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Isolation, Identification and Characterization of Bacillus sp., from Soil Sample

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Abstract

Bacillus is a genus of Gram-positive, rod-shaped bacteria, a member of the phylum Bacillota, with 266 named species[9]. The term is also used to describe the shape (rod) of other so-shaped bacteria; and the plural Bacilli is the name of the class of bacteria to which this genus belongs. Bacillus species can be either obligate aerobes which are dependent on oxygen, or facultative anaerobes which can survive in the absence of oxygen. Cultured Bacillus species test positive for the enzyme catalase[3] if oxygen has been used or is present. The organisms in soil sample were serially diluted and inoculated onto the agar plates. The isolated organisms were identified by means of various techniques. Gram staining technique was performed and small Gram positive bacilli were observed. Endospore staining technique was performed and red colour bacilli along with green colour spores were observed. Various Biochemical tests were done for the Identification of bacteria[1]. Further identification of bacterial strain was done by Sanger sequencing method. The microbial strain was identified as *Bacillus subtilis*.

Key words : *Bacillus, Serial* dilution, Inoculation, Identification, Gram staining, Endospore staining, Biochemical Tests, Sanger sequencing method, *Bacillus subtilis.*

Isolation of Bacteria :

Sterile Petri plates were taken and about 200ml of Nutrient agar medium was prepared and dispensed into the Petri plates and allowed to solidify. The organisms in soil sample are serially diluted[12] and inoculated onto the agar plates. And the plates were incubated at 37ºc for 24 hours. Following incubation, the plates were observed for the growth of bacterial culture.

Identification of bacteria
Microscopic observation

a. *Gram staining :*

A clean grease free glass slide was taken and a drop of saline was placed at the centre of the slide with a sterile inoculation loop, in an aseptic condition. Then an isolated

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colony from the freshly inoculated plate was taken and a thin film of smear was made and the slide was air dried. And it was heat fixed. Following the heat fixation method, gram staining was performed[6].

Procedure :

- Primary stain Crystal violet was added on the smear, left for 1 minute and washed with tap water.
- Mordant Gram's Iodine was added, left for 1 minute and washed with tap water.
- Smear was then decolourised with few drops of ethanol by placing the slide at the slanting position and washed with tap water.
- Finally the smear was flooded with the counter stain safranin for 2 minutes and washed with tap water.
- The slide was air dried and observed under 100x oil immersion objective.

b. *Endospore staining :*

A clean grease free glass slide was taken and a drop of saline was placed at the centre of the slide with a sterile inoculation loop, in an aseptic condition. Then an isolated colony from the freshly inoculated plate was taken and a thin film of smear was made and the slide was air dried. And it was heat fixed. Following the heat fixation method, Endospore Staining was performed[11].

Procedure :

- Cover the smear with a piece of paper towel within the border of the slide.
- Place the slide over a beaker of steaming water. Do not let the beaker boil dry.
- Flood the paper towel with malachite green and let the slide steam for 3-5 minutes.
- Remove the stained paper towel and discard it.
- Gently rinse the slide with tap water.
- Counterstain the slide with safranin for 1 minute and gently rinse the slide with tap water.
- The slide was air dried and observed under 100x oil immersion objective.

Biochemical Tests for the identification of bacteria :

Indole production test :

Peptone broth was prepared, sterilized, inoculated and incubated for 24 hours at 37°C. After incubation, few drops of Kovac's reagent was added and observed for the presence or the absence of cherry red colour ring at the top of the broth[4].

Methyl Red test :

MR–VP broth was prepared, sterilized, inoculated and incubated at 37°c for 18 to 24 hours. After incubation, few drops of MR indicator was added and observed for the presence or the absence of red colour[4].

Voges – Proskauer *test :*

MR – VP broth was prepared, sterilized, inoculated and incubated at 37°C for 18-24 hours[7]. Few drops of Solution A and few drops of Solution B of Barrit's reagents were added after incubation and then observed for the presence or the absence of red coloration.

