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Individually and Synergistic Degradation of Hydrocarbons by Biosurfactant Producing Bacteria

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Abstract

Background: Increasing worldwide contamination with hydrocarbons has urged environmental remediation using biological agents such as bacteria. Our goal here was to study the phylogenetic relationship of two crude oil degrader bacteria and investigation of their ability to degrade hydrocarbons.

Materials and Methods: Phylogenetic relationship of isolates was determined using morphological and biochemical characteristics and 16S rDNA gene sequencing. Optimum conditions of each isolate for crude oil degradation were investigated using one factor in time method. The rate of crude oil degradation by individual and consortium bacteria was assayed via Gas chromatography–mass spectrometry (GC-MS) analysis. Biosurfactant production was measured by Du Noüy ring method using Krüss-K6 tensiometer.

Results: The isolates were identified as *Dietzia cinnamea* KA1 and *Dietzia cinnamea* AP and clustered separately, while both are closely related to each other and with other isolates of *Dietzia cinnamea*. The optimal conditions for *D. cinnamea* KA1 were 35 °C, pH9.0, 510 mM NaCl, and minimal requirement of 46.5 mM NH4Cl and 2.10 mM NaH2PO4. In the case of *D. cinnamea* AP, the values were 30 °C, pH8.0, 170 mM NaCl, and minimal requirement of 55.8 mM NH4Cl and 2.10 mM NaH2PO4, respectively. Gas chromatography – Mass Spectroscopy (GC-MS) analysis showed that both isolates were able to utilize various crude oil compounds, but *D. cinnamea* KA1 was more efficient individually and consortium of isolates was the most. The isolates were able to grow and produce biosurfactant when cultured in MSM supplemented with crude oil and optimization of MSM conditions lead to increase in biosurfactant production.

Conclusion: To the best of our knowledge this is the first report of synergistic relationship between two strains of *D. cinnamea* in biodegradation of crude oil components, including poisonous and carcinogenic compound in a short time.

Keywords: Bioremediation; Biosurfactant; Carcinogenic; *Dietzia cinnamea* AP; *Dietzia cinnamea* KA1; Synergistic

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Introduction

Petroleum is a complex mixture of different hydrocarbons including aliphatic (linear or branched), cycloalkanes, mono- and polyaromatics, asphaltenes and resins and majority of these compounds are stable, toxic, and carcinogenic. The persistence of these compounds in the environment depends on the number of rings in the molecule and environmental factors such as pH, temperature, and salinity (1). Release of hydrocarbons into the environment due to human activities is the major cause of water and soil pollution (2).

Microbial communities play an important role in biological removal of pollutants from soils (3). Hydrocarbons in the environment are biodegraded

primarily by bacteria, yeast, and fungi. The reported efficiency of biodegradation ranged from 6% to 82% for soil fungi, 0.13% to 50% for soil bacteria, and 0.003% to 100% for marine bacteria. Many investigators reported that mixed populations of microorganisms with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in various environments. Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment (2).

Bioremediation technology utilizes microorganisms to degrade toxic pollutants to harmless products and these processes are environmentally safe and cost efficient (1, 4). Hydrocarbon microbial biodegradation in various environments is often limited primarily by the availability of nutrients, such as nitrogen and phosphorus. In fact, bioremediation technology speeds up degradation of the pollutant by optimization of environmental condition for microorganisms.

Among a variety of microorganisms, some species belonging to the genus Dietzia have been shown to be potential for bioremediation (5-10)Most of these isolates utilize hydrocarbons and have long-term viability in the environment even under dry and resource-limited conditions (11), therefore, they would be salutary in clean-up and bioremediation of oil pollutions (12). Also, they are believed to act as contributory factors to eliminate oil due to-contaminations by other hydrocarbon-degrading organisms (12-14). Further studies are needed to determine the optimal conditions if we intend to take advantage of these bacteria to accelerate bioremediation (15).

