isolated from Iranian crude oil with the ability to utilize long chain alkane substances as its sole source of carbon and energy , and the particular finding of this research that revealed the remarkable ability of the bacterium to use paraffin wax as the only source of all required chemicals for growth and convert it into a particular chain length hydrocarbon along with surfactant production, make this bacterium a unique option for industrial use , particularly in bioremediation, bio-upgrading and biorefining processes to provide a safe precursor to be used in candle producing industry.

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