Citrate utilization test :

Simmon's citrate agar slant was

prepared, sterilized, inoculated and incubated at 37ºC for 24 hours[7]. After incubation, the slant was observed for the presence or the absence of colour change from green to Prussian blue colour.

Urease production test :

Christensen's Urea Agar slant was prepared, sterilized, inoculated and incubated at 37ºC for 18- 24 hours[7]. The colour change from yellow to pink was observed after incubation.

H_2S *production test :*

SIM Agar deep tubes were prepared and inoculated with a straight inoculation needle to a depth of 1 cm and incubated at 30ºC for at least 4 days[7]. The tubes were observed for blackening due to the production of hydrogen sulphide.

Gelatin hydrolysis :

Nutrient Gelatin tubes were prepared and inoculated with test organisms. After inoculation, the tubes were incubated at 37ºC for 24-28 hrs. The tubes were observed for gelatin hydrolysis.

Oxidase test :

A single oxidase disc (1% Tetra methyl *p*- phenylene diamine), was taken and placed in a petridish using sterile forceps[7]. The colony to be isolated was picked up with a tooth pick. It is smeared over the disc and noted for the colour change into intense deep purple blue colour within 5 to 10 seconds for highly positive reaction, purple blue colour within 10 to 15 seconds for delay positive reaction and absence of coloration after 60 seconds for negative reaction.

Catalase test :

Peptone broth was prepared, sterilized, inoculated and incubated for overnight. After incubation, few drops of broth and few drops of Hydrogen peroxide were mixed well[7] and observed for the presence or the absence of Gas bubbles within in few minutes.

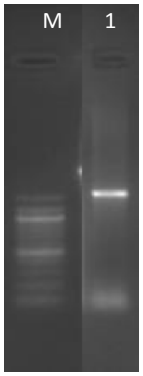
Identification of microbial strain by Sanger sequencing method

Project ID	ESP13D
Service type	Identification of given microbial strain by sanger sequencing method (4)
Sample type	Agar Plate
No of Samples	1
Name of the Samples	Bacteria (Ph.D)-10^{-6}
Target gene	16S rRNA Gene

Project Details Method :

1. 16S rRNA gene from given sample was PCR amplified[12].
2. Amplified DNA was analysed on 1% Agarose gel electrophoresis[2].
3. PCR products were purified and sequenced by sanger sequencing method using 16S rRNA forward primer.
4. Sequencing data were analysed by Blast Search Tool to identify the bacterial strain **PCR.**

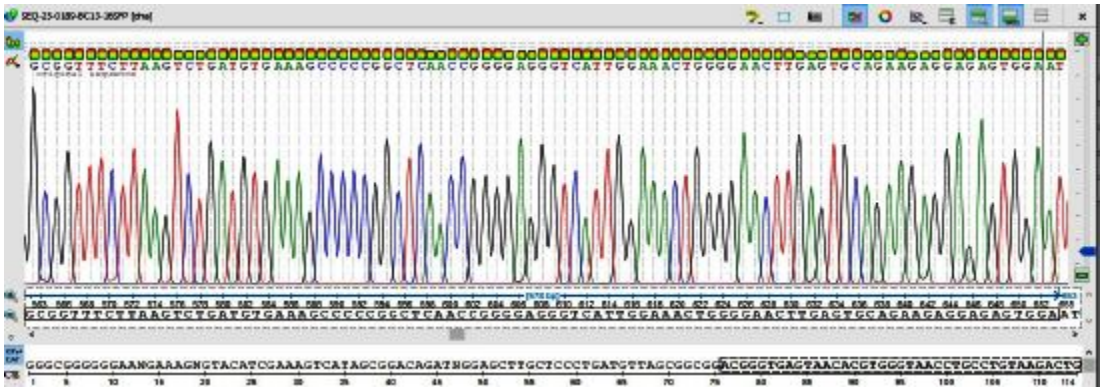
Amplification of 16S rRNA gene from the given sample



Loading order	
Lane no	**Sample Name**
M	100bp DNA marker
1	Bacteria (Ph.D)-10^{-6}

