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Full Length Article

Benzene Biodegradation by Novel Strain of *Caldibacillus*: Isolation, Characterization and Bioremediation Potential

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Abstract

Benzene pollution presents notable health hazards to individuals exposed in industrial settings or accidents, due to its persistent and water-insoluble nature. Eliminating benzene is crucial for safeguarding human health and environmental well-being. In this study, a soil sample was collected from a polluted semi-arid soil in the industrial estate of Al-mafraq city, Jordan. A new bacterial strain, designated as EJ2, was isolated from this polluted soil using mineral Stanier's medium (MSM) enriched with 1% benzene after multiple culture transfers. The isolated strain underwent biochemical and molecular identification for characterization. The isolate was cultured in enriched MSM with varying benzene concentrations (1, 2 and 3%), at pH values of 6.8 and temperature of 30°C. The strain displayed motile, Gram-positive bacilli with spore formation capability under light microscope examination. Biochemical testing indicated that this strain was positive for oxidase and catalase, while it exhibited the ability to hydrolyze carbohydrates and other organic compounds. 16S rRNA gene sequence analysis and BLAST searches against DNA sequence databases revealed that the new strain is a member of the genus *Cladibacillus* (Family *Bacillaceae*, Domain Bacteria) with the closest related species being *Cladibacillus* sp., showing a sequence identity of 99.09%. Most importantly, the strain exhibited growth when exposed to MSM containing benzene as the only carbon and energy substrate, within concentrations of 1 to 3%. Isolate EJ2 exhibited enhanced benzene degradation at a 1% concentration, with a generation time of 6.2 h, surpassing previously isolated *Lysinibacillus* strains (7.7-9.1 h) from the same soil source. The study identifies EJ2, a bacterium belonging to the *Caldibacillus* genus, as benzene-utilizing, suggesting promise for biodegradation. Therefore, additional research is crucial for understanding the metabolic pathways and processes of this novel isolate, thus optimizing its biodegradation capacity.

Keywords: Benzene; Biodegradation; *Caldibacilus; Lysinibacillus*; Polluted soil;16S rRNA gene

Introduction

Petroleum is a complex mixture containing various aromatic hydrocarbon compounds, including benzene, toluene, ethylbenzene and xylene, collectively referred to as BTEX (Irshaid and Jacob 2015). These BTEX chemicals are stable aromatic hydrocarbons that make up a sizeable portion of gasoline (Tunsaringkarn *et al.* 2012). The most fundamental aromatic hydrocarbon, benzene, is becoming more and more common in the environment. It comes from a variety of sources, including petrochemical manufacture, subterranean gasoline seepage and petroleum refining and might be hazardous to human health and the environment (Bamforth and Singleton 2005; Otenio *et al.* 2005; Yu *et al.* 2022).

Because of its tenacity and insolubility in water, benzene pollutes the environment and presents substantial health risks to people, animals and plants that come into contact with it accidentally or as a result of industrial use (Duarte-Davidso *et al*. 2001; Sanda *et al*. 2023). Moreover, benzene has been identified as a probable human carcinogen, mutagenesis agent and fetotoxic agent (Toth *et al.* 2021; Sanda *et al*. 2023). Inhalation or absorption of benzene at certain concentrations can have acute toxic effects on the central nervous system. The removal of benzene is, therefore, crucial for safeguarding human health and maintaining environmental well-being.

Benzene is a commonly utilized chemical across diverse industries, often subject to improper disposal practices. Consequently, such malpractice contributes to its pervasive presence as a substantial environmental contaminant. Bioremediation has emerged as a promising approach to tackle benzene pollution by utilizing microorganisms, primarily bacteria, to degrade and transform hazardous benzene compounds into less harmful forms (Kim and Jeon 2009; Vogt *et al*. 2011). The identification of bacterial species possessing the unique capability to catabolize benzene holds potential for effective bioremediation strategies to mitigate its detrimental impacts. Specific bacteria crucial for benzene breakdown have been identified in various studies, emphasizing their importance

(Fahy *et al.* 2008; Irshaid and Jacob 2015, 2016; Vogt *et al*. 2011). These bacteria employ diverse metabolic pathways and enzymes to convert benzene into less harmful intermediates (Genovese *et al.* 2008; Phan *et al.* 2021; Toth *et al*. 2021). Understanding the metabolic pathways and genetic regulation governing benzene catabolism in these bacteria provides valuable insights for the potential application of bioremediation technologies in remediating benzene-contaminated environments.

In our laboratory, a series of studies were conducted to explore the potential of various bacterial strains in the degradation of aromatic compounds present in the environment. *Enterobacter aerogenes*, isolated from diverse contaminated soils, sewage facilities and industrial locations, showed partial degradation and dechlorination of four chlorobenzoic acid compounds (Tarawneh *et al.* 2010). Moreover, aerobic xylene degrading bacteria were screened from gasoline-contaminated soil around gas stations, leading to the identification of four isolates with the ability to utilize *m*-xylene as a growth substrate (Irshaid and Jacob 2015). Moreover, toluene-utilizing bacteria were isolated from soil surrounding car body repair and painting workshops, resulting in the identification of four novel bacterial species with the capacity to utilize toluene as the sole source of carbon and energy (Jacob and Irshaid 2015). Lastly, in a study focusing on benzene degradation, five morphologically different bacterial isolates closely related to the genus *Lysinibacillus* were recovered from gasoline-contaminated soil samples, exhibiting potential for benzene degradation (Irshaid and Jacob 2016). These findings emphasize the importance of these specific bacterial strains as promising candidates for bioremediation in contaminated environments, especially concerning aromatic hydrocarbons like benzene.

As previously mentioned, a limited subset of bacterial species demonstrates favorable traits for benzene degradation. This limitation narrows the scope of viable bioremediation strategies applicable to benzene-contaminated settings. Investigating and isolating new bacterial strains with the capacity to degrade benzene holds the promise of yielding valuable knowledge to formulate efficient bioremediation techniques and environmentally sustainable measures for combating benzene pollution. These arguments give a strong foundation for future study in this area since they align with our urgent need to save the environment and encourage sustainable habits for a bright future. In this study, a novel benzene-degrading bacterial strain belonging to the genus *Cladibacillus* was isolated and identified from polluted semi-arid soil in the industrial estate area of Al-Mafraq city, Jordan. This discovery opens up new possibilities for bioremediation strategies in contaminated environments.

Materials and Methods

Sample collection

Soil specimens were gathered within the Al-Mafraq Industrial Estate located in Jordan during the initial period of August 2022. The sampled location, characterized by arid and elevated temperatures, exhibited extensive soil pollution due to longstanding contamination by petroleum-based fluids originating from diverse vehicular and machinery sources utilizing benzene fuel. Sterile 50 mL plastic tubes were used to collect samples, guaranteeing aseptic conditions. The obtained samples were immediately transported to the lab in ice boxes, where they were kept at 4°C for the enrichment and isolation processes that followed.

Enrichment and isolation of benzene-degrading bacteria

The enrichment experiment was carried out in mineral Stanier's medium (MSM). MSM was produced in the manner reported previously by Irshaid and Jacob (2016). In brief, ten grams of soil sample were enriched in sterile Erlenmeyer flasks (250 mL capacity) containing 50 mL of sterile MSM supplemented with 1% benzene as the only source of energy and carbon for enrichment. Benzene was purchased from Sigma-Aldrich Co. (USA) and had a purity of 99.8%. Benzene was aseptically sterilized through a 0.2 mm nitrocellulose membrane before being added to autoclaved MSM at the desired concentration.

The inoculated flasks were subjected to aerobic incubation within an orbital shaking incubator operating at 30°C and 180 rpm for a duration of 48 h. To curtail losses arising from volatilization and sorption phenomena, the flasks were sealed tightly with Teflon-coated stoppers. Furthermore, appropriate headspace was provided in the flasks to permit the maintenance of aerobic conditions during the experiment. Following an approximate incubation period of 48 h, 1 mL of the enriched medium was transferred to fresh flasks, and this process was repeated three times over a span of 48 h for each repetition. Subsequently, the culture was placed onto MSM agar plates containing 1% benzene and incubated at 30°C until separate colonies appeared. A single pure colony was isolated by a series of successive inoculations. The isolate was given the name EJ2 and was then grown on MSM agar plates with 1% benzene, allowing for future biochemical, molecular and biodegradation analysis. For the purpose of preservation, a 0.7 mL aliquot of the pure culture isolate was mixed with 0.3 mL of sterile glycerol within Eppendorf tubes, and the resulting mixture was stored at a temperature of -20°C.

Morphological and biochemical characterization of EJ2 isolate

Newly isolated EJ2 bacteria were identified using standard morphological and biochemical methods. Gram staining technique was employed for the identification process, following the procedure outlined by Cappuccino and Sherman (2021). Briefly, Isolate EJ2 was cultivated on MSM agar plates with 1% benzene at 30°C for 24 h aerobically to procure pure cultures. The resultant pure EJ2 cells underwent Gram staining and were subsequently

observed under a light microscope. Motility was evaluated using phase contrast microscopy, following the methodology outlined by Irshaid and Jacob (2016).

Oxidase and catalase tests were executed according to the methodology described in Cappuccino and Sherman's work (2021). For the oxidase test, viable cultures of each strain were applied with a small volume of oxidase reagent (2% N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride). A purple color change served as a positive outcome. The catalase examination involved the addition of a few drops of H_2O_2 onto fresh colonies of the isolated strains, with the presence of observable bubble formation indicating a positive reaction.

The EJ2 isolate was subjected to species identification using the qualitative RapID™ CB PLUS System (Remel, Thermo Scientific Inc., USA), which employs ERICTM software for identifying Gram-positive bacillus bacteria. Pure bacterial culture colonies were transferred to a test tube with sterile normal saline (2 mL) following the manufacturer's protocol. The bacterial suspension was introduced into wells of an identification kit within a transparent plastic tray containing reagents. This tray held 18 substrates for Gram-positive bacteria identification. After incubating the tray at 37°C for 4 h, color development indicated reactivity in each well. The reactions relied on detecting preformed bacterial enzymes, with pH changes reflecting substrate utilization. Positive and negative scores were analyzed to identify the test isolate by comparing results with stored reactivity patterns in a database or using computer-generated Code Compendium (ERIC-software).

Molecular identification of EJ2 isolate

For molecular identification and classification, DNA extraction, 16S rDNA PCR, and 16S rDNA sequencing were performed following the methods described by Jacob and Irshaid (2012). Genomic DNA from each pure isolate was extracted from freshly prepared cultures using the EZ-10 Spin Column Genomic DNA Isolation Kit (Biobasic, Ontario, Canada) according to the manufacturer's instructions. The 16S rRNA gene was PCR amplified and sequenced by GENEWIZ, Inc., USA, for pure DNA sample obtained from the EJ2 isolate.

The 16S rRNA gene sequence of isolate EJ2 was used to search for its closest relatives in the NCBI genetic sequence database using the Web BLAST Service (http://blast.ncbi.nlm.nih.gov/blast/ Blast.cgi). The partial 16S rRNA gene sequence of EJ2 from this study has been deposited in the GenBank database under the accession number KP297819 at the NCBI website: www.ncbi.nlm .nih.gov/genbank.

Phylogenetic tree construction for the EJ2 isolate

The molecular phylogenetic analysis for the EJ2 tree was performed using MEGA6 software following the method previously outlined by Tamura *et al*. (2021). This approach aims to identify the optimal tree fit for observed nucleotide sequences, considering substitution rate variations and uneven base frequencies.

A phylogenetic tree was constructed for the EJ2 strain, using 16S ribosomal DNA nucleotide sequences from five related bacterial strains obtained from the NCBI database through BLAST searches (NCBI website: www.ncbi.nlm.nih.gov/genbank). The analysis involved six nucleotide sequences, including 1st, 2nd and 3rd codon positions as well as noncoding regions, resulting in a final dataset of 1465 positions. Evolutionary history was inferred using the Maximum Likelihood method based on the Jukes-Cantor model. Sequence alignment and editing were performed using MEGA software, version 11 (Tamura *et al*. 2021). The resulting data from MEGA11 software were utilized to construct the phylogenetic tree representing the isolated bacteria.

Growth profiles of isolate EJ2 at 1%, 2% and 3% benzene concentrations

EJ2 cells were subjected to varying benzene concentrations (1, 2 and 3%) as the the sole carbon and energy substrate during incubation. The optimal benzene concentrations for the EJ2 isolate were determined by inoculating the cell of isolate with fresh cells in 15 mL of MSM supplemented with 1, 2 or 3% benzene in 50 mL tubes. The pH of the growth media was set to 6.8. The tubes were aerobically incubated in triplicate at 30°C with shaking at 180 rpm for 220 h. Furthermore, control experiments were prepared in a similar fashion and inoculated in tubes containing MSM without the addition of 0% benzene for all degradation experiments.

One-milliliter samples were aseptically extracted from the tubes at defined time intervals starting from inoculation (time zero). The growth rates of EJ2 isolate were measured by tracking the change in Optical Density (OD) at 600 nm using a UV-Vis Spectrophotometer (JENWAY, UK), with the abiotic enrichment medium serving as the blank for absorbance measurements. The experiments were replicated three times and mean values were recorded at different time points over a 220 h period.

Estimating generation times for EJ2 isolate and benzene-degrading *Lysinibacillus* strains

The experiment aims to assess the efficacy of benzene degradation by the EJ2 isolate in comparison to previously identified bacterial strains (1J1, 2J2, DJ2, 3J4 and 1J1-2), closely associated with the *Lysinibacillus* genus. Our lab previously isolated these benzene-degrading bacteria from contaminated soil in Al-Mafraq, Jordan (Irshaid and Jacob 2016). These five strains demonstrated benzene degradation capabilities. We are utilizing these strains alongside the newly isolated EJ2 strain to comparatively evaluate their benzene degradation potential.

Five selected isolates along with EJ2 were individually inoculated in 15 mL of minimal salt medium (MSM) containing 1 and 2% benzene in 50 mL tubes. The pH of the growth medium was adjusted to 6.8. The tubes were subjected to aerobic incubation in triplicate at 30°C with agitation at 180 rpm for a duration of 24 h. The generation times of all examine isolates were subsequently determined under these conditions. Additionally, the growth rates and generation times were continuously observed for a duration of 24 h across all experiments. Growth rates were assessed *via* OD measurements at 600 nm, while quantification of colony-forming units per mL (CFU/mL) was performed. Generation times were estimated utilizing the spread plate technique, with the calculation formula as g = t/n, where g = the generation time, n = number of generations, t = time. These experiments were conducted in triplicate, and the mean values were calculated.

Results

Isolation of pure strain utilizing benzenes as the only carbon source

A soil sample was collected from a polluted site within the Al-Mafraq Industrial Estate in Jordan. Microorganisms in this soil were gradually accustomed to a medium containing MSM supplemented with benzene, which was the sole source of carbon during this adjustment period. After the adaptation process with a benzene concentration of 1%, the microbial culture showed the predominance of a single bacterial strain, subsequently characterized and named as EJ2. Strain EJ2 underwent thorough microscopic inspection, followed by detailed biochemical, molecular, and genetic analysis.

Morphology and biochemistry analysis of isolate EJ2

Table 1 displays the distinctive characteristics of a newly discovered bacterial strain EJ2, isolated from contaminated soil within an industrial area in Al-Mafraq, Jordan. EJ2 cells exhibit a Gram-positive morphology with a rod-shaped structure and possess motility, along with the ability to form spores. Furthermore, this strain displays positive results for catalase and oxidase tests. Notably, EJ2 demonstrates the capacity to metabolize a variety of organic compounds, including glucose, sucrose, maltose, p-Nitrophenyl-*β*,D-glucoside, p-Nitrophenyl-n-acetyl-*β*, D-glucosaminide, p-Nitrophenyl-glycoside, O-Nitrophenyl-*β*,D-galactoside and p-Nitrophenyl phosphate.

Analyze EJ2 isolate on a molecular level

Table 2 presents the list of closely related culturable species identified in comparison to the new isolate. The 16S ribosomal DNA (rDNA) sequence of the isolate was acquired and has been archived in the GenBank database with the accession number KP297819. A BLAST search conducted on this 16S rDNA sequence indicated that the novel strain falls within the *Cladibacillus* genus, which is classified in the Bacteria domain and belongs to the *Bacillaceae* family. The most closely related known species is *Cladibacillus* sp. with a sequence similarity of 99.09%, followed by *Caldibacillus thermoamylovorans* and *C. hisashii* strain SML_I163, both sharing a sequence identity of 98.99%.

The Maximum Likelihood technique was used to identify the evolutionary connection between the EJ2 isolate and its closest relatives, as shown in Fig. 1. The phylogenetic tree for EJ2 was built using 16S ribosomal DNA nucleotide sequences from five separate *Cladibacillus* bacterial strains. This phylogenetic tree aims to shed light on the genetic relationships and evolutionary history of *Cladibacillus* strains, with a particular emphasis on the EJ2 isolate. The results of this analysis unveiled a pronounced genetic concordance between the EJ2 isolate and specific *Cladibacillus* species, particularly favoring *C. thermoamylovorans* and *C. hisashii*. This discovery highlights a strong genetic correlation between the EJ2 isolate and the designated *Cladibacillus* species, offering valuable insights into the evolutionary connections within this bacterial genus. Additionally, it provides fresh perspectives on the phylogenetic relationships of this bacterial species.

Effects of benzene concentration on the growth of EJ2 isolate

Fig. 2 shows the growth prolife of isolate EJ2 in MSM supplemented with benzene as sole carbon and energy source at various concentrations under pH 6.8 and 30°C and a control (0% benzene) is included as a reference for comparison. The data acquired from the experiments revealed a direct and proportional relationship between the concentration of benzene introduced at levels of 1, 2 and 3% and the subsequent increase in turbidity, which functions as an indicator of the proliferation of bacteria. However, it was observed that the rate of bacterial growth was notably lower when 2 and 3% benzene were present in the MSM compared to the growth rate observed when only 1% benzene was present. The experimental results also indicated that the process of biodegradation of 1 and 2% benzene commenced after a 20 h period of acclimation, while for 3% benzene, this process began after a 30 h period. In order to ascertain whether the observed variations in OD, a measure of turbidity, were solely attributed to the biodegradation process of benzene by the EJ2 strain, controlled experiments were meticulously conducted. Throughout these experiments, none of the control experiments exhibited growth in benzene-free MSM, confirming their reliance on benzene as the sole source of energy and carbon.

Comparison of generation times: EJ2 vs. other strains in benzene

Table 3 displays a comparison of the average generation

Table 1: Characterization of novel isolates EJ2: Morphological, biochemical and physiological Traits

Test	Result
Cell shape	*Bacillus*
Spore-formation	+
Motility	+
Gram-staining	+
CAT	+
OXD	+
GLU	+
SUC	+
RIB	-
MAL	+
*α*GLU	-
*β*GLU	+
NAG	+
GLY1	+
ONPG	+
PHS	+
EST	-
PRO	-
TRY	-
PYR	-
LGLY	-
LEU	-
URE	-
NIT	-
PIG	-

Legend (Abbreviations of chemical tests): +: Positive reaction, -: Negative reaction, CAT = Catalase Test, OXD = Oxidase Test, GLU: Utilization of Glucose, SUC: Utilization of Sucrose, RIB: Utilization of Ribose, MAL: Utilization of Maltose, *α*GLU: Hydrolysis of p-Nitrophenyl-*α*,D-glucoside, *β*GLU: Hydrolysis of p-Nitrophenyl-*β*,D-glucoside, NAG: Hydrolysis of p-Nitrophenyl-n-acetyl-*β*,D-glucosaminide, GLY1: Hydrolysis of p-Nitrophenyl-glycoside, ONPG: Hydrolysis of O-Nitrophenyl-*β*,D-galactoside, PHS: Hydrolysis of p-Nitrophenyl phosphate, EST: Hydrolysis of the fatty acid ester, PRO: Hydrolysis of Proline-*β*-naphthylamide, TRY: Hydrolysis of Tryptophan-*β*-naphthylamide, PYR: Hydrolysis of Pyrrolidine-*β*-naphthylamide, LGLY: Hydrolysis of Leucyl-glycine-*β*-naphthylamide, LEU: Hydrolysis of Leucine-*β*-naphthylamide, URE: Hydrolysis of Urea, NIT: Utilization of Potassium Nitrate and PIG: Yellow Pigmentation

Table 2: GenBank accession number of the 16S rRNA gene of the newly isolated strain EJ2 as well as its closest relatives based on its similarity

Closest Relative	% Identity	Accession Number
Caldibacillus sp.	99.09%	MZ984225.1
Caldibacillus thermoamylovorans	98.99%	MN733190.1
Caldibacillus hisashii strain SML_I163	98.99%	MG937587.1
Caldibacillus thermoamylovorans strain CB50-2	98.99%	MF361103.1
Caldibacillus thermoamylovorans strain Amp-choc19	98.99%	KR364739.1

Table 3: Comparison of mean generation times (Hours) in EJ2 bacterial strain and previously isolated strains in mineral Stanier's Medium with 1% and 2% Benzene

Isolate	Benzene concentration	
	1%	2%
Cladibacillus sp. EJ2	6.2	16.3
L. fusiformis 1J1	8.1	25.6
L. Xylanilyticus 2J2	9.1	28.8
L. odeysseyi DJ2	8.6	36.3
L. macrolides 3J4	7.9	39.7
L. boronitolerans 1J1-2	7.6	24.1

*Each value is the average of three separate experiments

times (in hours) between the EJ2 bacterial strain and previously isolated strains (1JI, 2J2, DJ2, 3J4 and IJ1-2). The comparison was conducted using MSM containing both 1 and 2% benzene concentrations. The presented table's data illustrates that EJ2 isolates demonstrated accelerated growth rates in benzene concentrations of 1 and 2%, outpacing previously isolated strains (1JI, 2J2, DJ2, 3J4 and IJ1-2). The generation time for EJ2 at 1% benzene concentration was 6.2 h, compared to 7.6-9.1 h for the aforementioned strains. Similarly, at 2% benzene concentration, EJ2 exhibited a generation time of 16.3 h, whereas the previous strains displayed generation times ranging from 24.1 to 39.7 h.

Discussion

In this study, a novel bacterial strain designated EJ2 was isolated from contaminated soil at an industrial site. The strain displayed small, motile, Gram-positive *Bacilli* in chains with spore formation, measuring between 0.4 to 0.5 μm. Biochemical analysis indicated the strain to be positive for oxidase and catalase activities, while it demonstrated the ability to hydrolyze carbohydrates and other organic compounds.

Isolate EJ2 exhibited morphological features and characteristics similar to strains of the genus *Caldibacillus*. Biochemical tests also yielded closely similar results, except for a few exceptions. The 16S rDNA sequence analysis confirmed that isolate EJ2 was closely related to *Caldibacillus*, with its closest relatives being *C. thermoamylovorans*, *C. hisashii* strain SML_I163, *C. thermoamylovorans* strain CB50-2, *C. thermoamylovorans* strain Amp-choc19. Based on literature data, a sequence similarity score of lower than 99% may represent a prototype strain of a different genospecies, while a similarity score of less than 97% could indicate a new genus (Drancourt *et al.* 2000; Stackebrandt and Ebers 2006). Considering the results of morphological, biochemical, physiological, phylogenetic, and 16S rDNA sequence analyses, isolate EJ2 is representatives of the genus *Caldibacillus*. *Caldibacillus* is a newly identified Gram-positive facultative anaerobic bacterial genus from *Bacillaceae*. It was initially isolated from a thermophilic aero-tolerant cellulolytic culture (Coorevits *et al.* 2012; Wushke *et al.* 2015, 2017). Through phylogenetic analyses, *Caldibacillus* was distinguished from *Bacillus* and new genera were established. The name *Caldibacillus* originates from Latin words denoting "warm, hot" and "a small rod," reflecting its thermophilic nature. *Caldibacillus* species, like *C. thermoamylovorans*, demonstrate both aerobic and anaerobic growth abilities (LPSN 2023; Yang *et al.* 2023). The phylogenetic analysis of isolate EJ2 was conducted following the methodology detailed by Tamura *et al.* (2021). This analysis allowed us to ascertain the evolutionary lineage of isolate EJ2. The resultant phylogenetic tree provides crucial information regarding

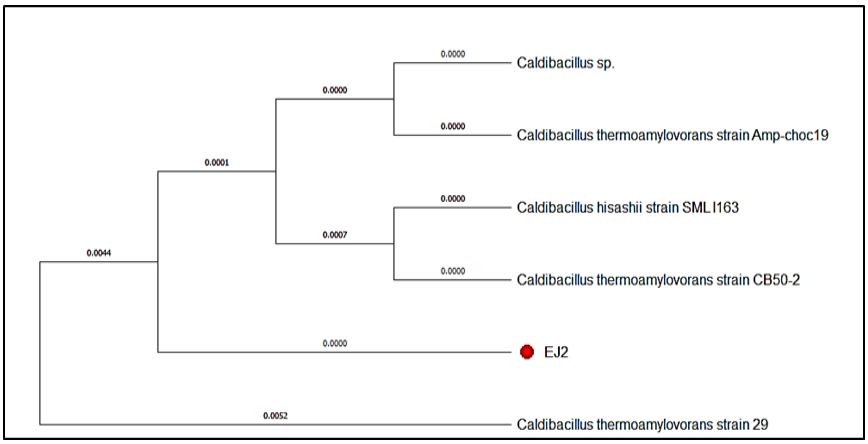

Fig. 1: A phylogenetic tree representing the isolate EJ2. This tree is constructed using nucleotide sequences derived from the 16S ribosomal DNA of five distinct bacterial strains that are categorized under the *Cladibacillus* genus. The specific names of these bacterial strains are indicated within the figure

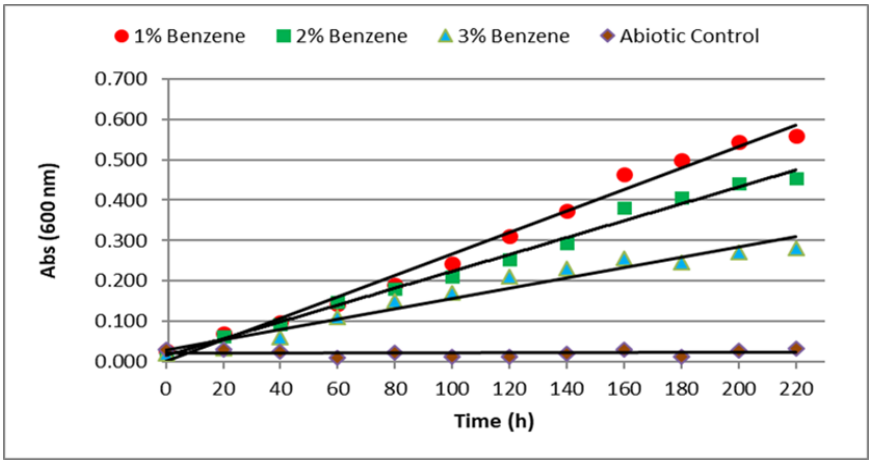

Fig. 2: Growth of isolate EJ2 in mineral Stanier's medium containing 1, 2 and 3% benzene as sole carbon and energy source. The outcome of the abiotic control experiment, featuring a 2% benzene concentration, is also displayed

shared ancestry, timelines of divergence and plausible evolutionary occurrences linked to this specific isolate. Furthermore, it aids in identifying the nearest relatives of isolate EJ2 within the *Cladibacillus* genus, thus offering significant insights into the genetic variability present within this particular group.

Our laboratory conducted research to identify bacterial isolates that have the ability to break down benzene, m-xylene, and toluene, which makes them attractive candidates for cleaning up polluted environments through bioremediation methods (Irshaid and Jacob 2015; Jacob and Irshaid 2015). Specifically, we focused on five strains (1J1, 2J2, DJ2, 3J4 and 1J1-2) related to *Lysinibacillus*, which we isolated from soil contaminated with gasoline, and investigated their potential to degrade benzene in a previous study (Irshaid and Jacob 2016). The results from that study indicated that these *Lysinibacillus*-related strains showed promise for benzene degradation. In our current study, we compared the efficiency of benzene degradation between the *Lysinibacillus*-related strains and a newly isolated strain called EJ2. The generation times for the five isolates at 1% benzene concentration ranged from 7.4 to 9.1 h, as determined by optical density measurements at 600 nm, showing strain-dependent variation. These findings closely align with previous research from our laboratory, which reported generation times of 7.2 to 8.7 h for these strains (Irshaid and Jacob 2016). Notably, isolate EJ2 displayed a faster benzene degradation rate, achieving 1% benzene degradation within a generation time of 6.2 h, outperforming the *Lysinibacillus* strains in benzene utilization. Furthermore, data from supplementary controls consistently indicated constant optical density over the study period, supporting the inference that fluctuations in turbidity resulted primarily from EJ2 strain-mediated biodegradation of benzene. These findings highlight EJ2's potential as a candidate for benzene bioremediation in contaminated environments.

It is important to note that only a small or limited

bacterial species have been identified and garnered attention due to their remarkable ability to rely solely on benzene as their exclusive carbon and energy source (Fahy *et al.* 2008; Irshaid and Jacob 2015; Irshaid and Jacob 2016; Toth *et al.* 2021; Yu *et al.* 2022). Here, we report the isolation of a new benzene-utilizing strain that belongs to *Caldibicillus* genus from a polluted hot environment under aerobic conditions. The EJ2 strain shows potential as a candidate for benzene-contaminated bioremediation strategies. Understanding its distinct biochemical pathways and enzymes involved in benzene metabolism within this bacterial strain holds considerable potential for advancing effective bioremediation techniques to minimize the environmental impact of this toxic aromatic compound.

The Genus *Caldibicillus* members have been observed to possess crucial thermophilic and biodegradation characteristics, rendering them valuable in biodegradation and bioremediation processes (Huang and Monk 2004). This study not only contributes to existing knowledge but also reveals previously undisclosed aspects of *Caldibacillus* bacterial strains, particularly their distinctive abilities to break down and utilize benzene. This finding underscores the significance of comprehending bacterial diversity and potential applications across various genera. Additionally, it emphasizes the continued significance of research in this field and a requirement for environmental protection against dangerous pollutants like benzene and other carcinogenic, mutagenic and fetotoxic substances. This demonstrates the importance of sustainable practices and the broad effects that research has on our environment and the future.

Conclusions

To summarize, a novel bacterial strain named EJ2, belonging to the genus *Caldibacillus*, was isolated from contaminated soil at an industrial site. Genetic analysis, specifically of the 16S rRNA gene, confirmed its close relationship to *Caldibacillus* species. EJ2 shared morphological, biochemical and physiological traits with related species and other strains of *Caldibacillus*, while also displaying unique abilities, such as utilizing benzene as its primary carbon and energy source. Phylogenetic analysis further supported its association with *Caldibacillus*. The isolate was found to be able to grow with benzene at levels of 1, 2 and 3% as a sole source of carbon and energy under aerobic condition. More importantly, EJ2 exhibited a faster benzene degradation rate compared to previously isolated *Lysinibacillus* strains from the same site, making it a promising candidate for benzene bioremediation in polluted environments. *Caldibacillus* species' thermophilic properties and biodegradation capabilities are valuable for bioremediation strategies. Moreover, this investigation represents the initial report of such capability within *Caldibacillus* bacterial strains, highlighting their distinctive capacity for benzene catabolism and assimilation.

The aerobic isolation of EJ2 underscores its potential in benzene-contaminated bioremediation, emphasizing the necessity of understanding its metabolic pathways for effective bioremediation approaches. The aerobic isolation of EJ2 emphasizes its potential for biodegrading benzene-contaminated material, needing knowledge of its metabolic pathways for efficient approaches. Therefore, understanding the metabolism of EJ2 is crucial for understanding its potential for benzene biodegradation, providing understanding of its function, and assisting in the development of remediation strategies.

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Author Contributions

Conceptualization, JJ and FI; methodology, FI, JJ, and QA; resources QA, data curation, JJ, FI and QA; original draft preparation, FI and JJ; review and editing, JJ, FI and QA. All authors have read and approved the final version of the manuscript.

Conflicts of Interest

All authors declare no conflict of interest.

Data Availability

The study's data will be available upon request to the corresponding author.

Ethics Approvals

There are no animals used in this work.

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