Research Paper

Optimization of crude oil degradation by *Dietzia cinnamea* KA1, capable of biosurfactant production

Amirarsalan Kavynifard¹, Gholamhossein Ebrahimipour¹ and Alireza Ghasempour²

¹ Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran
² Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran

The aim of this study was isolation and characterization of a crude oil degrader and biosurfactant-producing bacterium, along with optimization of conditions for crude oil degradation. Among 11 isolates, 5 were able to emulsify crude oil in Minimal Salt Medium (MSM) among which one isolate, named KA1, showed the highest potency for growth rate and biodegradation. The isolate was identified as *Dietzia cinnamea* KA1 using morphological and biochemical characteristics and 16S rRNA gene sequencing. The optimal conditions were 510 mM NaCl, pH 9.0, 35 °C, and minimal requirement of 46.5 mM NH₄Cl and 2.10 mM NaH₂PO₄. Gravimetric test and Gas chromatography–Mass spectroscopy technique (GC-MS) showed that *Dietzia cinnamea* KA1 was able to utilize and degrade 95.7% of the crude oil after 5 days, under the optimal conditions. The isolate was able to grow and produce biosurfactant when cultured in MSM supplemented with crude oil, glycerol or whey as the sole carbon sources, but bacterial growth was occurred using molasses with no biosurfactant production. This is the first report of biosurfactant production by *D. cinnamea* using crude oil, glycerol and whey and the first study to report a species of *Dietzia* degrading a wide range of hydrocarbons in a short time.

Abbreviations: GC – gas chromatography; MSM – minimal salt medium; MS – mass spectroscopy technique

Keywords: Biosurfactant / *Dietzia cinnamea* KA1 / Hydrocarbon / GC-MS / Gravimetric test

Received: June 20, 2015; accepted: November 11, 2015

DOI 10.1002/jobm.201500386

Introduction

The genus *Dietzia* was first proposed by Rainey et al. [1] to accommodate an actinomycete previously classified as *Rhodococcus maris* [2]. Since then, the members of the genus *Dietzia* (order *Actinomycetales*, suborder *Corynebacterineae*, family *Dietziaceae*) have been isolated from widely different environments with various functions. Several strains are potential human pathogens [3, 4] but others have found their ways for industrial applications due to their diverse metabolic activities [4, 5].

The emerging issues caused by oil exploration, industrialization and urbanization have urged environmental remediation using the indigenous biological agents. Among a variety of microorganisms suggested as biological oil and oil-related pollution scavengers, some species belonging to the genus *Dietzia* have been shown to be potential for bioremediation. They have been reported as alkane degraders [6–10], but more importantly, their ability to degrade recalcitrant materials such as polycyclic aromatic compounds, including naphthalene, phenanthrene, benzoate, fluoranthene signifies its importance. In addition, it has been reported that they act as contributory factors to elimination of oil-related contaminations by other hydrocarbon-degrading organisms [11, 12, 5].

Biosurfactants are surface-active compounds synthesized by a wide range of microorganisms [13]. 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 [5]. This helps bacteria attach oil droplets and also helps other microorganisms use hydrocarbons as the source of carbon and energy. Recently, it was reported that two isolates of *Dietzia* could use alkanes and produce different kinds of biosurfactants [8]. *Dietzia cercidiphylli* Strain 3372 was isolated by
Chen et al. [7] which was capable of biosurfactant production using paraffin as the sole carbon source. *D. maris* WR-3 has been reported to produce wax ester-like compounds as biosurfactants [14]. Members of this genus as well as other genera with biosurfactant-producing and oil-degrading characteristics are of great interest. Further studies are needed to determine the optimal conditions if we intend to take advantage of these bacteria to accelerate bioremediation [15].

Since most Dietzia isolates utilize hydrocarbons and have long-term viability in the environment even under dry and resource-limited conditions [16], they would be salutary in clean-up and bioremediation of oil pollutants [5]. The aim of this study was isolation and salutary in clean-up and bioremediation of oil pollution. These bacteria to accelerate bioremediation [15].

Optimal identifications [5]. The aim of this study was isolation and salutary in clean-up and bioremediation of oil pollution. These bacteria to accelerate bioremediation [15].

**Materials and methods**

**Sampling**

Samples including water and sediments were collected from oil polluted seasonal ponds of Khami located at northeast of Ahvaz, Iran. The sampling site had been naturally exposed to oil pollutions because of natural oil seepage over decades. [17]. Temperature, salt concentration, and pH of the sampling site were 40 °C, 513 mM and 7, respectively. The samples aerated every 20 min and then, left unshaken to settle down. Thereafter, 5 ml supernatant from each flask was transferred into 100 ml sterilized pond water supplemented with 1% (v/v) crude oil (obtained from National Iranian Oil Company), and incubated for further 48 h at 40 °C with orbital shaking of 95 rpm, and subcultures were performed three times. At the end of the enrichment period, 100 μl of serial dilutions (10^-1–10^-8) from the media were cultivated on PYAM plates and incubated at 40 °C. After 14 days, colonies were purified and their ability to degrade crude oil and their growth was assessed by incubating at 40 °C while shaking at 95 rpm in Mineral Salts Medium (MSM) containing 1% (v/v) crude oil. As control, a non-inoculated flask was incubated in the same conditions. MSM was a modified medium of Schlegel, H. G. (18), composed of 3.65 mM NH₄Cl, 0.169 mM Na₂HPO₄, 16.2 mM MgSO₄·7H₂O, 0.67 mM KCl, 0.18 mM CaCl₂, 51 mM NaCl, 0.065 mM FeSO₄ and 1 ml of trace element solution. Trace element solution was: 0.147 mM ZnCl₂, 1.06 mM NiCl₂, 1.54 mM CoCl₂, 0.138 mM Na₂SeO₄, 0.242 mM Na₂MoO₄, 0.118 mM Na₂WO₄, 0.082 mM NaVO₃, and 7 mM HCl. pH of the mineral medium was adjusted to 8.0 by adding 0.1 M Tris/HCl. Based on the rate and efficiency of emulsification and oil consumption, the isolates were screened and the most potent isolate, named KA1, was selected for the following studies.

**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 L of the modified Schlegel’s medium. Trace element solution was: 0.147 mM ZnCl₂, 0.77 mM NiCl₂, 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 L 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 hydroxymethyl 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 (Islambad sugar factory, Iran) and Crude oil (National Iranian Oil).

**Bacterial characterization**

Phylogenetic analysis of 16S rRNA was performed for characterization of the KA1 isolate, as follow: genomic DNA was extracted by phenol/chloroform/isoamyl alcohol according to Sambrook et al. [19] method. 16S rDNA gene was amplified by polymerase chain reaction (PCR) with a pair of universal primers [20], 8F (5'-AGAGTTTGATCCTGCGCTGAG-3') and 1525R (5'-AAGGAGGTGATCCTGCC-3'). The PCR amplification mixture of 16 μl, contained 0.5 μl of Taq DNA polymerase, 5 μl of Taq buffer (TAPS, pH 8.8, 3 mM MgCl₂, 50 mM KCl), 0.2 mM dNTP mix and 3.1 μM of each primer. 200 ng of the bacterial DNA were added to the reaction. Amplification was performed by using the following conditions: 94 °C for 3 min, followed by 30 cycles; each consisted of a denaturation at 94 °C for 10 s, an annealing at 50 °C for 10 s and an extension step at 72 °C for 4 min, followed by a final extension step at 72 °C for 5 min. Subsequently, PCR products were electrophoresed on a 1% agarose gel.
and visualized under UV light after staining with ethidium bromide. DNA fragments were sequenced by a sequencer (SEQLAB, Germany). Phylogenetic analysis was carried out using 16S rDNA sequence alignment by Blast software with closest species deposited in NCBI GenBank. A rooted phylogenetic tree was constructed using neighbor-joining method with bootstrap value of 1000 replications (MEGA, version 4). Finally, for accurate characterization of the KA1 isolate, morphological observation and biochemical tests were performed according to the experiments described in “Bergey’s Manual of Determinative Bacteriology” [21].

**Optimization of oil degradation**

To optimize crude oil degradation, environmental parameters such as salt concentration, pH and temperature 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 1 ml crude oil was assessed. After optimization of each parameter, the optimized value was considered fixed for optimization of other parameters. 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 the bacterial culture with turbidity equivalent to 0.5 McFarland (1.5 × 10^6 cell/ml). The control flasks remained un-inoculated.

In order to optimize salt concentrations, mineral medium with concentrations of 0–1530 mM NaCl (with 170 mM intervals) were prepared while other parameters were as: 36 mM NH₄Cl, pH 8, 1.69 mM Na₂HPO₄ and 40 °C.