Strain identification by Sanger sequencing

Chromatogram of Sanger sequencing data – Bacteria (Ph.D)-10^{-6}



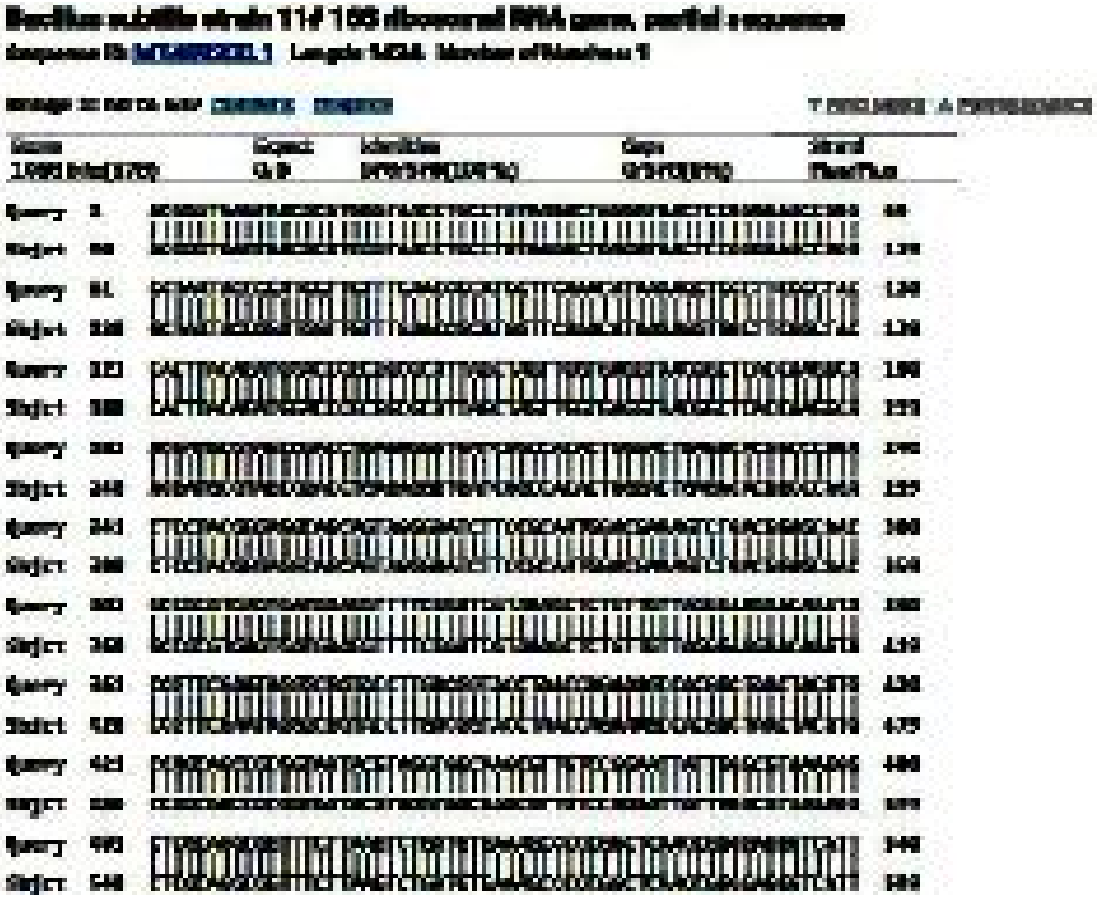
General Blast Search Result – Bacteria (Ph.D)-10^{-6}

Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank — Graphics — Distance tree of results — MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus subtilis strain 11# 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1068	1068	100%	0.0	100.00%	1434	MT459200.1
Bacillus subtilis strain RSE165 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1068	1068	100%	0.0	100.00%	1489	JQ687562.1
Bacillus subtilis strain PVR_13 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1068	1068	100%	0.0	100.00%	782	MT813518.1
Bacillus subtilis strain AY12 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1068	1068	100%	0.0	100.00%	1415	OQ362862.1
Bacillus subtilis strain PHRCD7 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1068	1068	100%	0.0	100.00%	1512	ON748653.1
Bacillus subtilis strain LZ045 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1068	1068	100%	0.0	100.00%	1450	JQ023605.1
Bacillus sp. CIFE_HT35 16S ribosomal RNA gene, partial sequence	Bacillus sp. CIF...	1068	1068	100%	0.0	100.00%	1373	KM016989.1
Bacillus subtilis strain S20 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1068	1068	100%	0.0	100.00%	1450	OQ564768.1
Bacillus tequilensis strain 281 16S ribosomal RNA gene, partial sequence	Bacillus tequilen...	1068	1068	100%	0.0	100.00%	961	PQ780576.1
Bacillus subtilis strain HBUAS69243 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1068	1068	100%	0.0	100.00%	1472	OP420642.1
Bacillus tequilensis strain S3-P9 16S ribosomal RNA gene, partial sequence	Bacillus tequilen...	1068	1068	100%	0.0	100.00%	1489	KC851828.1
Bacillus subtilis strain Tao2 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1068	1068	100%	0.0	100.00%	828	OR775745.1

General Blast Alignment result- Bacteria (Ph.D)-10^{-6}



Blast against Lactobacillus taxid: 1578 Search Result – Bacteria (Ph.D)-10^{-6}

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Lactobacillus sp. strain labmurCatfish02-13 16S ribosomal RNA gene, partial sequence	Lactobacillus sp.	1062	1062	100%	0.0	99.83%	1154	MH250204.1
Lactobacillus sp. strain labplantCatfish02-13 16S ribosomal RNA gene, partial sequence	Lactobacillus sp.	1051	1051	100%	0.0	99.48%	1161	MH250205.1
Uncultured Lactobacillus sp. clone HL-D61 16S ribosomal RNA gene, complete sequence	uncultured Lacto...	1040	1040	100%	0.0	99.13%	1245	KU588048.1
Uncultured Lactobacillus sp. clone HL-D61 16S ribosomal RNA gene, partial sequence	uncultured Lacto...	1040	1040	100%	0.0	99.13%	1083	OR453104.1
Uncultured Lactobacillus sp. clone HL-D61 16S ribosomal RNA gene, partial sequence	uncultured Lacto...	1040	1040	100%	0.0	99.13%	943	OR453200.1
Lactobacillus helveticus strain bcpca-gj-10 16S ribosomal RNA gene, partial sequence	Lactobacillus hel...	905	905	100%	0.0	94.99%	1510	KX247766.1
Lactobacillus sp. TLMP1 16S ribosomal RNA gene, partial sequence	Lactobacillus sp....	863	863	100%	0.0	93.61%	921	KF406344.1
Lactobacillus sp. TLMP4 16S ribosomal RNA gene, partial sequence	Lactobacillus sp....	863	863	100%	0.0	93.61%	899	KF406347.1
Uncultured Lactobacillus sp. clone lac1 16S ribosomal RNA gene, partial sequence	uncultured Lacto...	863	863	100%	0.0	93.61%	1423	OQ519650.1
Uncultured Lactobacillus sp. clone RT501 16S ribosomal RNA gene, partial sequence	uncultured Lacto...	822	822	98%	0.0	92.79%	576	MN752871.1
Lactobacillus sp. enrichment culture clone PSR03 16S ribosomal RNA gene, partial sequence	Lactobacillus sp....	821	821	93%	0.0	94.41%	614	JF292453.1
Lactobacillus sp. SWM_Isolation_3 16S ribosomal RNA gene, partial sequence	Lactobacillus sp....	752	752	100%	0.0	89.98%	1409	DQ298155.1

Blast against Lactobacillus taxid: 1578 Alignment Result – Bacteria (Ph.D)-10^{-6}

Lactobacillus sp. strain labmurCatfish02-13 16S ribosomal RNA gene, partial sequence

Sequence ID: MH250204.1 **Length:** 1154 **Number of Matches:** 1

Range 1: 69 to 647 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1062 bits(575)	0.0	578/579(99%)	1/579(0%)	Plus/Plus

