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Optimization of Phenanthrene-Degradation by *Dietzia cinnamea* AP for Bioremediation Applications

Zahra Fathi [1], Golamhossein Ebrahimipour [1], Amirarsalan Kavyanifard [2*], Ziba Najmi [1]

[1] Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran.
[2] Department of Science, Payam Noor University (PNU), P.O. Box, 19395-3697, Tehran, Iran.

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Corresponding Author:
Amirarsalan Kavynifard
Department of Science, Payam Noor University (PNU), Tehran, Iran.
Phone: +98 212 243 1664
E-mail: kavyanifard@gmail.com

Abstract

Background: This study focused on isolation and identification of a Phenanthrene (Phe) degrader bacterium and optimization of environmental conditions for Phe degradation.

Materials and Methods: Enrichment technique was used for isolation and the most effective isolate; named AP was selected based on its Phe biodegradation abilities. The isolate was identified using morphological and biochemical tests as well as16S rDNA sequencing. The effects of various factors such as temperature, pH and C/N on bacterial growth and Phe degradation were investigated using protein assay (Bradford) and Gas Chromatography (GC), respectively.

Results: The selected isolate was identified as *Dietzia cinnamea* AP. It was able to degrade Phe at pH 6-10 (optimum at 8), temperatures of 25 -45 °C (optimum at 35 °C) and NH4Cl concentrations of 0.5-2.5 gL-1 (optimum at 2 g L-1). By optimization of environmental parameters, within 10 days of fermentation, Phe degradation rate increased by more than 1.2 fold (from 60% to 73%).

Conclusion: *D. cinnamea* AP was found to be an appropriate candidate for bioremediation applications. To the best of our knowledge, this is the first report of *D. cinnamea* species that can degrade Phe.

Keywords: Biodegradation, Phenanthrene, *Dietzia cinnamea* AP, Gas Chromatography

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Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are organic compounds containing two or more benzene rings. They are hazardous environmental contaminants that have noticeable biological effects, mutagenicity, carcinogenicity and toxicity (1). Phenanthrene (Phe) is a tri-cyclic aromatic hydrocarbon that is found in high concentrations in PAH contaminated surface soils, sediments and waste sites. Due to its potential severe hazards, Phe is of significant environmental concerns (2). Although Phe may undnnergo volatilization, photolysis, adsorption and chemical degradation but microorganisms play an effective role in its degradation in various ecosystems and microbial biodegradation is the main process in natural bioremediation (3). There are many bacterial species capable of degrading Phe and most of them are isolated from contaminated sediment or soils (4-6). It was found that microorganisms isolated from hydrocarbon contaminated soils are as active as or even more active than those isolated from uncontaminated soils (1). Phe biodegradation varies with the type and number of the microorganisms and the rate of biodegradation depends on pH, temperature, accessibility of nutrients and chemical partitioning in growth medium (7, 8).

Natural biodegradation of hydrocarbons in polluted environments is extremely slow, therefore, for more efficient and rapid elimination of hydrocarbon pollutants, bioaugmentation strategy (adding bioscavengers to the polluted sites) can be applied (9). So, isolation of new bioscavengers and optimization of environmental condition have gained interests for

their better performance. Although many Phe degrading bacteria have been isolated (10-13), more efficient Phe-degraders are always favored in bioremediation of Phe-contaminated sediments and soils (14). The main objective of this paper was to isolate bacterial strains which are able to degrade Phe and optimization of environmental parameters for more efficient removal of this contaminant.

Materials and methods

Sampling

Hydrocarbon contaminated soil samples were taken from oil exploration sites around Ahvaz, south of Iran; and transferred to laboratory in sterile bottles on ice while aerated every 20 min. The sampling site pH and temperature were 7 and 40 °C, respectively.

Media and chemicals

1- A stock solution of Phe was achieved by dissolving 2 g of phe in 100 ml acetone (200 mg L-1). It was stored at 4 °C for further uses. Specific amounts of the stock were used, if necessary, for Phe and acetone solvent was first evaporated at any stage.