Biosurfactants are surface-active compounds synthesized by a wide range of microorganisms (16). They are amphiphilic molecules containing hydrophobic and hydrophilic moieties and tend to interact with surfaces of different polarities and reduce the surface and interfacial tensions of solutions (12). This helps bacteria attach oil droplets and also helps other microorganisms use hydrocarbons as the source of carbon and energy.

Our goal here was to study the phylogenetic relationship of two crude oil degrader bacteria and investigation of the ability of these isolates to degrade crude oil and compare their performance for bioremediation individually and in consortium. Optimal conditions for each isolate were determined and the effect of environmental optimization on biosurfactant production was additionally investigated.

Materials and Methods

Microorganism

Two hydrocarbon degrading bacteria (named KA1and AP) were isolated in our laboratory from water and sediments collected from oil polluted seasonal ponds of Khami located in northeast of Ahvaz (17). Phylogenetic analyses of 16S rDNA along with complementary morphological and biochemical tests were performed for exact classification of the isolates.

Media and Chemicals

Two media were used: 1- Mineral Salt Medium (MSM) was a modified Schlegel's medium (18) composed of 36 mM NH4Cl, 1.69 mM Na2HPO4, 0.066 mM FeSO4, 16 mM MgSO4.7H2O, 0.67 mM KCl, 1.8 mM CaCl2, 513 mM NaCl and 1 ml of trace element solution added to 1 liter of the modified Schlegel's medium. Trace element solutionincluded 0.147 mM ZnCl2, 0.77 mM NiCl, 1.54 mM CoCl2, 1.38 mM Na2SeO4, 0.24 mM Na2MoO4, 0.118 mM Na2WO4, 0.054 mM NaVO3 and 0.007 mM HCl. pH of mineral medium was adjusted to 8.0 by 100 mM Tris/HCl.

2- PYAM (Peptone Yeast-extract Agar Medium) contained Peptone (3 g), Yeast extract (5 g), Agar (12 g) in 1 liter of water from sampling site with final pH of 8.0.

All the media and chemicals were purchased from Merck (Darmstadt, Germany), except for the followings: CaCl2, glucose, starch, arabinose, Tris (Tris hydroxy- methyl amino methane) and Folin Phenol reagent (Sigma-Aldrich, Germany); Peptone, SIM and Agar (Difco), MRVP and TSI (Oxid); MgSO4.7H2O (Riedel-de Haën); NH4Cl (Chemlab, Belgium); Molasses (Islamabad sugar factory, Iran) and Crude oil (National Iranian Oil).

Optimization of crude oil degradation by bacteria

To evaluate oil degradation by individually isolates and consortium, environmental factors such as pH (5-12 with 1 unit intervals), temperature (25-45 °C with 5 °C intervals) and salt concentration (0-1530 mM NaCl with 170 mM intervals) were monitored in MSM by analyzing one factor at a time. In addition, to attain minimum bacterial requirement for N and P in the process of degrading 1ml crude oil, NH4Cl (in the range of 9.3-65.1 mM with 9.3 mM intervals) and Na2HPO4 (in the range of 0.42-2.96 mM with 0.42 mM intervals) were tested. Briefly, 100 ml mineral medium supplemented with 1% (v/v) crude oil as the sole carbon source was prepared and inoculated with 1 ml of each bacterial culture (separately) with turbidity equivalent to 0.5 McFarland (1.5×106 cell/ml). The control flasks remained uninoculated. The flasks were incubated for 5 days with orbital shaking at 95 rpm. As a factor for oil degradation and cell growth, total protein was measured according to the method described by Lowry et al. (19). The experiments were conducted in triplicates.

Comparison between consortium and individual bacteria for crude oil degradation

In order to comprise individual and consortium bacteria for degradation of crude oil, single bacteria were cultured in MSM flasks at their optimum conditions, separately. In the case of bacterial consortium, the averages of different factors were calculated by summing of optimum values of each factor (from both bacteria) divided by two. Thus bacterial consortium was incubated in MSM flasks with following factors: pH 8.5, 340 mM NaCl, 51.15 mM NH4Cl, 2.10 mM Na2HPO4 and 32.5 °C. The flasks were incubated for 5 days with orbital shaking at 95 rpm. Control flasks were also used. Finally, the residual values of crude oil were measured as described in next parts.