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Query  1    ACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGG-AAACCGG  59
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Sbjct  69   ACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGTAAACCGG  128

Query  60   GGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTA  119
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Sbjct  129  GGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTA  188

Query  120  CCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC  179
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Sbjct  189  CCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC  248

Query  180  AACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG  239
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Sbjct  249  AACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG  308

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Sbjct  549  GCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCAT  608
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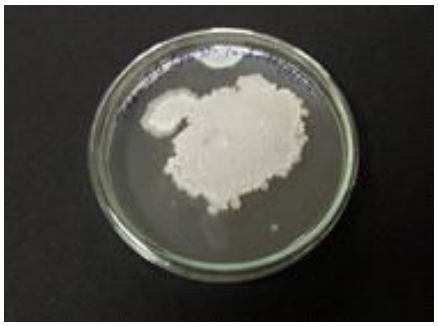
Isolation of Bacteria

The culture was isolated by inoculating it into the fresh Nutrient agar plate. And the plate was incubated at 37°C for about 24 hours. Following incubation, the bacterial culture was stored for the further use[7].

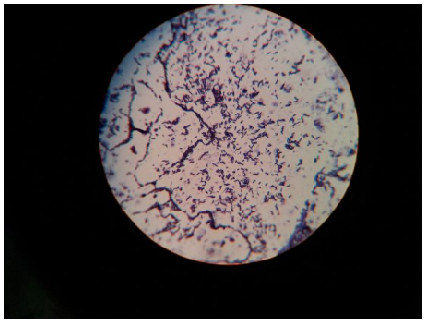


Colonies on Nutrient Agar (10-6 dilution)

Identification of bacteria
Microscopic observation

a. Gram Staining

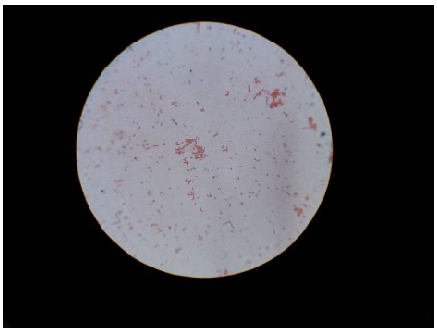
Small Gram positive bacilli were observed.



Gram staining

b. Endospore Staining

Red colour bacilli along with green colour spores were observed[10].



Endospore Staining

Biochemical Test Results for the Identification of bacteria

Indole production test - Result

No cherry red coloured ring was formed.

Methyl Red test – Result

Light red colour was observed.

Voges – Proskauer test - Result

No pink colouration was observed.

Citrate Utilization test - Result

Growth was observed on the slant along with Prussian blue colour formation.

Urease Production test - Result

Growth was observed on the slant along with pink colouration in the inoculated region.

H_2S Production test - Result

No black colouration was observed and no motility was observed.

Gelatin Hydrolysis Test – Result

No solidification were observed.

Oxidase test – Result

Intense deep purple blue colour within 5 to 10 seconds was observed.

Catalase test - Result

Gas bubbles were observed.

Table-1. Biochemical Test Results for the Identification of bacteria

S.No	Biochemical Tests	Results
1.	Indole	-
2.	Methyl red	+
3.	Voges proskauer	-
4.	Citrate utilization	+
5.	Urease	+
6.	H_2S Production	-
7.	Gelatin hydrolysis	+
8.	Oxidase	+
9.	Catalase	+

Identification of microbial strain by Sanger sequencing method :

Nucleotide General Blast analysis of 16S rRNA gene sequencing data matches with the bacterial strain ***Bacillus subtilis strain*** with 100% query coverage and identity.

The identified bacterial strain from sample **Bacteria (Ph.D)-10^{-6}** is **Bacillus subtilis.**

Summary

- The organisms in soil sample were serially diluted and inoculated onto the agar plates.
- The isolated organisms were identified by means of various techniques.
- Gram staining technique was performed and small Gram positive bacilli were observed.
- Endospore staining technique was performed and red colour bacilli along with green colour spores were observed.
- Various Biochemical tests were done for the Identification of bacteria.
- