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Molecular characterization of Indigenous Bacillus Isolates from Agricultural Soils

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Abstract:
. Five species—*B. subtilis* (S1), *B. licheniformis* (S2), *B. tequilensis* (S3), *B. velezensis* (S4), and *B. persicus* (S5)—were identified through 16S rRNA gene sequencing. Gene-specific PCR revealed the presence of OPA and MPD genes, whereas OPD and OPDA were not detected. Phylogenetic analysis demonstrated considerable genetic diversity among the isolates and identified S1 (*B. subtilis*) and S2 (*B. licheniformis*) as the most promising candidates for the bioremediation of chlorpyrifos-contaminated soils.

Introduction:
Pesticides are chemical compounds or combinations of chemicals that are primarily employed in agriculture to combat, prevent, eliminate or kill pests, this increases the yield of agricultural plants and the production of food (Dar et al., 2020; Siddiqui et al., 2022). Billions of pounds of pesticides are used annually in agriculture. Their use sharply increased after World War II with the introduction of chemicals like DDT, BHC, aldrin, dieldrin, endrin, and 2,4-D, which were popular due to their effectiveness, ease of use, and low cost. (Pimentel, D. and Greiner, A., 1997). Pesticides are classified by their chemical composition—such as organochlorines, organophosphates, carbamates, and pyrethroids—with toxicity levels ranging from low to high. They are also commonly categorized by target pest, including insecticides, herbicides, and fungicides. (Kim et al., 2017; World Health Organization [WHO], 2010; Capoferri et al., 2018) . These substances are transported into plants, animals and humans, and then move up the food chain over time. Various natural and human-caused processes can release these toxic substances into the environment (Chaurasia et al., 2023). The WHO and FAO define pesticides as substances or microorganisms used to control pests, including disease vectors (Eduardo et al., 2019). Short-term exposure can cause nausea, headaches, respiratory and skin irritation, and endocrine disruption, while long-term exposure may lead to birth defects, infertility, thyroid issues, neurological disorders, diabetes, and cancer (Bourguet & Guillemaud, 2016; Mostafalou & Abdollahi, 2013). Modern pesticide development focuses on compounds that degrade quickly, target pests specifically, and minimize harm to non-target species, with precise dosing (Umetsu & Shirai, 2020). However, some pesticides persist in soil and sediment, enter the food chain, and contaminate groundwater, posing risks to water quality and human health (Lykogianni et al., 2021). Chlorpyrifos is widely used as a broad-spectrum insecticide in various pest control application in both urban and agricultural settings (Lemus, R. and Abdelghani, A., 2000). Chlorpyrifos (O, O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate) is an organophosphorus insecticide with the molecular formula $C_9H_{11}C_{l3}NO_3PS$, which has been detected in surface waters in the U.S. (Giesy, J. P. & Solomon, K. R. 2014). Chlorpyrifos is a stable, low-volatility insecticide (melting point 41.5–42.5°C)

that can persist in water due to slow decomposition and long photolysis half-life (Mostafalou & Abdollahi, 2013).

Materials and Methods:

The study area included sites of different agriculture soil and determination their locations by GPS in Babylon province, s1(Al Mahaweli village N=32 º 45. 467, E=044 º 24.264) s2 (Abu Gharaq village, N=32º34.333, E=044º20.414), s3 (Alanyl village N=32 º 32.510, E=044 º 27.527), s4 (AlKifl village N=32 º 14.388, E=044 º 23.218), s5(Al Hashemite village N=32 º 35.231, E=044 º 60.762) Hilla City, Iraq. These samples included five sites from for this study in Zigzag line Babylon province as following. site number, area name and their coordinate mentioned in Map

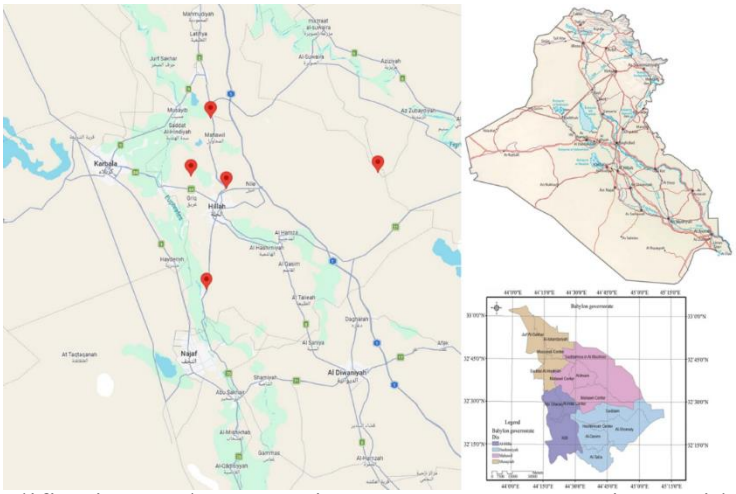

Fig (1): Location Map of Babylon Province, Hilla City

Identification of Bacterial species

Bacterial isolates were initially identified by cultural and Gram characteristics and the Vitek-2 system, then confirmed molecularly by 16S rRNA gene amplification and sequencing. Sequence comparison with the NCBI database (≥97–99% similarity) verified their taxonomic identity. (Cheesbrough, 1991).

Inoculum preparation

The biodegradation experiment of chlorpyrifos was conducted using selected Bacillus isolates under controlled laboratory conditions. Mineral Salt Medium (MSM) was employed as the basal medium, composed per liter of distilled water of KH_2PO_4 (1.5 g), K_2HPO_4 (1.5 g), $(NH_4)_2SO_4$ (1.0 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (0.1 g), $CaCl_2$ (0.02 g), and $FeSO_4 \cdot 7H_2O$ (0.001 g). Chlorpyrifos was added to the medium served as the sole source of phosphorus and/or carbon. (Kumar, H. et al., 2022; Mauldin, R. E. et al. 2006). Cultures were incubated in 250 mL Erlenmeyer flasks containing 100 mL of medium, maintained at 30 ± 2 °C and pH 7.0 ± 0.2, and shaken at 150 rpm in a rotary shaker incubator to ensure adequate aeration. The incubation period lasted for seven days, during which samples were collected daily for analysis

Chlorpyrifos degradation in liquid medium

Erlenmeyer flasks (250mL) containing mineral salt medium(100mL) supplemented with (0.5144578 mg) of chlorpyrifos were inoculated with bacterial cell suspension in triplicates. The flasks were incubated at 30 ◦C with shaking at 150 rpm and an uninoculated flask was used as control. CP residue extraction and estimation by HPLC was done as described previously. (Zafar, S., & Sikander, S. 2016).

DNA extraction and purification

Bacterial isolates were extracted according (Cheng, H.R & Jiang, N. 2006)

PCR technique

Table 1: Gene primers used in this study

Name of gene	Sequence of Primers (F and R)	Size	References
OPDA	5-- TGTTCCGGTAACCACTCACA-3- 5-- CACTCTCAGAGGGACGAAGG-3-	412bp	Ali, M.et al 2012
OPD	5-- AGGGTTGTGCTCAAGTCTGC-3- 5-- CAATAAACTGACGTCGCGAC-3-	327bp	Ali, M.et al 2012
MPD	5-- AGCAGGTCGACGAGATCTAC-3- 5-- TTGACGACCGAGTAGTTCAC-3-	574bp	Ali, M.et al 2012
OPA	5-- TCACACTGACTCACGAGC-3- 5-- CGGCCAATAAACTGACGT -3-	192bp	Kwak, Y. et al 2012

Table 2: PCR and DNA Sequencing Primers

Sequence of primers	Size	References
5′- CTACGGGGGGCAGCAG-3′ 5′-GGACTACCGGGGTATCT-3′	**465bp**	**(Mori *et al.*,2014)**

Results:

Molecular Identification of Bacterial Isolates

A total of 15 bacterial isolates were identified using 16S rRNA gene sequencing. Genomic DNA was extracted, and successful amplification of the ~500 bp 16S rRNA fragment was confirmed by clear single bands on 1% agarose gel. The purified PCR products were sequenced, and the resulting sequences were analyzed using BLAST against the NCBI GenBank database to determine species. identity based on the highest sequence similarity. BLAST analysis showed that the isolates belonged to two main phyla: Firmicutes and Proteobacteria. Most isolates (15) were Firmicutes, mainly of the genus Bacillus, while one was identified as Pseud Escherichia vulneris (Proteobacteria). Six representative Firmicutes strains were identified as *B. subtilis (S1), B. licheniformis (S2), B. tequilensis (S3), B. velezensis (S4), and B. persicus (S5)* Niallia circulans, and with >98% sequence similarity to reference strains.

Extraction and Purity Assessment of Genomic DNA

High-quality genomic DNA was extracted from all tested isolates. DNA purity was evaluated spectrophotometrically by measuring absorbance at 260 nm and 280 nm, yielding an A260/A280 ratio of approximately 1.8, indicative of pure DNA free from protein contamination (Green and Sambrook, 2012). Agarose gel electrophoresis further confirmed DNA integrity, showing distinct, intact bands with no evidence of degradation.

The identification of obtained isolates

The 16S rRNA gene sequences of all bacterial isolates were analyzed using the BLAST tool to compare them with reference 16S rRNA sequences available in the GenBank database. A single primer pair was employed to amplify the target gene, consisting of the forward primer 5′-CTACGGGGGGCAGCAG-3′ and the reverse primer 5′-GGACTACCGGGGTATCT-3′ (Mori et al., 2014). These primers were used to amplify the 16S rRNA genes from the selected isolates, which included *S1 (Bacillus subtilis), S2 (B. licheniformis), S3 (B. tequilensis), S4 (B. velezensis) and S5 (B. persicus)*. Bacterial isolates were collected from five main sampling sites within the study area, with three isolates obtained from each site, resulting in a total of 15 isolates designated as S1 to S15. These isolates were analyzed using the 16S rRNA gene sequence to determine their evolutionary relationships with closely related reference species. However, the phylogenetic tree displays only 14 isolates, suggesting that one isolate may have been excluded during the analysis, possibly due to poor sequence quality or failure to meet the alignment criteria. The variety of the 16S rRNA's sequence can be utilized to differentiate these bacteria, this is because it can adapt to the various genetic diversity patterns that are present in different bacteria. After the NCBI BLAST in program's alignment of these PCR amplicons, the sequencing reactions showed the specific identification. The NCBI BLAST in engine showed that the similarity between the sequenced samples and the intended target was between 99 and 100 percent for both sets of primers (500 base pairs of amplified sequence). In this study, two full trees with the composition of nucleic acids in the 500bp segments derived from the 16S rRNA sequences were constructed. These data indicate that the 16S rRNA sequence-based amplicons have the ability to recognize these bacterial sequences with a high degree of specificity, they also lack any significant similarity to other species' sequences. This evolutionary tree was comprised of 69 nucleic acid sequences that were aligned. The evolutionary tree that was derived from the DNA samples that were analyzed was employed to categorize the samples into three groups of evolutionary origin, each of which had a distinct history of evolution in the bacterial domain. The presented evolutionary tree is based on the 16S rRNA gene sequence, which is highly specific and used to deduce evolutionary relationships between bacteria. The tree includes the reference strains database sequences and 15 isolated bacterial strains (labelled S1 to S15). The sequences were phylogenetically aligned neighboring with their closest reference species based on similarity. It is worth mentioning that the Notably, isolates S2, S6, S8, and S9 grouped with Bacillus tequilensis (Ref: OL626994.1), indicating a high genetic similarity. Isolates S13 and S14 showed closer phylogenetic relationships to Bacillus velezensis (Ref: MW126937.1). A few more Isolates were placed near the reference strains of Bacillus subtilis (Ref: CP121748.1, isolates S1, S3, S7, and S15), Bacillus licheniformis (Ref: CP157731.1, isolate S14), and Bacillus persicus (Ref: MK849324.1, isolate S12), further demonstrating proved the diversity of the bacillus Bacillus species present among the studied samples. Outgroup species Escherichia coli and Staphylococcus aureus were included in the analysis to validate the phylogenetic tree and serve as external points of comparison. The phylogenetic analysis displayed distinct clades for different bacillus species; a handful of isolates were in close proximity to the reference strains The first group, linked to Bacillus tequilensis (Ref:

OL626994.1), comprised isolates S2, S6, S8, and S9, which grouped closely with the reference strain. This great level of sequence similarity shows a strong genetic tie and suggests that these isolates are probably members of the same species. In addition to the major Bacillus clades, a number of isolates were found to be affiliated with non-Bacillus species, highlighting the microbial diversity present within the samples. Isolate S5 was closely aligned with Pseudomonas aeruginosa (Ref: JQ178515.1). Similarly, isolate S10 clustered with Staphylococcus aureus (Ref: CP002643.1). Additionally, reference sequences of Escherichia coli and Pseudomonas vulneris were incorporated into the phylogenetic analysis. Although no isolates. grouped directly with these species, their inclusion served as outgroups to root the phylogenetic tree.

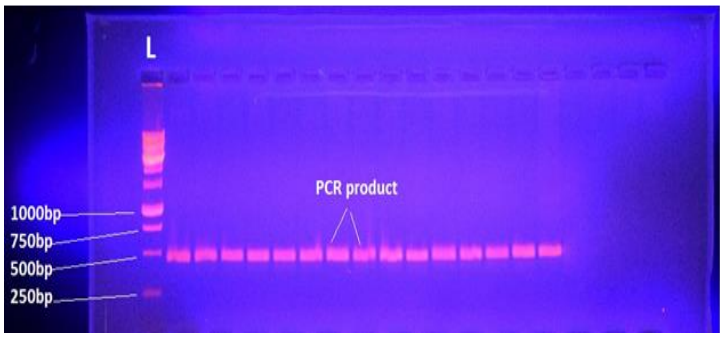

Fig (2): Amplification of ITS region by PCR, the PCR product was subjected for direct sanger sequencing for species identification, lane L 250 DNA step ladder, other lane represents 15 bacterial isolates.

Isolate code	(GenBank accession number)	Identity
1	**Bacillus subtilis** PQ319632.1	99 %
2	**Bacillus licheniformis** KF879257.1	99 %
3	**Bacillus tequilensis** MF540451.1	100 %
4	**Bacillus velezensis** MN233381.1	99 %
5	**Bacillus persicus** KJ676970.1	100 %
6	**Bacillus subtilis** PQ319632.1	99 %
7	**Niallia circulans** KT720357.1	99 %
8	**Pseudescherichia vulneris** NR_119109.1	100 %
9	**Bacillus velezensis** MN233381.1	99 %
10	**Bacillus subtilis** PQ319632.1	100 %
11	**Bacillus licheniformis** KF879257.1	100 %
12	**Bacillus persicus** KJ676970.1	99 %
13	**Bacillus licheniformis** KF879257.1	100 %
14	**Bacillus tequilensis** MF540451.1	99 %
15	**Bacillus subtilis** PQ319632.1	100 %

Table 3: Phylogenetic affiliation of 16S rRNA partial sequences of Thirteen bacterial isolates

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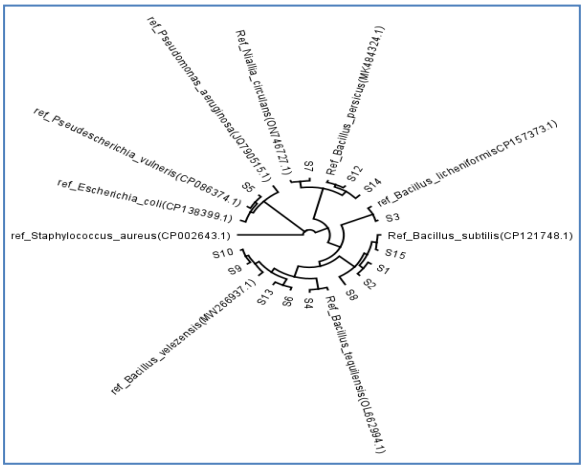

Fig (3): Phylogenetic Analysis of Bacterial Isolates Based on 16S rRNA Gene Sequences in Comparison with Reference Strains.

Detection of target genes responsible for biodegradation.

To improve the accuracy of genetic characterization and to determine the genes involved in the biodegradation process, primers specific to these genes were evaluated. These genes play a central role in the biodegradation pathways of the bacterial isolates under study. In addition, the extracted genomic DNA was of high quality and suitable for downstream molecular analyses, including PCR amplification and 16S rRNA gene sequencing.

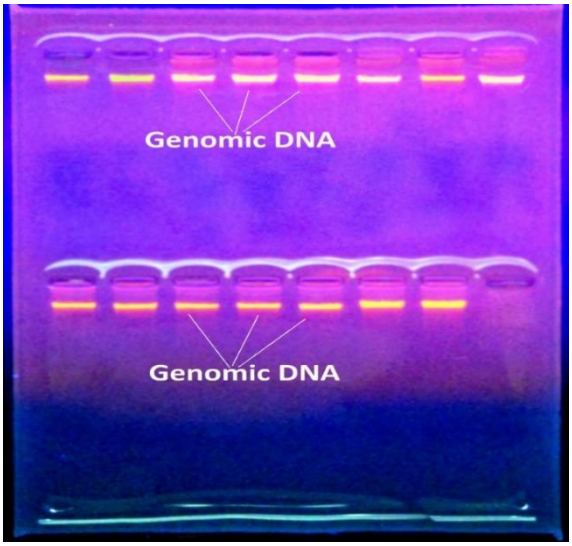

Fig (4): agarose gel electrophoresis of extracted bacterial genomic DNA, the extracted DNA was resolved on 1% agarose and run with 100 V for 60 minutes gel.

Detection of biodegradation genes by polymerase chain reaction technique (PCR).

The genomic DNA of *B. subtilis* (S1), *B. licheniformis* (S2), *B. tequilensis* (S3), *B. velezensis* (S4), and *B. persicus* (S5) were isolated and fourth genes coding for biodegradation of PAH compound were amplified by PCR. The products of PCR were electrophoresed by agarose gel electrophoresis. Genomic DNA was used to amplify the gene coded for the biodegradation. The results were showed in (Figure 5,6,7 and 8) respectively, gene OPA that positive in 2 isolates (Bacillus tequilensis and Bacillus subtilis) that have molecular weight 192 pb. The second gene MPD that gave positive results in 2 isolates (Bacillus subtilis and Bacillus licheniformis) with molecular size 574 pb. Third gene OPD and the fourth gene OPDA which all samples showed a negative result for the presence of the gene, as no bands were observed at the expected size of 327, 412 bp indicating the absence of the gene in the tested samples.

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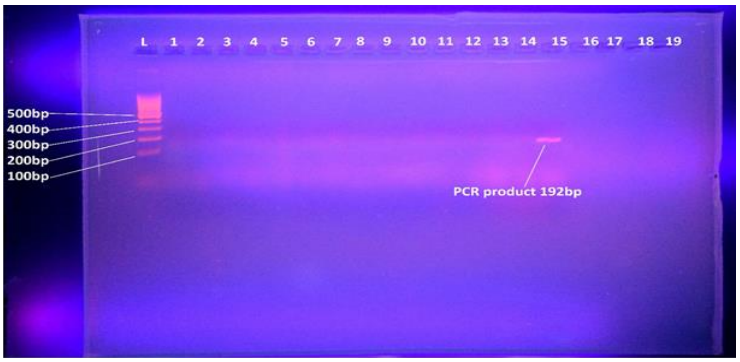

Fig (5) : detection of OPA gene by gene specific PCR , the PCR product was resolved on 2% agarose gel ; lane L 100bp step DNA ladder ; other lanes represent different samples , only one sample (s15) show positive detection .

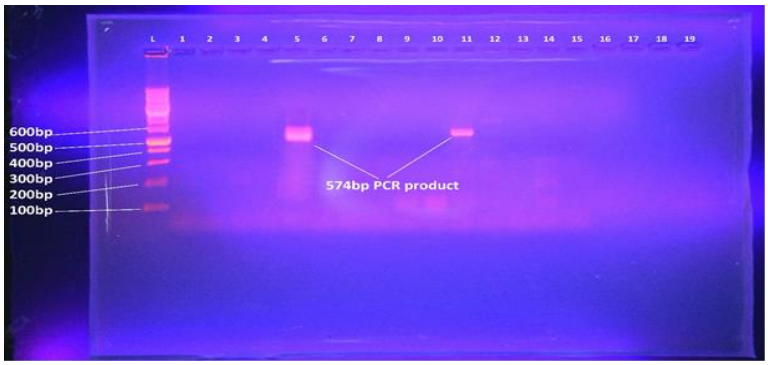

Fig (6): detection of MPD gene by gene specific PCR, the PCR product was resolved on 2% agarose gel, lane L 100bp step ladder, other lanes different samples, only samples 5 and 11 show positive detection (574bp), other samples are negative.

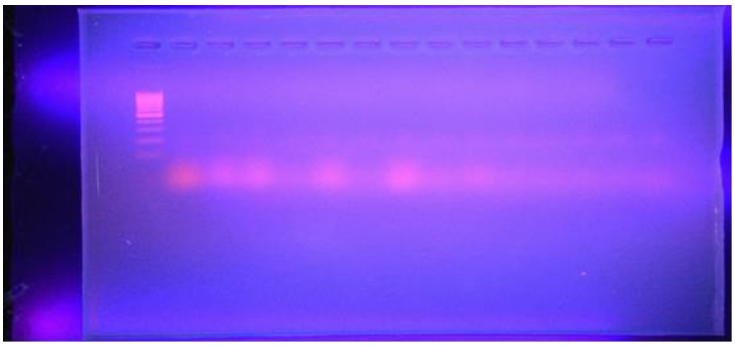

Fig (7): detection of OPD gene by gene specific PCR, the PCR product was resolved on 2% agarose, lane L 100bp step ladder, other lanes different samples, all samples show negative result for OPD gene (327bp PCR product).

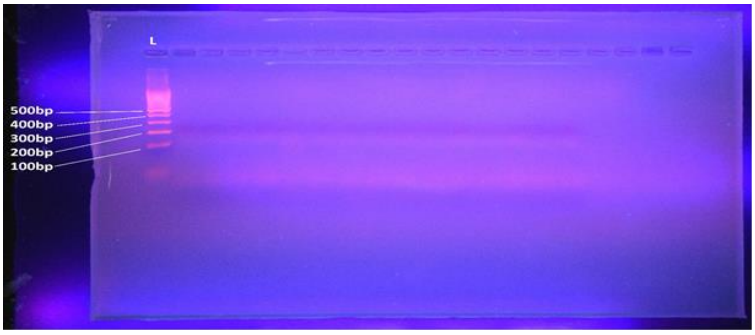

Fig (8): detection of OPDA gene by gene specific PCR, the PCR product was resolved on 2% agarose, lane L 100bp step ladder, other lanes different samples, all samples show negative result for OPD gene (412bp PCR product).

Discussion:

Molecular Confirmation of Bacterial Isolates through DNA Extraction and ITS-PCR Analysis:

The study included 15 bacterial strains, and PCR-based screening was performed to detect biodegradation-related genes using gene-specific primers. This approach aimed to identify target genes that theoretically indicate the presence of catabolic pathways responsible for the breakdown of specific environmental pollutants, based on primer specificity. The primers were validated using genomic DNA from reference bacterial strains previously reported to degrade organophosphorus and organochlorine compounds. The selected isolates belonged to the same taxonomic groups as known degraders, and/or demonstrated the ability to degrade chlorinated pesticides. Such diversity strengthens the overall microbial capacity to withstand environmental stressors and enhances contaminant degradation potential. (Kwak, et al 2012; Chakka, et al 2015). The OPA and MPD genes are key biodegradative markers associated with the breakdown of organophosphorus pesticides, including chlorpyrifos. These genes encode enzymes that catalyze the hydrolysis of organophosphate bonds, converting chlorpyrifos into less toxic intermediate compounds. The detection of OPA and MPD genes in bacterial isolates indicates their potential metabolic capacity to degrade chlorpyrifos and utilize it as a carbon or phosphorus source. Therefore, the presence of these genes provides valuable molecular evidence supporting the biodegradation ability of specific strains and highlights their suitability for bioremediation applications in pesticide-contaminated environments. (Li, et al 2008; Sharma, et al 2021). The primary intermediate formed is 3,5,6-trichloro-2-pyridinol (TCP), followed by further degradation into simpler organic compounds, including dihydroxy pyridine derivatives, pyruvate, and acetate, which can be assimilated into central metabolic pathways such as the TCA cycle. The detection of OPA and MPD genes offers clear molecular support for the biodegradation results, confirming that strains carrying these genes possess enhanced ability to degrade chlorpyrifos. This agrees with previous studies showing that organophosphate-degrading bacteria often utilize chlorpyrifos as a nutrient source, facilitating microbial adaptation in contaminated environments (Parmar, et al 2015; Ahmad, et al 2022).

Implications for Bioremediation:

The MPD and OPA genes were detected in isolates S1 and S4, which correlates with their strong ability to degrade chlorpyrifos observed in the biodegradation assays. This finding confirms the genetic basis underlying the degradative activity exhibited by these strains. Such results support their potential application in biotechnological approaches for the remediation of pesticide-contaminated environments. In contrast, the absence of the OPD and OPDA genes in the remaining isolates suggests that these strains may rely on alternative enzymatic pathways or yet unidentified genes involved in chlorpyrifos degradation. This highlights the importance of applying metagenomic and transcriptomic analyses to identify novel genes and metabolic routes that may not be detected using conventional PCR primers. Also, the differences in genes found among the isolates underline the need for choosing microbial groups with matching abilities to break down things, rather than depending on just one type.

This cooperative method can improve how well complex polluted mixtures, often found in farm soils get cleaned up (Aislabie & Lloyd-Jones, 1995).

Molecular Phylogeny of Environmental Bacterial Isolates:

The obtained results are consistent with previous studies reporting that members of the genus Bacillus are ubiquitous and highly diverse in environmental and agricultural ecosystems, largely due to their capacity to form endospores and adapt to various ecological niches (Logan & De Vos, 2009). In contrast, isolates S10 and S11 exhibited close phylogenetic relationships with Staphylococcus aureus (Accession No. CP006243.1), suggesting their affiliation with the genus Staphylococcus. This finding is noteworthy since S. aureus is a well-known opportunistic pathogen commonly associated with clinical infections and foodborne diseases (Tong et al., 2015). Meanwhile, isolates S15 and S16 showed high sequence similarity to Bacillus subtilis (Accession No. CP121748.1), a species recognized for its non-pathogenic nature and its industrial and biotechnological significance owing to its ability to produce a wide range of bioactive metabolites (Earl et al., 2008). The presence of such beneficial Bacillus strains among the isolates highlights their potential application as biocontrol agents, biofertilizers, or sources of industrially important enzymes.

The observed bacterial diversity reflects both the variety of environmental samples analyzed and the inherent genetic heterogeneity within and between bacterial species. The use of intergenic spacer (IGS) regions, which evolve more rapidly than conserved coding sequences, has proven useful in differentiating closely related taxa and inferring microbial phylogeny (González al., 2025 ; Garcia, et al 2024). Although the constructed phylogenetic tree provides a robust framework for classification, additional molecular analyses such as full-length 16S rRNA sequencing and/or whole-genome sequencing (WGS) are recommended to confirm taxonomic assignments and elucidate potential functional traits. Complementary phenotypic characterization would further enhance understanding of the metabolic capabilities and ecological roles of these isolates.

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