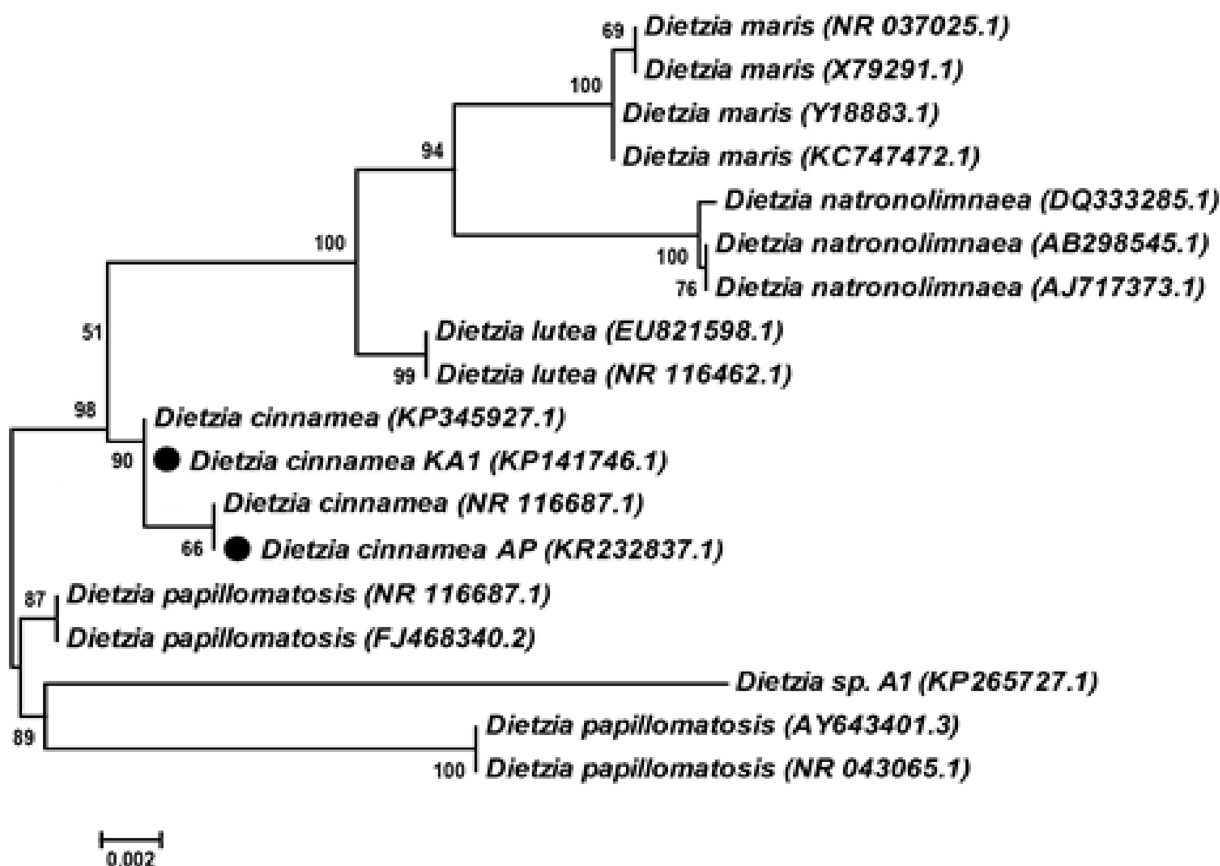

Figure 1. Phylogenetic comparison of 16S rDNA gene sequences from KA1 and AP strains and species in the genus Dietzia as determined by the neighbor-joining method. Bootstrap values expressed as percentages of 1,000 replications. Scale bar represents 0.1 substitutions per nucleotide position.