In the case of pH, mineral medium with pH ranging from 5 to 12 and 510 mM NaCl, 36 mM NH₄Cl, 1.69 mM Na₂HPO₄, and 40 °C was prepared.

To analyze the bacterial requirement for N, mineral medium was supplemented with 9.3–65.1 mM NH₄Cl (with 9.3 mM interval), while other parameters were as 510 mM NaCl, pH 9, 1.69 mM Na₂HPO₄, and 40 °C.

Similarly, Na₂HPO₄ in the range of 0.42–2.94 mM (with 0.42 mM interval) was applied to determine the bacterial requirement for P, while other parameters were as 510 mM NaCl, pH 9, 46.5 mM NH₄Cl, and 40 °C.

In order to optimize the temperature, mineral media were incubated at the range of 25–45 °C (with 5 °C intervals) and other parameters were as 510 mM NaCl, pH 9, 46.5 mM NH₄Cl, and 2.10 mM Na₂HPO₄.

The flasks were incubated for 5 days with orbital shaking at 95 rpm.

Since the total produced protein or bacterial growth is a result of crude oil consumption (crude oil was the sole source of carbon and energy), we concluded that protein production corresponds with crude oil degradation. Thus, for optimization of crude oil degradation, we measured and compared total produced protein values under various environmental parameters. Total produced protein was measured according to the method described by Lowry et al. [22]. The experiments were conducted in triplicates.

**Protein measurement**

For estimating growth based on total protein production, at 24 h intervals, bacteria were harvested by centrifugation of 1 ml of the inoculated MSM at 8000 g for 10 min. The pellet was then suspended in 1 ml of Ringer’s solution (111.2 mM NaCl, 5.7 mM KCl, 2.25 mM CaCl₂, and 2.4 mM NaHCO₃), vortexed and centrifuged (8000 g for 10 min) again. For cell lysis, the pellet was re-suspended in 1 ml of distillated water and 0.5 ml of 0.3 M NaOH and then vortexed. The suspension was incubated at 90 °C for 60 min. This cell lysate was used for protein staining that was done by Lowry method [22]. Briefly, Lowry’s solution was prepared by mixing solution A (4 mg/ml NaOH and 20 mg/ml Na₂CO₃) and solution B (10 mg/ml sodium taurate and 5 mg/ml CuSO₄). Folin’s reagent was prepared, as follow: 1 volume of Folin’s Ciocalteus Phenol reagent in 2 volume of distilled water.

Five milliliter of Lowry’s solution was added to each glass tube containing cell lysis and a blank glass tube (containing 1 ml of distilled water and 0.5 ml of 0.3 M NaOH) while stirring. After incubation in the dark for 20 min, 0.5 ml of Folin’s reagent was added to each tube and mixed immediately and incubated in the dark for 30 min. Tubes were then centrifuged at 8000 g for 10 min. Absorbance of the supernatant was measured at 623 nm (Shimadzu, UV-120-02; Japan). The results were checked against the standard curve of serial dilutions of 0, 10, 50, 150, and 200 μg/l bovin serum albumin (BSA).

**Biosurfactant production**

Following incubation in optimal conditions for 1 week, the production of biosurfactant was investigated via oil spreading test according to the method described by Youssef et al. [23]. More ever, emulsification index (EI) was measured as follow: 2 ml of the culture medium was centrifuged (8000 g for 10 min); 2 ml of kerosene (2 ml) was mixed with the supernatant by vortexing for 1 min. After maintaining at room temperature for 24 h, emulsification activity was calculated with by measuring emulsified layer [24]. Emulsification activity was determined using the following formula:

\[ EI = \frac{\text{height of emulsion}}{\text{height of total solution}} \times 100\% \]

**Efficacy of crude oil removal**

In order to assess the efficacy of the isolate for oil degradation, amount of the remaining oil in the whole
medium, including biomass, was determined via preliminary gravimetric test followed by gas chromatography coupled with mass spectroscopy (GC/MS) analysis.