2- Mineral Salt Medium (MSM) included 36mM NH4Cl, 1.69mM Kh2PO4, 0.066mM FeSO4, 16mM MgSO4.7H2O, 1.8mM CaCl2, 0.67mM KCl, 513mM NaCl, along with 1ml of trace element solution. Trace element solution composed of 0.147mM ZnCl2, 1.54mM CoCl2, 0.77mM NiCl, 1.38mM Na2SeO4, 0.24mM Na2MoO4, 0.118mM Na2WO4, 0.054mM NaVO3, and 0.007mM HCl. Final pH of MSM was adjusted at 7.0 using 100mM Tris/HCl (15).

3- MSM agar contained 15g agar in 1L MSM. After distribution of the medium in plates and consolidation, whole surface of plates were coated by spreading a layer of Phe (2 ml of Phe stock).

All the materials were supplied from Merck (Darmstadt, Germany); but Tris (Tris hydroxymethyl-amino methane), CaCl2, arabinose, starch, glucose, and Folin Phenol reagent were purchased from Sigma–Aldrich, Germany; Peptone, SIM and Agar from Difco, MRVP and TSI from Oxid; MgSO4.7H2O from Riedel-de Haën and NH4Cl from Chemlab, Belgium.

Isolation of Phe-degrading bacteria

In order to isolate Phe-degrading bacteria, 10 g of samples were mixed with 100 ml aseptic distilled water in 250 ml flasks, shacked at 100 rpm for 10 min and then, kept unshaken to precipitate. For enrichment of Phe degrading bacteria, 10 ml of the supernatant was transferred into the conical flask containing 100 ml MSM and supplemented with 200 mg/L Phe as the sole carbon and energy source (pH7) and finally incubated at 30 °C for two weeks (40 rpm). Phe consumption was monitored by increasing in bacterial biomass (absorbance at 600 nm). Then, subcultures (four times) were performed by transferring 1ml of the enriched culture to the same fresh medium.

At the end of enrichment process, a 10-fold serial diluted concentration of inoculated MSM were made, cultured on MSM agar and incubated at 30 °C. Colonies with clearing zone were purified and tested for Phe degradation ability as follow: equal concentration of any of colonies (an absorbance of 0.3 at 600 nm) were inoculated in MSM and incubated at 30 °C for 10 days (140 rpm). MSM with no bacterial inoculums was used as control. The experiments were conducted in triplicate. The amount of produced protein was considered as a criteria for bacterial growth and Phe degradation (8).

Protein measurement

Total produced protein was done as follows: at 48h intervals, 1ml of fermented MSM was centrifuged at 8000 g for 10 min. The harvested bacteria were then suspended in 1ml of Ringer's solution (5.7mM KCl, 111.2mM NaCl, 2.4mM NaHCO3, and 2.25mM CaCl2), vortexed and centrifuged again (8000 g for 10min). In order to cell lysing, the pellet was re-suspended in 0.5ml of 0.3M NaOH and 1ml of distillated water and then vortexed. The suspension was incubated at 90°C for 1h. The achieved cell lysate was used to the total produced protein measuring that was done by dye-binding assay of Bradford. Bradford protein assay protocol was as follows: for Bradford reagent preparation, 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml 95% ethanol, then after 100 ml, 85% (w/v) phosphoric acid was added. The final volume of mixture was taken to 1 liter using dH2O, when the dye had completely dissolved, and filtered through Whatman 540 paper just before use. Finally, 20µl of the cell lysis was added to 1ml of the diluted reagent and vortexed. Absorbance of the formed blue color was measured at 595nm (Shimadzu, UV-120-02; Japan). The results were checked against the standard curve of a serial dilution series (0.1-1.0 mg/ml) of bovine serum albumin (16).

Characterization of isolated bacterium

To identify selected isolate, biochemical and morphological characterization were done according to Bergey's manual of determinative bacteriology (17). Phylogenetic identification was performed using 16S rDNA analysis. Genomic DNA was extracted using phenol/chloroform/isoamyl alcohol referring (18) method. 16S rDNA gene fragment was amplified by PCR using a set of universal primer including Rd1 (5'-AGGAGGTGATCCAGCC-3') and Fd1 (5'-AGAGTTTGATCCTGGCTCAG-3'). The PCR

amplification mixture of 16 µl, contained the bacterial DNA (of about 200 ng), 5 µl of Taq buffer (TAPS, pH 8.8, 3mM MgCl2, 50 mM KCl), 1 µl (3 units) of Taq DNA polymerase, 5 µl of 2 mM dNTP mix and 5 µl of each primer (10 pM/µl). The amplification program was set for 30 cycles; all included a denaturation at 96oC (10 s), an annealing at 50 °C (10 s) and an extension step at 60 °C (4 min); with a final extension step at 72 °C for 5 min. Subsequently, PCR products were electrophoresed on 1% agarose gel and visualized using ethidium bromide. DNA fragments were sequenced using a sequencer (SEQLAB, Germany). Finally, all the sequences were compared with reference sequences in NCBI databases (http: //WWW.ncbi.nlm.nih. gov/Genbank) using Blast software. Phylogenetic analysis was done by MEGA software version 4 and genetic relationship was found based on the 16S rDNA gene sequence using neighbor-joining tree with bootstrap value of 1000 replication.

Optimization of Phe degradation

Effects of environmental parameters such as temperature, pH and Carbon/Nitrogen (molar) ratio on Phe degradation and growth of the isolate was investigated. To determine the effect of temperature, log-phase cells (in 5ml MSM) were cultured at a range of temperatures (25 °C to 45 °C by 5 °C intervals) and shaken at 140 rpm. After determination of optimal temperature, in order to determine the effect of pH parameter, the pH of the MSM was adjusted from 6 to10, and temperature was adjusted at 35 °C with shaking at 140 rpm. Finally to determine the effect of C/N ratio, various concentrations of NH4Cl of 0.5 to 2.5 g/L (with 0.5 g/L intervals) were used while other condition was 35 °C, pH8 and shaking at 140 rpm. The experiments were performed in triplicate. Sampling for protein assay was done at two-day intervals.

Extraction and analysis of Phe by GC

A 10-day-old fermented flask (containing 100 ml MSM) under optimal conditions and a control flask (containing 100 ml MSM) were extracted separately by ethyl acetate, and Phe amounts were measured by GC. The concentration of Phe was determined according to Wu et al. (19) 50 ml ethyl acetate were added to the flask and was shaken for 15 min. After forming two phases, Phe-containing organic phase was collected and aqueous phase was re-extracted by 50 ml ethyl acetate. Eventually, the organic phase was dried by Na2SO4 (anhydrous) and the volume was adjusted to 100 ml. Extract samples were analyzed using GC-FID equipped with a HP-5MS fused silica capillary column (60m × 0.25 mm ID × 0.25 lm thicknesses, Agilent Technologies, USA) with the detector and injector temperature of 300 °C and 280 °C, respectively. We used nitrogen as carrier gas. Oven temperature program was as following: 80 °C (for 2 min) to 120 °C at a rate of 10 °C/min and 120 °C to 300 °C at a rate of 4 °C/min and held at 300 °C for15 min. The quantification and identification of chemicals were performed by matching their retention times of standards (20).

Statistical analysis

All experiments were done in triplicate and the values were expressed as means. In order to reveal any significant differences between the parameters and variables we used Kruskal–Wallis one-way analysis of variance, Kolmogorov–Smirnov test, U Mann–Whitney Test, or by one-way ANOVA and the Tukey test (SPSS software, version 19). Results were considered significant if $p<0.05$.

Results

Isolation and selection of the most efficient Phe-degrading bacterium

Three Phe degrading bacteria, named AP, BP and CP were isolated and purified by aforementioned enrichment and screening techniques. Compared with other isolates, the clear zone formed around AP isolate on MSM agar was the biggest, indicating that the isolate was the most efficient in Phe biodegradation. On the other hand, the amount of protein produced by AP strain was more than that of other isolates in MSM medium (Table 1). Thus, AP isolate was selected as the best Phe degrader strain and other next tests and analyses were done using that.

Table1. Results of screening and selection for most efficient Phe degrader bacterium.

Isolate	AP	BP	CP
Produced protein (mg/g Phe)	*29	16	19
Clear zone diameter (mm)	5	2.3	3.1

*showed significant difference than the other isolates statistically (using U Mann Whitney test).