Evaluation of Biosurfactant production

To evaluate the effect of optimization of environmental factors on biosurfactant production, following incubation of the isolates (individually) in non-optimized MSM and optimized MSM for one week, surface tension of cell-free MSM was measured by Du Noüy ring method using Krüss-K6 tensiometer (Germany). The reduction in surface tension of MSM to a lower value means biosurfactant production by the bacteria.

Efficacy of crude oil removal

In order to assess the efficacy of the isolates (in the consortium and singly) for oil degradation, amount of the remained oil in the whole medium, including biomass, was determined via Gas Chromatography Mass Spectroscopy (GC/MS) analysis. GC/MS chromatography is widely used for comparing and analysis of crude oil (20, 21). GC/MS analysis was performed using Agilient 7890A GC system, equipped with a 5973N mass selective detector, split/splitless injector and column DB-5MS, 30 m × 0.25 mm × 0.25 µm. The injector was set at constant temperature of 300 °C and programmed incrementally starting from 80 °C to 300 °C with the rate of 3 °C min-1 for oven plus final retention time

of 15 min at 300 °C, and H_2 as the carrier gas at the constant flow of 1.5 ml min^{-1}. Detection conditions were as ionization mode (EI, MS mode): scan 40-500 Da, 5 Hz acquisition rate, MS/MS mode: scan 40-500 Da and CE: 10 eV, source temperature of 280 °C, Quad temperature of 150 °C.

Prior to injection, the following procedure was conducted for the samples: 100 mg of each sample (for control and the inoculated crude oil) was extracted in 10 ml hexane using sonication and centrifugation (12000×g, 10 min). Subsequently, amended with 1mg Linalool (as standard) and then aliquots of the clear supernatant were diluted 10-fold in hexane (final oil concentration: 1 mg/ml), from which 5 µl was injected. Finally, the results were compared with the reference spectra's of NIST & WILEY libraries.

Statistical analysis

All experiments were conducted in triplicate and the values were expressed as means. The means were compared using one-way ANOVA and the Tukey test (in SPSS software, version 19) to indicate any significant difference among parameters and variables. The results were considered significant if $p<0.05$.

Results

Determination of phylogenetic relationship between bacteria

Briefly, 1416 bp (from KA1 isolate) and 1442 bp (from AP isolate) nucleotide DNA fragments of 16S rDNA were amplified and sequenced using specific primers and deposited in NCBI database. The isolates were identified as *Dietzia cinnamea* KA1 (KP141746) and *Dietzia cinnamea* AP (KR232837) by morphological, biochemical and 16S rDNA sequence analysis.

BLAST analyses were performed and a neighbor joining tree of the isolates was constructed along with the closest species as determined by BLAST. Results of phylogenetic analysis indicated that the KA1 and AP isolates clustered separately, while both are closely related to each other and other isolates of *Dietzia cinnamea*. Other species of Dietzia such as *D. maris*, *D. natronolimnaea*, *D. lutea* and *D. papillomatosis* clustered as outliers, further supporting that the KA1 and AP isolates belongs to *Dietzia cinnamea* (Figure 1).

Optimization of crude oil degradation by isolates

In order to optimize oil degradation, the impact of many environmental factors on each bacterial growth and crude oil degradation were tested at different ranges and the optimal condition for each factor was determined separately.

Both bacteria grew and degraded crude oil at a wide range of pH from 6 to 11, but no growth and oil degradation occurred at high acidic (pH5) and high alkaline (pH12) meaning the isolates preferred alkaline to neutral values of pH than acidic (Figure 2A).

In the case of *D. cinnamea* KA1 although there were slight differences between pH7 to 9, statistical analysis showed that the isolate at pH9 had a significant difference for crude oil degradation, so it was selected as optimum pH for oil degradation.

In the case of *D. cinnamea* AP there were no statistically significant differences between pH7 and pH8, but the latter was considered optimum since more value of protein was produced at this pH (Figure 2A).

Both bacteria, except in 1530 mM NaCl at which the logarithmic phase took place on the third day, entered the logarithmic phase on the second day of incubation (Figure 2B). The amounts of produced protein systematically dropped at values higher than 680 mM NaCl along with increase in concentration.

In the case of *D. cinnamea* KA1, although the peak of 510 mM NaCl was slightly higher than that of 340 and 680 mM NaCl, statistical analysis did not indicate any significant difference for growth and crude oil degradation between these concentrations. Highest amount of protein (as criteria for growth and crude oil degradation) was produced at 510 mM NaCl, therefore, this concentration was considered as optimum for *D. cinnamea* KA1. The optimum concentration had a significant difference with 0 and 170 mM NaCl (Figure 2B).

In the case of *D. cinnamea* AP, there was no significant difference for growth and crude oil degradation between 170, 340 and 510 mM NaCl concentrations, but maximum value of protein was achieved at 170 mM NaCl, so, it was considered as optimum (Figure 2B).

We studied the effect of N and P factors in crude oil degradation and aimed at determining the minimum requirement of these elements for growth and crude oil degradation by isolates. For *D. cinnamea* KA1, the best results of crude oil degradations were obtained at values more than 4.65 mM NH_4Cl, but statistical analysis showed no significant differences in growth and crude oil degradation between 4.65 and 5.58 mM NH_4Cl and 6.51 mM NH_4Cl. Therefore, 4.65 mM NH_4Cl was considered as the optimum concentration for *D. cinnamea* KA1 (Figure 2C).

Similar procedure was carried out for *D. cinnamea* AP and concentration of 55.8 mM NH_4Cl was determined as optimum. Here, 65.1 and 55.8 mM NH_4Cl had identical effects on crude oil degradation by *D. cinnamea* AP statistically; but differ from other concentrations of NH_4Cl (Figure 2C).