Gravimetric determination of crude oil fractions was performed as described by Thouand et al. [25]. A full biodegraded flask (containing 100 ml MSM under optimized parameters) was extracted, three times with chloroform (3 × 20 ml). The organic fraction was dried with sodium sulfate, filtered, and concentrated. To avoid any loss of the light fractions, all solvent evaporation steps were done under vacuum not exceeding 40 °C. The obtained organic fraction was fractionated with n-hexane into two main fractions: (I) a hexane-soluble fraction and (II) a hexane-insoluble fraction. Fraction I and II were separated by filtration through a Millipore SC 8-mm filter, followed by rinsing with hexane. After drying, fraction II was weighed and considered as asphaltene. Fraction I was dissolved in 200 ml of chloroform and 7 g of silica gel (Geduran Si 60, Merck). After stirring for 15 min, chloroform was distilled off, leaving a dry silica gel and the absorbed oil. The silica gel was used to make a column (0.5-cm diameter), which was successively eluted with three 20-ml fractions of cyclohexane, benzene, and methanol. The three fractions were weighed after evaporation of the solvent and considered as saturated hydrocarbons, aromatic hydrocarbons, and resins, respectively. To gain accurate results, three flasks were used and mean of each value was reported. To compare biodegraded fractions with fractions of intact crude oil, same procedure repeated with three control flasks (100 ml of MSM containing 1 ml crude oil). Remaining value of each fraction was calculated by deduction of biodegraded fraction from the equivalent control fraction.

We took advantage of GC/MS Chromatography as it is widely used for comparing and analysis of crude oil [26, 27]. 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 μm. The injector was set at constant temperature of 300 °C and programmed incrementally, starting from 80 to 300 °C with the rate of 3 °C per min⁻¹ for oven plus final retention time of 15 min at 300 °C, and H₂ 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 1 mg 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. Disappearance of the corresponding peaks of the spectra for experimental group as compared to those for control group denotes the efficacy of the isolate for degradation capability in diversity.

**KA1 growth and biosurfactant production with urea**

To evaluating of KA1 isolate for growth and biosurfactant production in the presence of urea as the sole nitrogen source, MSM was supplemented with 0.66 g/L urea (equivalent to 46.5 mM NH₄Cl). Other parameters were as 1% (v/v) crude oil, 510 mM NaCl, pH 9, 2.10 mM Na₂HPO₄ and 35 °C.

**KA1 growth and biosurfactant production on different carbon sources**

For this purpose, molasses (10 g/L) and glycerol (10 g/L) were used separately as the sole carbon sources in MSM. Other parameters were as 510 mM NaCl, pH 9, 46.5 mM NH₄Cl, 2.10 mM Na₂HPO₄ and 35 °C. In the case of whey, the growth and biosurfactant production of the isolate was investigated without any supplementation of carbon, nitrogen and phosphorus. Other parameters were as 510 mM NaCl and 35 °C.

**Statistical analysis**

All experiments were conducted in triplicate and the values were expressed as means. The means were compared using Kolmogorov–Smirnov test, Kruskal–Wallis one-way analysis of variance, U Mann–Whitney Test, or by one-way ANOVA and the Tukey test (SPSS software, version 19) to indicate any significant differences among the parameters and variables. Results were considered significant if p < 0.05.

**Results**

**KA1 isolate as the most efficient oil degrader**

Using enrichment technique, 11 isolates (named KA1 to KA11) were isolated on PYAM plates. Five isolates (KA1, KA4, KA5, KA8, and KA11) were able to grow on crude oil while degrading it, among which one single superior isolate, KA1, with the highest growth rate (based on the total protein produced after 5 days) and biodegradation ability was chosen for further investigation (Table 1). The KA1 isolate was able to emulsify crude oil in MSM faster than the other isolates (after 2 days of incubation) and
almost completely consumed crude oil within 5 days and rendered the medium clear.

Following phylogenetic analysis of 16S rRNA, complementary morphological and biochemical tests, were performed for classification of the KA1 isolate. Briefly, a 1416 bp nucleotide DNA fragment of 16S rRNA was amplified from the KA1 isolate and sequenced using specific primers and deposited in NCBI database (GenBank accession number: KP141746). A BLAST analysis was performed and a neighbor joining tree of the isolate was constructed (Fig. 1) along with the closest species as determined by BLAST. Results of our phylogenetic analysis indicated that the KA1 isolate clustered with Dietzia sp. 158 × A1, with 98.81% nucleotide identity. In addition, the KA1 isolate was also found closely related to other isolates of Dietzia cinnamea with 94–98.5% nucleotide identity. Other species of Dietzia such as Dietzia lutea and Dietzia maris clustered as outliers with 97.37 and 91.26% nucleotide identity, respectively, further supporting that the KA1 isolate belongs to Dietzia cinnamea. Other species of Dietzia were found more distant with very low nucleotide identity and therefore were not included in the phylogenetic tree. Results of morphological and biochemical analyses of KA1 are listed in Table 2. Based on results of the phylogenetic analysis and complementary morphological and biochemical tests; the strain was named as Dietzia cinnamea KA1.

### Optimization of crude oil degradation

In order to achieve the maximal oil degradation, environmental conditions including salt concentrations, pH, temperature, nitrogen and phosphate concentrations were tested at different ranges and the optimal condition for each factor was determined separately.

Growth on crude oil and its degradation as the only source of carbon took place by D. cinnamea KA1 in a wide range of NaCl concentrations, however, the values varied considerably (Fig. 2a). Except for the concentration of 1153 mM NaCl at which the logarithmic phase took place on the third day, the bacterium entered the logarithmic phase on the second day of incubation. The amounts of produced protein systematically dropped at values higher than 850 mM NaCl along with increasing in concentration. 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 total produced protein (and therefore crude oil degradation) between these concentrations. Since the highest amount of protein was produced at 510 mM NaCl, this concentration was considered optimal for NaCl. The optimal concentration had a significant difference with 0 and 170 mM NaCl. As Gilmour [28] and Margesin et al. [29] demonstrated, halophiles are organisms that live in highly saline environments, and require the salinity to survive, while halotolerant organisms can grow under saline conditions, but do not require elevated concentrations of salt for growth. Since the isolate was able to grow in the absence or presence of salt (the range of 0–12.5%, Table 2), it is considered as halotolerant bacterium.

When the KA1 isolate was tested for growth at different ranges of pH, it grew and degraded crude oil at a wide range of pH from 6 to 11, however, entering the logarithmic phase at values of 6 and 11 occurred with a more gentle slope and amounts of the produced protein were less (Fig. 2b). Although there were slight differences between pH 7 to 9, statistical analysis showed that at pH 9 the KA1 isolate had a significant difference for crude oil degradation and therefore this pH was selected as the optimal pH for oil degradation. By contrast, there was no significant difference between pH7 and pH8 or between pH6 and pH11 (Fig. 2b). No growth and oil degradation occurred when the pH was highly acidic or alkaline, pH 5 and pH 12, respectively. In general, the isolate preferred alkaline to neutral values of pH than acidic.

In order to optimize NH₄Cl concentration, results showed that amount of produced protein (or growth) dropped down (Fig. 3a) as NH₄Cl concentration decreased (from 37.2 to 9.3 mM NH₄Cl). This reduction was because of the nitrogen required for protein production. Best results were obtained at values more than 46.5 mM NH₄Cl. Concentrations of 46.5, 55.8, and 65.1 mM NH₄Cl had no significant difference, statistically. Since our goal was to determine the minimal required NH₄Cl for growth and crude oil degradation, the amount of 46.5 mM NH₄Cl was considered the optimal concentration.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>KA1</th>
<th>KA2</th>
<th>KA3</th>
<th>KA4</th>
<th>KA5</th>
<th>KA6</th>
<th>KA7</th>
<th>KA8</th>
<th>KA9</th>
<th>KA10</th>
<th>KA11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsifying crude oil ability</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Starting day of emulsification</td>
<td>2</td>
<td>−</td>
<td>−</td>
<td>4</td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>4</td>
</tr>
<tr>
<td>Total protein (mg/ml oil)</td>
<td>6.4ᵃ</td>
<td>0</td>
<td>0</td>
<td>1.32ᵇ</td>
<td>2.73ᵇ</td>
<td>0</td>
<td>0</td>
<td>3.21ᵇ</td>
<td>0</td>
<td>0</td>
<td>1.55ᵇ</td>
</tr>
</tbody>
</table>

ᵃ: Showed significant difference statistically with all other isolates (using U Mann–Whitney test).
ᵇ: Showed no significant difference statistically with each other (using U Mann–Whitney test).

---

Table 1. Results of screening for crude oil degrader bacteria.
In the case of Na$_2$HPO$_4$, similarly to NH$_4$Cl, the amounts of produced protein (or growth) were reduced as Na$_2$HPO$_4$ concentration decreased (from 1.68 to 0.42 mM Na$_2$HPO$_4$). By contrast, the highest amounts of protein were produced at concentrations higher than 2.1 mM. At 2.1, 2.52, and 2.95 mM Na$_2$HPO$_4$ there was no significant differences in the amount of the produced protein (Fig. 3b). Similarly to NH$_4$Cl, we aimed to determine the minimal concentration of Na$_2$HPO$_4$ and therefore 2.1 mM Na$_2$HPO$_4$ was considered optimal.

For optimization of temperature, the amounts of produced protein were monitored at temperatures ranging from 20 to 50 °C (with 5 °C intervals). As shown in Fig. 3c, D. cinnamena KA1 grew and degraded crude oil in the range of 20 to 45 °C, but not at 50 °C. Not only were the amounts of the produced protein at 20, 25, and 45 °C lower than those of 30, 35, and 40 °C, but also entering the logarithmic phase was delayed. At 35 °C, entering the logarithmic phase was posterior than 30 and 40 °C. The amounts of produced protein and slope of the logarithmic phase at 35 °C were the highest and statistical analysis also supported the observation. But statistical analysis showed no significant differences between temperatures of 35 °C and 40 °C (Fig. 3c). Because of production of more protein at 35 °C, this temperature was considered optimal for crude oil degradation.

**Crude oil gravimetric test analysis**

Gravimetric test analysis indicated that the saturated fraction of crude oil decreased from starting concentration 73.27% to remaining 0.40%, the aromatic fraction decreased from starting concentration 22.05% to remaining 0.85%, and the resin fraction decreased from starting concentration 2.17% to remaining 0.40% (Table 3). These reductions suggest biodegradation of these fractions by the bacterium. Statistical analyses affirmed these reductions, too. Despite the, slightly increased asphaltene fraction from starting concentration 2.50% to remaining concentration 2.65%, statistical analyses didn’t approve the increase. In general, the isolated strain used in this study degraded 95.70% of crude oil.

**GC/MS Chromatography Analysis**

We applied GC/MS Chromatography to compare changes in abundance of compounds in the control oil and the biodegraded oil, using NIST & Wiley Library. The biodegraded oil lost more hydrocarbons compared to the un-inoculated oil. Because the same quantity of control and inoculated samples were injected for each chromatogram, the response for each sample was proportional to the amount of degraded and un-degraded hydrocarbons. Thus, a decrease in peaks from inoculated sample to control reflected a reduction in the GC/MS-detectable hydrocarbons. The results demonstrated that the KA1 isolate efficiently degraded hydrocarbons with 10–22 carbons in their molecule, C$_{10}$–C$_{22}$ molecules, but it was found less efficient for degradation of shorter (C$_{8}$–C$_{9}$) and longer molecules (C$_{23}$–C$_{27}$). Conversely, in the case of C$_{26}$–C$_{27}$, it was observed that the residual of fractions were increased after incubation (Fig. 4).
KA-1 growth and biosurfactant production with urea as the sole nitrogen source
As shown in Table 4, the isolate was able to grow and degrade crude oil in the presence of urea as the sole nitrogen source and the growth value (by OD$_{650}$) was slightly more than that of NH$_4$Cl (0.85 for urea and 0.82 for NH$_4$Cl). Biosurfactant production also occurred in the presence of urea, (33.06% EI). Considering urea as an inexpensive substrate, this feature is an excellent ability for industrial applications.

Figure 2. The effects of different salt concentrations (a) and different pH (b) on total produced protein by $D$. cinnamea KA1, as criteria for crude oil degradation. The constant parameters were 36 mM NH$_4$Cl, pH 8, 1.69 mM Na$_2$HPO$_4$ and 40 °C for (a) and 510 mM NaCl, 36 mM NH$_4$Cl, 1.69 mM Na$_2$HPO$_4$, and 40 °C for (b).

Figure 3. The effects of different NH$_4$Cl concentrations (a), different Na$_2$HPO$_4$ concentrations (b) and different temperatures (c) on total produced protein by $D$. cinnamea KA1, as criteria for crude oil degradation. The constant parameters were 510 mM NaCl, pH 9, 1.69 mM Na$_2$HPO$_4$ and 40 °C for (a), 510 mM NaCl, pH 9, 46.5 mM NH$_4$Cl and 40 °C for (b) and 510 mM NaCl, pH 9, 46.5 mM NH$_4$Cl and 2.10 mM Na$_2$HPO$_4$ for (c).
KA-1 growth and biosurfactant production on different carbon sources

As shown in Table 4, KA1 isolate was able to grow with molasses as the sole carbon source; but biosurfactant production was not established (EI was only 1.87%). Although growth took place, it was less than the other carbon sources (OD$_{650}$ of 0.53 for molasses while 0.82 and 0.91 for crude oil glycerol, respectively). By contrast, the maximal growth value of the isolate was observed in whey medium (0.96) and biosurfactant production was positive with EI of 30.62%. Finally, in the case of glycerol medium, the isolate was able to grow (OD$_{650}$ of 0.91) and produce biosurfactant with EI of 38.55.

Discussion

Hydrocarbons are the major components of crude oil of which the clean-up of polluted environments is of great importance [30]. Microbial degradation appears to be the most environmental-friendly method for removal of oil pollutant since other methods such as surfactant washing

Table 3. Results of gravimetric analysis test.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Saturated$^a$</th>
<th>Aromatic$^a$</th>
<th>Resin$^a$</th>
<th>Asphaltene</th>
<th>Total$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact crude oil (%)</td>
<td>73.28</td>
<td>22.05</td>
<td>2.17</td>
<td>2.50</td>
<td>100</td>
</tr>
<tr>
<td>Biodegraded crude oil (%)</td>
<td>0.40</td>
<td>0.85</td>
<td>0.40</td>
<td>2.65</td>
<td>4.30</td>
</tr>
</tbody>
</table>

$^a$Intact crude oil and biodegraded crude oil showed significant difference statistically (using U Mann–Whitney test).

Figure 4. GC/MS chromatogram indicating crude oil biodegradation by D. cinnamenea KA1. Control crude oil (above) and biodegraded (below) crude oil.
and incineration lead to introduction of more toxic compounds to the environment.

In this study, we isolated a halotolerant bacterium characterized and named *D. cinnamea* KA1 that had biosurfactant production and oil degradation abilities. Comparing with previous studies [31, 32], only one difference was found between our isolate and other members of *Dietzia* species: our isolate was able to utilize glycerol as the sole carbon and energy source, exceptionally. The results of morphological and biochemical tests (Table 2), were accompany to 16S rRNA gene sequencing. These finding supported that the strain belonged to the genus *Dietzia* and characterized and named *D. cinnamea* KA1. It was notable that the growth rate of isolate and the produced protein (which is equivalent to the amount of crude oil degradation) were increased gradually after optimization of any of environmental parameters. Our results showed that conditions for the optimal growth of the *D. cinnamea* KA1 isolate (including salt concentration, pH and temperature) were close or similar to the environmental parameters of sampling site. It is expectable for the KA1 isolate to have optimal growth and crude oil degrading activity in conditions in which it evolved to grow.

Other studies have reported efficiency of biodegradation ranging from 6 to 97.2% by various microorganisms including soil fungi, soil bacteria and marine bacteria [33, 34]. Wang et al. [6] reported a novel *Dietzia* strain, DQ12-45-1b, which was capable of using a broad range of crude oil components, but the strain needed a long period of time (8–34 days). Regarding the results, *D. cinnamea* KA1 indicated a high efficiency for degradation of crude oil (95.70%) in a shorter period (5 days). Furthermore, compared to other *Dietzia* species, *D. cinnamea* KA1 could use a broader range crude oil components as the sole carbon sources, including saturated fraction, resins and aromatic hydrocarbon in a shorter period (5 days).

Not only *D. cinnamea* KA1 was able to grow with crude oil and glycerol as well as in whey medium, but also produced biosurfactant. Only a few species of *Dietzia* such as *D. maris* WR-3 [14] and *Dietzia* sp. CBMAI 705 [35] have been reported to produce biosurfactants using hydrocarbons. But for *D. cinnamea* species, there is no report of biosurfactant production using hydrocarbons and this is the first study reporting biosurfactant production using crude oil, whey and glycerol as carbon sources by *D. cinnamea* KA1.

The ability of biosurfactant production with inexpensive sources of carbon and the highly efficient for degradation of various hydrocarbon components found in crude oil, the ability of proliferation with inexpensive sources of carbon and nitrogen, and the wide range of salinity, pH and temperature tolerance; suggest *D. cinnamea* KA1 as a novel candidate for bioremediation applications in various environmental conditions, especially for biodegradation in alkaline soils.

### References