Characterization of selected bacterium

For phylogenetic characterization of AP isolate, a fragment of 1442 bp nucleotide of 16S rRNA was amplified and sequenced using universal primers and deposited in NCBI database (GenBank accession number: KR232837). As determined by BLAST analysis, a neighbor joining tree of the isolate was constructed (Figure 1). Results of NCBI blasting revealed that AP isolate clustered with *Dietzia cinnamea* strain DSM 44904, with 99% nucleotide identity. AP isolate was also found to be closely related to other strains of *D. cinnamea* including

D. cinnamea IMM (NR 042390.1) and *D. cinnamea* BC07 (KF052080.1) with 98% nucleotide identity.

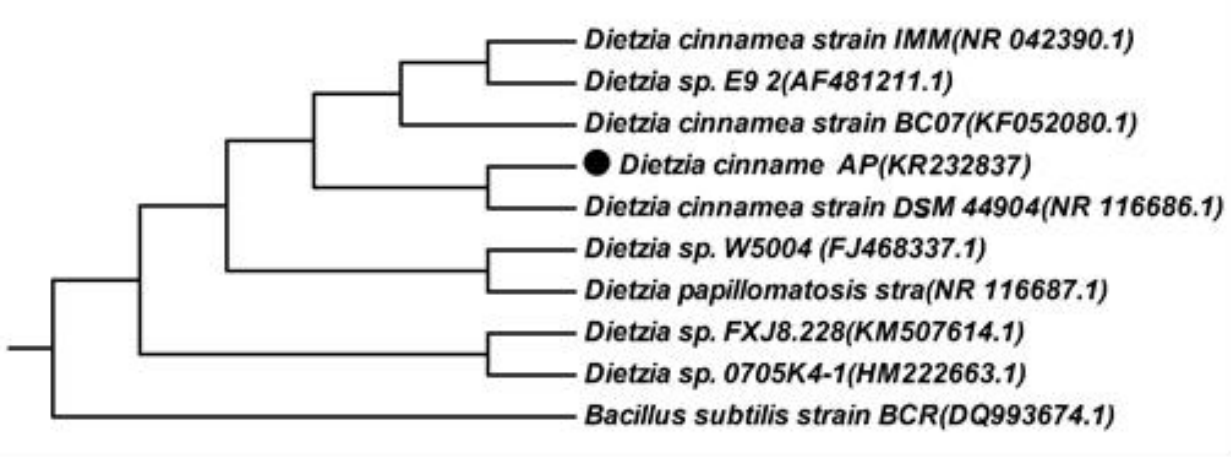

Figure 1. Phylogenic trees according to 16S rRNA gene sequences illustrating the relationships between strain AP and other members of Dietzia genus and Bacillus subtilis strain BCR (DQ993674.1).

By comparing the phylogenetic analysis and results achieved from morphological and biochemical tests (Table 2), the isolate was named as *Dietzia cinnamea* AP.

Table 2. Biochemical and morphological characteristics of isolate AP.

Cell shape and properties	Gram-positive, short rod and coccoid with size of 1.0–1.2×1.1–2.4 (in single, double and multiple), non-spore or capsule forming, non-motile
Colony Morphology	convex, smooth-edge, circular, glistening and deep orange
Positive reactions	oxidase, urease , catalase, arobic, nitrification, nitrate reduction and methyl-red
Negative reactions	denitrification, acid-fast, H_2S production, Voges-Proskauer test and indole production
Utilized	d-glucose, acetate, ethanol, d-fructose, glycerol, *n*-hexadecane, *n*-decane, maltose, d-xylose and starch
Not utilized	d-arabinose, citrate, d-galactose, gelatin, lactose, mannose, raffinose and sucrose
GC content	71.6%
Tolerated	pH (5-10), temperature (15-48 °C) and NaCl (0-9%)

Optimum conditions for Phe degradation by D. cinnamea AP

Environmental parameters including temperature, pH and NH4Cl concentration were optimized to achieve maximal Phe degradation. As shown in Figure 2A, *D. cinnamea* AP was able to grow and degrade Phe at temperatures from 25 °C to 45 °C, but with low efficiency at 25 °C and 45 °C. Statistical analysis showed no significant differences between the amounts of protein produced at 35 °C and 40 °C. Since more protein was produced at 35 °C, the temperature considered as optimum for Phe degradation. In the case of pH, it was observed that Phe degradation took place at the range of 6 to 9 by *D. cinnamea* AP (Figure 2B). Based on statistical analysis, *D. cinnamea* AP at pH8 showed a significant difference for Phe degradation compared with other values of pH and therefore selected as the optimum pH for Phe degradation. Neither of growth or Phe degradation occurred at acidic pH (pH5). Overall, the isolate preferred alkaline values of pH than acidic values. The selected bacterium grew and degraded Phe in concentration ranges of 1 to 2.5g/L NH4Cl. No significant differences were observed btween concentrations of 2 and 2.5g/L NH4Cl, statistically, but these concentrations differed from other concentrations. Since minimum amount of NH4Cl is desired in industrial experiences, therefore the amount of 2g/L NH4Cl was considered as optimum concentration for NH4Cl (Figure 2C). At lower concentrations of NH4Cl, the rates of growth and Phe degradation decreased as NH4Cl concentrations decreased. Figure 2D shows that optimization of environmental parameters resulted in increasing of total produced protein more than 1.5 fold (from 78.24 to 125 µg/200 mg Phe). Since increase in protein

