Isolation and selection of highly degrading chlorpyrifos *bacillus* spp. from tea plantation soils

Pham Quoc Cuong[1], Tran Minh Bao[2], Le Đuc Trung[2], Nguyen Thanh Lam[3,4] and Nguyen Duong Tam Anh[1*]
1. Faculty of Biology and Biotechnology, University of Science, Vietnam National University Ho Chi Minh City (VNU-HCMC), VIETNAM
2. Institute for Environment and Resources – IER, Vietnam National University Ho Chi Minh City (VNU-HCMC), VIETNAM
3. Ministry of Natural Resources and Environment, Hanoi, VIETNAM
4. Faculty of Environmental Sciences, University of Science, Vietnam National University, Hanoi, VIETNAM
*ndtanh@hcmus.edu.vn

Abstract

Bacillus is a genus widely used in agriculture as well as in environmental remediation. This study aimed to isolate and select Bacillus strains with high chlorpyrifos (CP) degradation ability from five tea growing areas in Da M'Bri, Bao Loc city, Lam Dong province, Vietnam where pesticides are commonly used in cultivation. Fifty-seven bacterial strains were isolated from 25 tea growing soil samples using minimal salt medium (MSM) agar containing 300 µg/ml chlorpyrifos as the sole carbon source. These bacterial isolates were evaluated for their ability to grow in MSM agar with increasing CP concentrations from 100 to 500 µg/ml and their CP degradation ability was quantified.

Three strains, designated as L1D3.3, L4D2.2 and L4D1.1, exhibited the highest chlorpyrifos degradation efficiencies of 88.04%, 88.78% and 91.74% respectively. These strains were identified and characterized as Bacillus megaterium (L1D3.3), Bacillus aerius (L4D2.2) and Bacillus altitudinis (L4D1.1) followed by Bergey's manual and 16S rRNA full length gene sequence analysis.

Keywords*:* *Bacillus altitudinis*, *Bacillus megaterium*, *Bacillus aerius*, chlorpyrifos, bioremediation, pesticide.

Introduction

Tea is an industrial crop that plays an important role in Lam Dong province, Vietnam. In 2022, the tea growing area of this province is 11,287.4 hectares, accounting for 21% of the tea growing area nationwide[7]. However, the uncontrolled pesticide abuse in the intensive cultivation of tea plants has led to Lam Dong tea products being contaminated with pesticide residues, leading to difficulties in tea consumption and a sharp decrease in tea export output[6]. This pesticide pollution directly affects the economy, especially the health of humans, livestock and crops and reduces biodiversity in agricultural areas. Most chemical pesticides are involved in this pollution. Among them, the notable pesticides currently used most are organophosphate compounds. Specifically, the rate of use of this group of pesticides in the total pesticide base in the Mekong delta is 5.9% of organophosphate compounds[9].

**** Author for Correspondence***

Chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] (CP) is an organophosphate pesticide that is widely used in the world. In Vietnam, the chemical pesticide has been banned from circulation since 2019. It can kill a very wide range of insects including mosquitoes, caterpillars, brown planthoppers, fruit worms, cockroaches, flies, termites, fire ants, lice etc. through the mechanism of inactivating the enzyme acetylcholinesterase, an enzyme that plays an important role in nerve conduction in animals. CP is toxic not only to harmful insects but also to beneficial insects such as honeybees and many other animals such as earthworms, fish, frogs, birds, mammals including humans. The effects of this pesticide on humans when exposed for a long time include acute poisoning, birth defects and a high risk of autoimmune diseases[4].

Chlorpyrifos has been reported to be degraded by many bacterial and fungal species. In the UK and Australia, the bacteria groups with the ability to strongly degrade CP are bacteria of the genus *Pseudomonas* and *Bacillus*[2]. The group of *Bacillus* spp. showed a CP decomposition rate of over 80%[3]. In Vietnam, there have been a number of studies on isolating and screening these microbial strains with the aim of applying them to treat pesticide-contaminated soils. The results of this study would serve as a basis for a set of biological recovery solutions for chlorpyrifos-contaminated cultivated soils and for bioremediation.

Material and Methods

Materials: Twenty-five soil samples were collected from twenty-five tea growing sites in Da M'bri, Bao Loc city, Lam Dong province, Vietnam, with uncontrolled pesticide use. Chlorpyrifos (CP) was purchased from Beyond Industries (China) Limited with 95% purity. Chlorpyrifos stock solution (40000 µg/ml) ws prepared in 10% DMSO.

Soil sample collection: Soil samples were collected according to TCVN 4046-85 cultivated soil sampling method. These samples were collected from 5 areas with twenty-five sample collecting sites. The representative soil areas were determined according to the "perpendicular line" or "zigzag line" rule to evenly distribute the sample locations on the soil areas. Then, the sample soils on the same area were mixed well in the same bag representing each different sampling area. Sampling depth was from 0 - 15 cm around the root zone. The soil was stored at room temperature until used for bacterial isolation.

Isolation of chlopyrifos degrading bacteria: 10 g of soil

samples were ground and suspended in 100 ml of mineral salt medium (MSM) containing 300 µg/ml of CP as the sole carbon source. These suspensions were incubated at room temperature for 7 days[8]. Then, serial dilutions of these bacteriral suspensions from 100 to 108 consisting of 0.1 mL of the suspension and 0.9 mL of the MSM supplemented with CP (300 µg/ml) were made and incubated at room temperature for 24 hours. 100 µl of each bactreial suspension was streaked onto the MSM agar supplemented with CP (300 µg/ml) and the plates were incubated at room temperature for 24 hours. The growing bacterial colonies on the plates were sub-cultured onto the MSM agar containing the same concentration of CP until pure cultures were obtained.

Selecting bacterial strains capable of highly degrading chlorpyrifos: Petri assay method was applied to select the bacterial strains with high chlorpyrifos-degrading ability. Pure bacterial strains were inoculated onto MSM agar plates containing increasing concentrations of CP from 100 - 500 µg/ml at room temperature for 48 hours. The size of bacterial colonies will be recorded to evaluate their growth as well as CP degrading ability.

The selected bacterial strains with the highest CP degrading ability were subjected to morphological, cultural and biochemical studies followed by Bergey's manual[11] and identified by 16S rRNA full length gene sequencing. The 16S rRNA gene gene was carried out by Nam Khoa Biotek Company Limited, Ho Chi Minh city, Vietnam. The nucleotide sequences were used for Blast analysis against the NCBI data base to obtain related sequences of related organisms. These sequences were aligned using BLASTN and a phylogenetic tree was constructed using the MEGA11.0.13 analysis programme.

Quantitative determination of chlopyrifos by UV spectrophotometrical method: Analysis of CP using UV was performed to assess CP degrading ability of the selected bacterial strains growing in the liquid MSM supplemented with CP at different concentrations from 100 to 500 µg/ml. Absorbance of chlorpyrifos dissolved in the MSM was proportional to its concentration at 295 nm.

Data processing method: The experiment was repeated at least 3 times and conducted in a randomized manner. The data were averaged and processed using Microsoft Excel 365 software.

Results and Discussion

Chlorpyrifos residues in tea plantation soil samples: The representative soil samples collected at different weather periods showed different soil chlorpyrifos concentrations (Table 1).

Table 1
Chlorpyrifos residue in team plantation soil samples in Da M'bri, Bao Loc city, Lam Dong province, Vietnam

Area	Site	Location	Collecting time	Chlorpyrifos (µg /ml)
1	L1D1	11.637116,107.741200	Light sunny afternoon	0.014 ± 0.03
	L1D2	11.637118,107.741577	Light sunny afternoon	0.079 ± 0.05
	L1D3	11.637131,107.741939	Light sunny afternoon	0.041 ± 0.06
	L1D4	11.637239,107.742340	Light sunny afternoon	0.025 ± 0.03
	L1D5	11.637410,107.742960	Light sunny afternoon	0.084 ± 0.03
2	L2D1	11.594552,107.744398	Early morning	0.706 ± 0.08
	L2D2	11.594632,107.744123	Early morning	0.106 ± 0.07
	L2D3	11.594711,107.743900	Early morning	0.081 ± 0.09
	L2D4	11.594784,107.743636	Early morning	0.823 ± 0.06
	L2D5	11.594838,107.743438	Early morning	0.976 ± 0.05
3	L3D1	11.594359,107.742971	Afternoon after the rain	0.606 ± 0.04
	L3D2	11.594262,107.743373	Afternoon after the rain	0.609 ± 0.02
	L3D3	11.594248,107.743852	Afternoon after the rain	0.07 ± 0.05
	L3D4	11.594318,107.744333	Afternoon after the rain	0.298 ± 0.08
	L3D5	11.594493,107.744800	Afternoon after the rain	0.94 ± 0.03
4	L4D1	11.598079,107.743257	Afternoon after the rain	0.829 ± 0.03
	L4D2	11.598050,107.743805	Afternoon after the rain	0.064 ± 0.02
	L4D3	11.598109,107.744304	Afternoon after the rain	0.197 ± 0.04
	L4D4	11.598193,107.744728	Afternoon after the rain	0.601 ± 0.09
	L4D5	11.598306,107.745200	Afternoon after the rain	0.773 ± 0.1
5	L5D1	11.595438,107.740766	Hot noon	0.63 ± 0.04
	L5D2	11.595201,107.740591	Hot noon	0.852 ± 0.07
	L5D3	11.595022,107.740463	Hot noon	0.114 ± 0.07
	L5D4	11.594819,107.740299	Hot noon	0.915 ± 0.04
	L5D5	11.594664,107.740184	Hot noon	0.018 ± 0.03

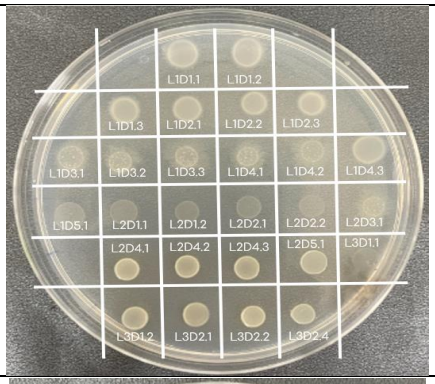
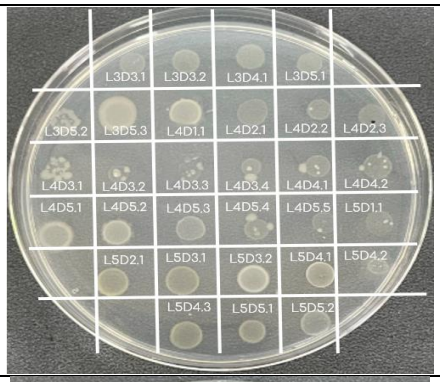
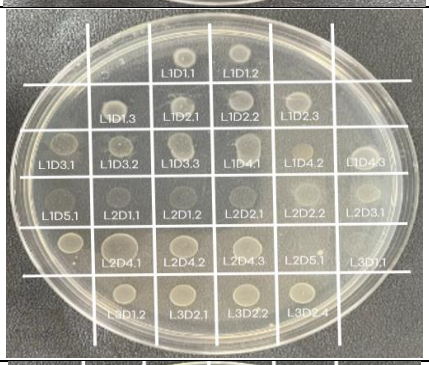
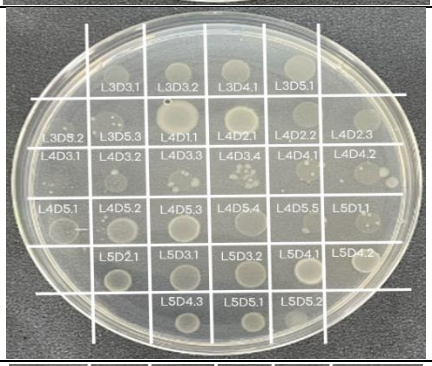
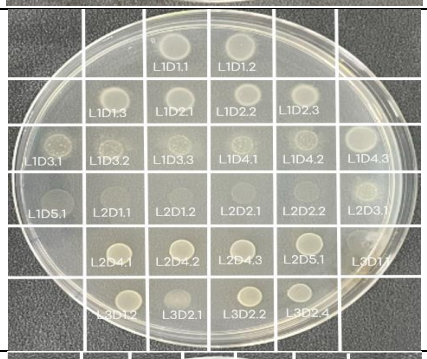
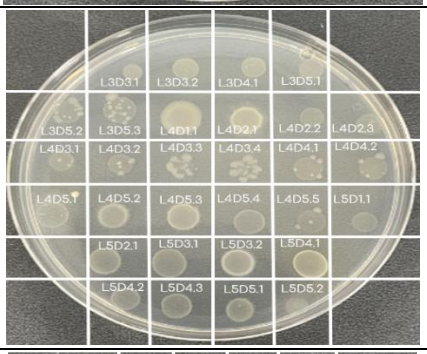
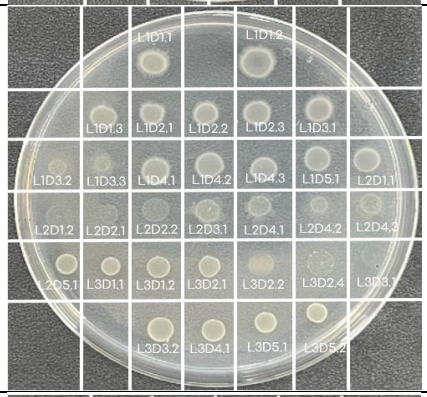
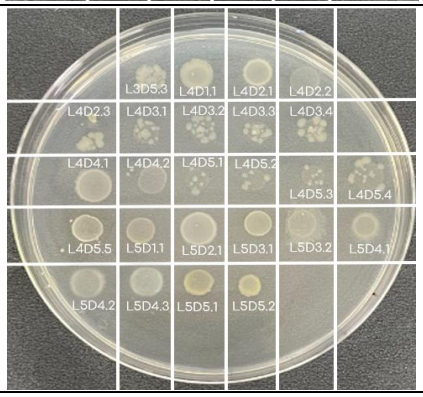
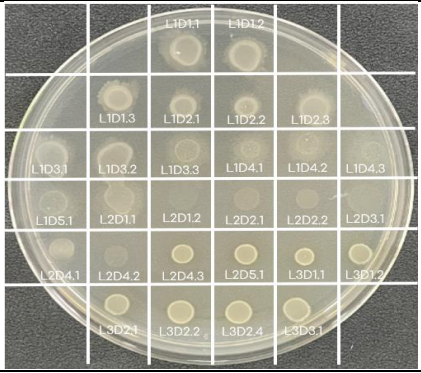
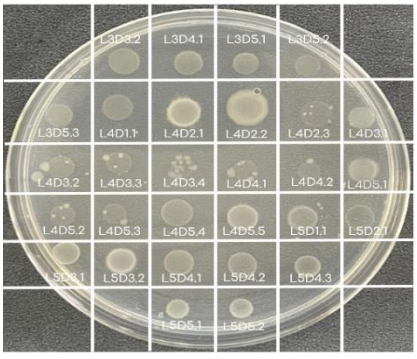
100 µg/ml		
200 µg/ml		
300 µg/ml		
400 µg/ml		
500 µg/ml		

Figure 1: Growth ability of 57 bacterial isolates on MSM agar containing chlorpyrifos concentration increasing from 100 to 500 µg/ml

The tea plantation areas were known to have been sprayed with the pesticides containing CP 10 days before. The results of the analysis of chlorpyrifos (-ethyl) residue in the soil samples were at low levels.

Isolation and selection of bacteria capable of degrading chlorpyrifos: Fifty-seven bacterial strains with chlorpyrifos-degrading potential were isolated from 25 tea plantation soil samples. Among them, 13 bacterial strains were isolated from area 1, 9 bacterial strains from area 2, 11 bacterial strains from area 3, 15 bacterial strains from area 4 and 9 bacterial strains from area 5. The fifty-seven isolated bacterial strains showed distinct growth on MSM agar media containing increasing concentrations of chlorpyrifos from 100 to 500 µg/ml (Figure 1). The results showed that the different growth abilities of the bacterial strains reflected their different tolerance or ability to degrade chlorpyrifos.

In particular, bacterial strains from area 3 and 4 had the ability to grow very strongly even at chlorpyrifos concentrations from 500 µg/ml. This result suggests the application of bacterial strains with the strong ability to degrade and tolerate high toxicity in the soil environment.

The fifty-seven isolated bacterial strains were further quantified for their ability to degrade chlorpyrifos on liquid MSM medium by UV spectrophotometrical method. The results showed that 57 bacterial strains in liquid MSM medium supplemented with chlorpyrifos concentration of 300 µg/ml had the ability to degrade chlorpyrifos from 1% to 91.74% (Figure 2). 3 bacterial strains including L1D3.3, L4D2.2 and L4D1.1 showed the highest percentage of chlorpyrifos degradation 88.04%, 88.78% and 91.74% respectively. Therefore, strains of L1D3.3, L4D2.2 and L4D1.1 were subjected to further studies for bacterial identification.

Identification and taxonomic characterization of selected bacterial strains L1D3.3, L4D1.1 and L4D2.2: Colony morphology, cell morphology and physiological and biochemical characteristics of the three selected bacterial strains including L1D3.3, L4D2.2 and L4D1.1 are described in table 2. Based on the criteria of Bergey's manual, the results showed that the three selected bacterial strains all belonged to the genus *Bacillus* spp.

The degradation of chlorpyrifos by strains belonging to the genus *Bacillus* has been reported in many previous studies. Abd et al[1] reported that strains belonging to the genus *Bacillus* such as *Bacillus safensis*, *Bacillus subtilis* and *Bacillus cereus* strain ATCC14579T isolated from pesticide-contaminated soil in Sudan, were capable of biodegrading pesticides containing chlorpyrifos.

Bacillus cereus species isolated from agricultural soil was reported by Kavitha et al[5] to have a maximum CP degradation capacity of 89%. A *Bacillus megaterium* species isolated from rice plants was able to degrade chlorpyrifos, although its degradation capacity was not strong[9]. Potential CP-degrading *Bacillus* spp. strains would be effective, dual-benefit agents for bioremediation of chlorpyrifos-contaminated agricultural soils and associated aquatic environments, also supporting enhanced plant growth and resistance and thereby preventing pollution and health hazards and improving crop yields.

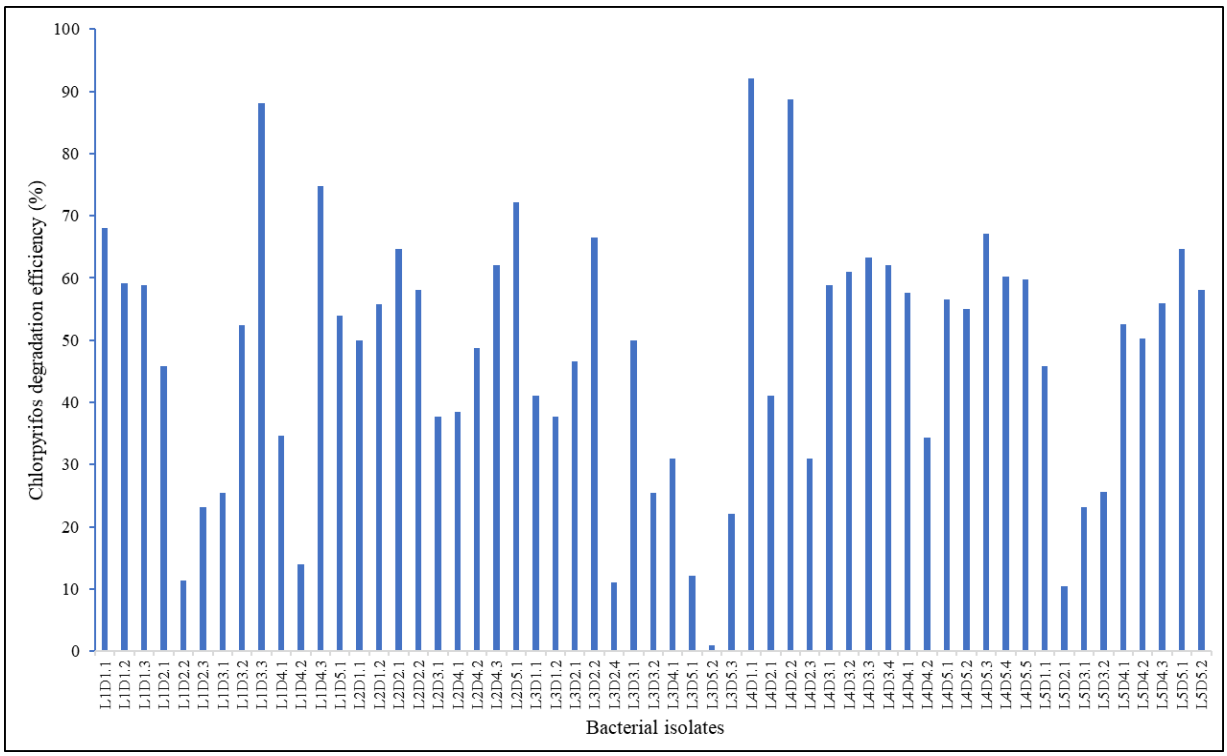


Figure 2: Chlorpyrifos degradation efficiency of 57 bacteria isolated from tea plantation soil

Based on Bergey's manual, the three selected bacterial isolates designated as L1D3.3, L4D2.2 and L4D1.1 were identified as members of the genus *Bacillus*. The taxonomic identification was further confirmed by full length sequencing of the 16S rRNA gene (1430 bp) and Blast analysis. The results identified the three strains L1D3.3, L4D1.1 and L4D2.2 as belonging to the species *Bacillus megaterium*, *Bacillus altitudinis* and *Bacillus aerius* respectively, with a similarity of 99.78%, 99.86% and 100% respectively (Figure 3).

Conclusion

This study dealt with chlopyrifos-degrading *Bacillus* strains that have potential applications in pesticide-contaminated agricultural soil treatment. The results showed that there were many chlopyrifos-degrading bacteria present in tea plantation soil. Three isolates belonging to the genus *Bacillus* were selected including *Bacillus megaterium* (L1D3.3), *Bacillus aerius* (L4D2.2) and *Bacillus altitudinis* (L4D1.1) with very high chlopyrifos decomposition efficiency of 88.04%, 88.78% and 91.74% respectively. They were capable of growing in the media with high CP concentrations from 100 - 500 µg/ml or higher. These three strains have great potential applications to remove pesticide residues in cultivated soil environments.

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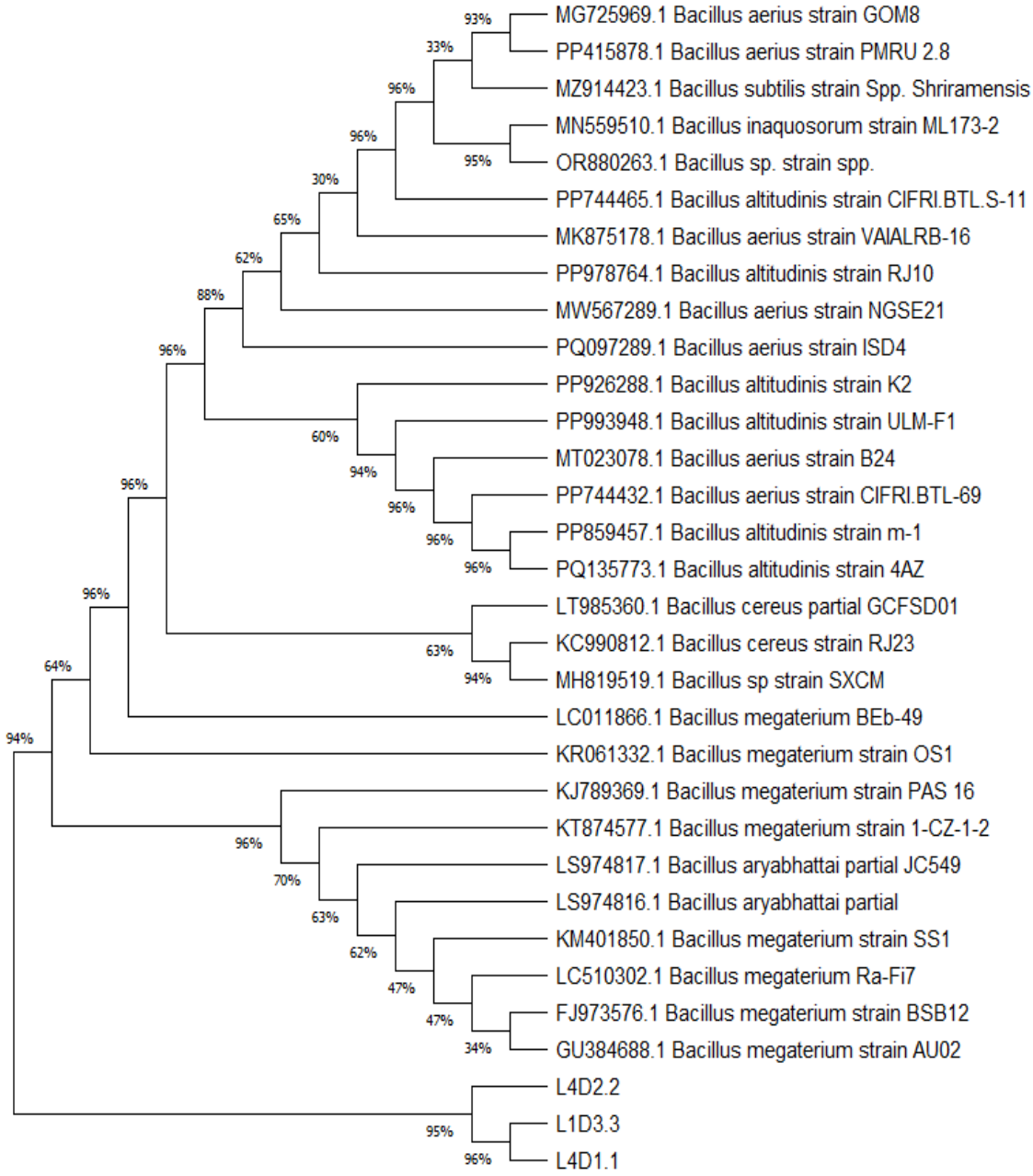
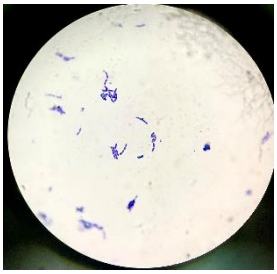
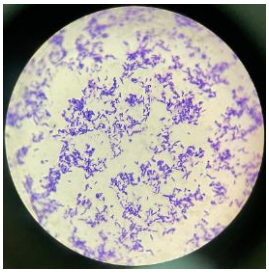
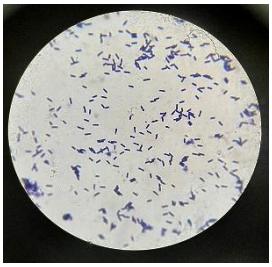


Figure 3: Phylogenetic tree construction based on the 16S rRNA gene region

Table 2
Morphological and biochemical characteristics of selected bacterial strains L1D3.3, L4D2.2 and L4D1.1

Characteristics	**Strain L1D3.3**	**Strain L4D1.1**	**Strain L4D2.2**
Colony morphology	Round, 2-3 mm in diameter, white, irregular, smooth, slightly convex	Round, 0.5-1 mm in diameter, white, with a regular margin, convex	Round, 3-4 mm in diameter, white, irregular, raised
			
Cell morphology	Rods	Rods	Rods
Gram	+	+	+
Microscopic morphology			
Motility test	-	-	-
Glucose test	+	+	+
Sucrose test	+	+	+
Manitol test	+	+	+
Mio-inositol test	+	+	+
Galactose test	+	+	+
Xylose test	+	+	+
Arabinose test	-	-	-
Maltose test	+	+	+
Lactose test	+	+	+
Citrate test	-	-	-
H_2S test	-	-	-
Urease test	-	-	-
Oxidation test	+	+	+
Fermentation test	+	+	+
Voges – Proskauer test	-	-	-
Methyl red test	-	-	-
Catalase test	+	+	+
Gelatinase test	+	+	+
Indol test	-	-	-
Predicted bacterial strains	*Bacillus sp.*	*Bacillus sp.*	*Bacillus sp.*

(-): negative, (+): positive

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