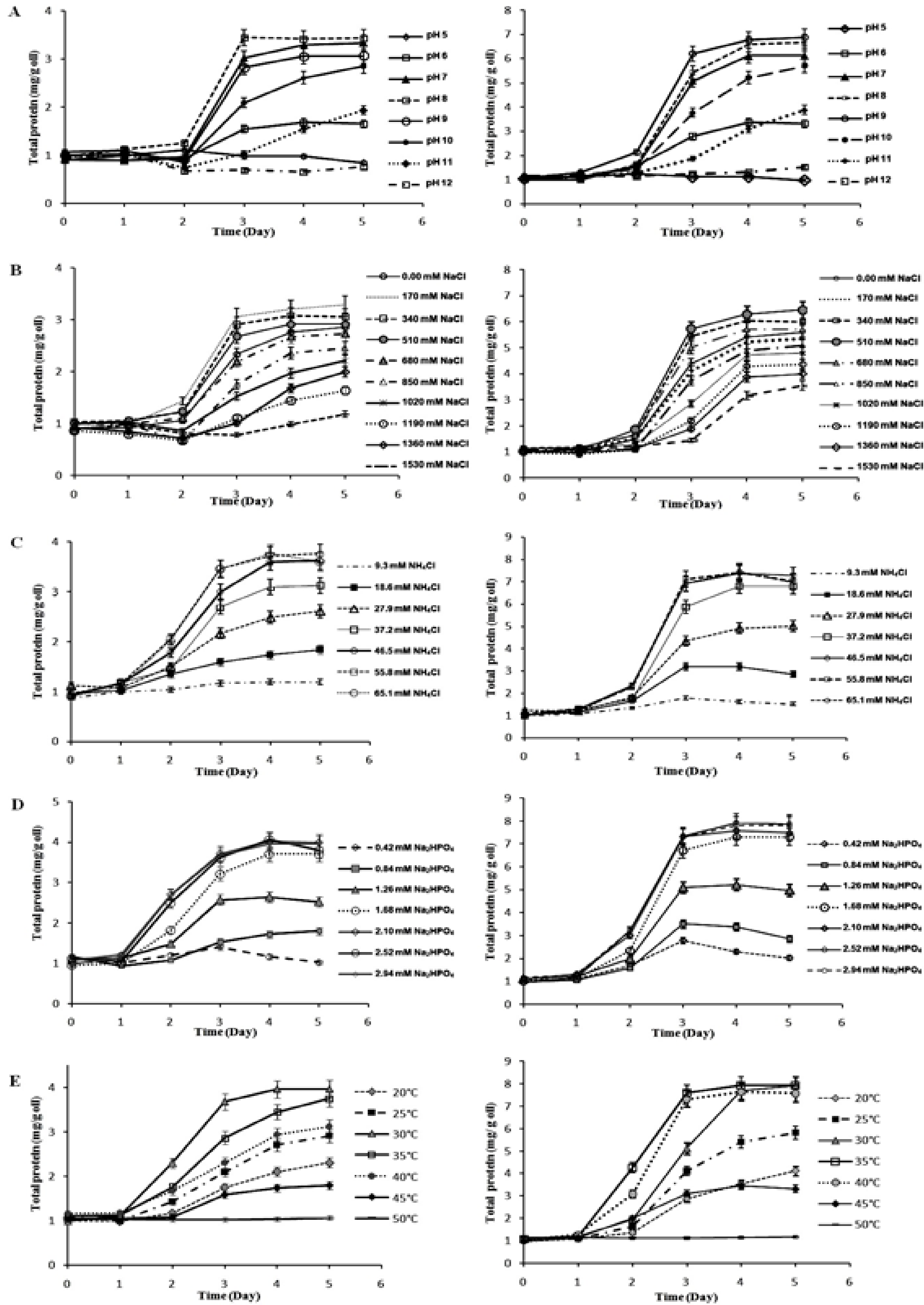

Figure 2. Optimization of crude oil degradation by *D. cinnamea* KA1. The effects of: a) different pH, b) different salt concentrations, c) different NH4Cl concentrations, d) different Na2HPO4 concentrations and e) different temperatures on oil degradation by *D. cinnamea* AP (right) and *D. cinnamea* KA1 (left).

Interestingly, the isolates showed identical paradigm for Na2HPO4 concentrations. The amounts of produced protein were reduced as the concentration of Na2HPO4 decreased (from 1.68 to 0.42 mM Na2HPO4); but the highest amounts of protein were produced at values higher than 2.1 mM. Statistical analysis showed no significant differences for growth and crude oil degradation between 2.1, 2.52 and 2.94 mM Na2HPO4 (Figure 2D) and so, 2.10 mM Na2HPO4 was considered optimal for both of them.
Both bacteria grew and degraded crude oil in the range of 20 to 45 °C, but not at 50 °C. In the case of *D. cinnamea* KA1, entering to logarithmic phase at 35 °C was posterior than 30 and 40 °C. Statistical analysis showed significant difference of 35 °C with other temperatures and therefore this temperature was considered optimal for crude oil degradation (Figure 2E). Selection of 30 °C for *D. cinnamea* AP as optimum temperature was supported by statistical analysis and more production of protein at this temperature.
At the end of environmental factors optimization, compared to *D. cinnamea* AP, the total produced protein by *D. cinnamea* KA1 was almost twice (Figure 2 and table 1).

Table 1. Evaluate of the effect of MSM optimization on total protein and biosurfactant production by isolates.