produced was a criterion for Phe degradation, it can be concluded that the rate of Phe degradation increased by environmental parameters optimization.

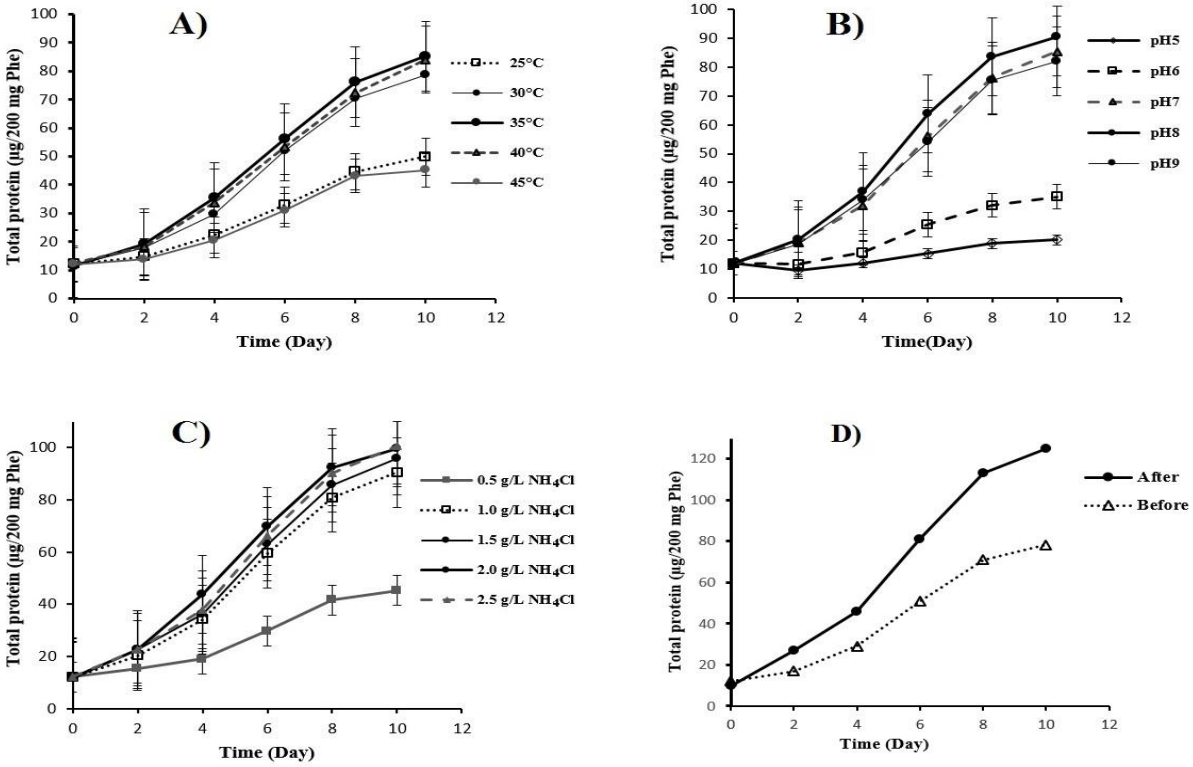

Figure 2. Optimization of Phe degradation by *D. cinnamea* AP. A) Effect of different temperatures on bacterial growth in MSM at pH 7 and 1 g/l NH4Cl. B) Effect of different pH on bacterial growth in MSM at 35 °C and 1 g/l NH4Cl. C) Effect of different NH4Cl concentrations on bacterial growth in MSM at 35 °C and pH 8. D) Effect of optimization on amounts of protein produced by *D. cinnamea* AP.

Before optimization of environmental factors, *D. cinnamea* AP was able to degrade only 60% of Phe at 10-day incubation; but after optimization of environmental factors the value of Phe degradation raised to 73%. These were proved by GC analysis of remained Phe in 10-day-old fermented MSM under optimized (Figure 3).

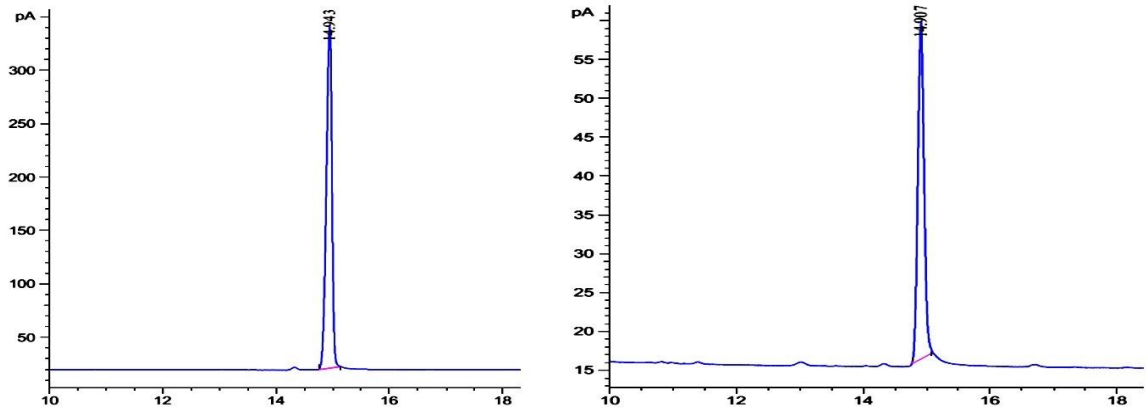

Figure 3. GC analysis showing Phe consumption by *D. cinnamea* AP. Phe values in control MSM (left) and in optimized MSM after 10-days of incubation (right).

Discussion

High concentrations of Phe in PAHs contaminated sediments and their potential bioaccumulation and severe hazards, calls for developing a safe procedure for their removal from contaminated sites. Therefore, great attention has been paid to develop effective methods to remediate PAHs contaminated environments in the past decades (14).

We successfully isolated three Phe degrader bacterial strains, amongst which one (named AP) was the most efficient and selected for Phe degradation. Based on biochemical morphological and tests as well as 16S rDNA sequencing, our isolate was characterized as *Dietzia cinnamea* AP. Some members of Dietzia species have been revealed to degrade aromatic compounds (21) and Phe degrader members in Dietzia genus have been reported (22-24). For example, two *D. cinnamea* strains were found to have tendency for n-C11 to n-C36 (25, 26) and n-C10 to n-C26 (17) alkanes hydrocarbon biodegradation. Despite the ability of these strains to degrade alkanes hydrocarbon, they were unable to degrade Phe as the sole carbon and energy source; while *D. cinnamea* AP could use Phe as the sole carbon and energy source. Based on our current knowledge we can claim that present study is the first report of Phe degradation by *D. cinnamea* species.

Since environmental parameters affect various activities of organisms (27, 28) including their ability to degrade and utilize various compounds, additional studies were made to determine optimal conditions for maximum Phe degradation by the bacterium. Total protein produced was considered as a criterion for growth and therefore Phe degradation. Our results showed that *D. cinnamea* AP degraded Phe at a wide range of pH (6 to10), temperature (25-45 °C) and NH4Cl concentration (0.5-2.5 gL-1). Optimization of environmental parameters led to increase in performance of Phe degradation by our isolate. This increase was proven by total protein produced and GC analysis. Precise measurements achieved from GC analysis showed that Phe degradation rate increased more than 1.2 fold (from 60% to 73%) by optimization of environmental parameters. Acceptable Phe degradation rates were achieved by the bacterium under wide ranges of environmental parameters, therefore, it could be suggested as a versatile candidate for bioremediation applications in various media including acidic or alkaline environments at variable temperatures.

To the best of our knowledge this is the first report of Phe degradation by *D. cinnamea* species as sole carbon and energy source. According to the results, *D. cinnamea* AP could be valuable in bioremediation of Phe-contaminated environments.

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Authors' contributions

All the authors performed the experiments. EG supervised the study. NZ advised the study KA wrote the manuscript. All authors discussed the data and commented on the manuscript.

Conflict of interest

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

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