Strain	*D. cinnamea* KA1		*D. cinnamea* AP	
	Total protein (OD_{650})	Surface tension ($mN\ m^{-1}$)	Total protein (OD_{650})	Surface tension ($mN\ m^{-1}$)
Before optimization	6.86	45	3.43	61
After optimization	7.92	33	3.96	44

Evaluation of Biosurfactant production
As shown in table 1, the surface tension of optimized MSM reduced to lower values than the un-optimized MSM, which means that biosurfactant production was increased. It can be concluded that the rate of biosurfactant production had accompany with optimization of crude oil degradation for isolates. Biosurfactants play numerous roles including increasing of bioavailability of hydrophobic compounds for owner bacteria. Here, biosurfactant production increased by MSM optimization, thus the bioavailability of crude oil for isolates increased therefore the growth values of bacteria have been increased.

Table 2. Evaluating of crude oil consumption by *Dietzia cinnamea* KA1, using GC/MS analysis. (Normal alkanes: NA, Iso and Branched alkanes: IBA, Alkyl Cyclopentanes: ACP, Alkyl Cyclohexanes: ACH, Bicycloparaffins: BCP, Alkylbenzenes: AB, Aromatic cycloparaffins: ACPF, Fluorenes: FN, Binuclear aromatics: BA, Polynuclear aromatics: PNA).

Sample	Paraffin hydrocarbons		Cycloparaffins (Naphtenes)			Aromatics					Total
	Normal	IBA	ACP	ACH	BCP	AB	ACPF	FN	BA	PNA	
Control (%)	16.86	24.68	12.67	5.84	5.97	14.53	3.12	2.30	11.5	2.53	100
***D. cinnamea* KA1 (%)**	0.62	0.46	0.79	0.68	0.91	1.29	0.17	0.43	1.97	0.55	7.87
D. cinnamea AP (%)	2.02	3.15	4.65	3.29	3.76	8.12	1.58	0.90	9.32	1.04	37.83
Consortium (%)	0.00	0.00	0.24	0.19	0.43	0.81	0.00	0.21	0.69	0.17	2.74

Crude oil consumption capability
We applied GC/MS chromatography to compare changes in abundance of fractions in the control oil and the biodegraded oil, using NIST & Wiley library. The results are shown in table 2. The findings demonstrated that the isolates were able to degrade various compounds of crude oil efficiently; including paraffin hydrocarbons (normal, iso and branched alkanes), cycloparaffins (alkyl cyclopentanes, alkyl cyclohexanes and bicycloparaffins), and aromatics (alkylbenzenes, aromatic cycloparaffins, fluorenes, binuclear aromatics and polynuclear aromatics). In general, paraffin hydrocarbons reduced from 41.54% to 1.08, 5.17 and 0.00%; cycloparaffins hydrocarbons from 24.48% to 2.38, 11.70 and 0.86%, and aromatic hydrocarbons from 33.98% to 4.41, 20.96 and 1.88

by *D. cinnamea* KA1, *D. cinnamea* AP and bacterial consortium, respectively. Because the same quantity of controls and inoculated samples were injected for each measurement, the response for each sample was proportional to the amount of degraded and undegraded hydrocarbons. It is noteworthy that any reduction is caused by biodegradation because any loss by nonbiological processes would affect the control sample to the same extent as the inoculated sample. To facilitate the comparison, the results are summarized in Figure 3.

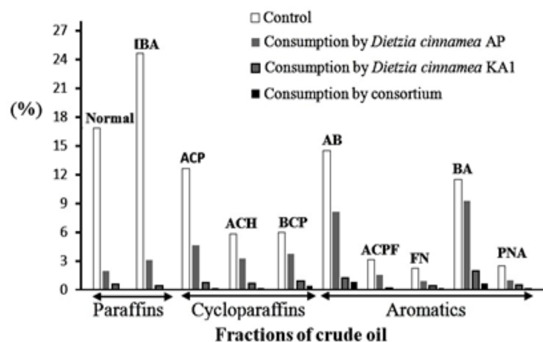

Figure 3. Comparison of crude oil degradation by individually and consortium of isolates based on GC/MS chromatography analysis.

D. cinnamea KA1 was found to be more efficient than *D. cinnamea* AP in degradation of all fractions of crude oil, individually. In consortia, the amounts of degradation of all fractions increased, meaning that there was synergistic relationship between the isolates for crude oil degradation.

Discussion

The threat of oil contamination in the world is growing rapidly due to increasing human activities, which requires the development of appropriate response methods to protect the environment. Microbial bioremediation of hydrocarbon contaminated soil and water has emerged as a promising technology in recent years (22).

The results of morphological and biochemical tests were consistent with16S rDNA gene sequencing. These findings supported that the strains belonged to the genus Dietzia and species cinnamea characterized and named *D. cinnamea* KA1 and *D. cinnamea* AP.

The isolates were able to grow and degrade crude oil at a wide range of pH from 6.0 to 11.0, temperature 20-45 °C and salinity from 0 to 1530 mM NaCl. Considering the wide range of tolerations, both isolates, especially *D. cinnamea* KA1which showed more ability to crude oil degradation can be used for treatment of polluted environments having variable conditions. Treatment of such environments using biochemical custom methods could be difficult and expensive (23).

Bioavailable nitrogen and phosphorus elements are limiting in the case of large oil spills. To overcome this limitation careful application of fertilizers to accelerate bioremediation could be helpful (24). In this study, bacterial isolates showed different nitrogen requirement: *D. cinnamea* KA1 and *D. cinnamea* AP required minimal amounts of 4.67 mM NH4Cl and 55.8 mM NH4Cl, respectively. But in the case of phosphorus, both bacterial minimal requirements were the same (2.10 mM NaH2PO4).

Consortium of various bacterial genera leads to increase in gene diversity, which results in increase in the ability of consortium to consume more different compounds. Microbial populations that consist of strains that belong to various genera have been used in hydrocarbon-contaminated soil treatment (25), but here we used consortium of two bacteria which belong to one genus and even one species. Members of a species are expected to have the same abilities in consumption of various compounds. Since, here we used a consortium comprised of bacteria belonging to one genera and even one species; and because of increase in crude biodegradation than the individual bacteria, it was possible that our isolates may had different plasmids or even different chromosomal genes involving in crude oil degradation.

Wang et al. (5) reported a novel Dietzia strain, DQ12-45-1b, which was capable of using a broad range crude oil components, but the strain needed a long period of time (8-34 days). According to other studies (12-14), consortium of *D. cinnamea* KA1 and *D. cinnamea* AP had a suitable efficiency to degrading a broad range of compounds contained in crude oil as sole carbon and energy source (97.26%) in a shorter period (5 days). Furthermore, compared to other Dietzia species, consortium of *D. cinnamea* KA1 and *D. cinnamea* AP could use a broader range crude oil components as the sole carbon sources, including saturated fraction, resins, and aromatic hydrocarbon in a shorter period.

It is notable that aromatic compound existing crude oil (such as Naphthalene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, and Chrysene), are poisonous and carcinogenic. Thus, the ability of isolates in degradation of these dangerous materials and converting them to microbial biomass could be helpful for environmental health.

Conclusion

Despite isolation of isolates from a single environment and although they belonged to one species, the amounts of crude oil degradation by bacteria showed significant differences. This difference may be due to having different plasmids or even having different chromosomal genes.

Environmental parameters on the other hand, affected the growth rate, biosurfactant production and crude oil degradation values of isolates; so the growth rate of isolates (which is equivalent to the amount of crude oil degradation) increased gradually after optimization of any environmental factors. It must be considered that environmental factors optimization could not decrease egregious differences between growth rates of isolates. Comparing to genetically effects on crude oil degradation, the effects of environmental factors were insignificant, meaning that genetic factors play major role in crude oil degradation.

Because of synergistic relationship between *D. cinnamea* KA1 and *D. cinnamea* AP isolates, the values of degradation for various fractions of crude oil increased when the isolates were used in consortium. Extraordinary ability of this consortium to crude oil degradation, suggested an efficient candidate for bioremediation applications in various environmental conditions.

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Conflict of interest

The authors declare that they have no conflict of interest in this work.

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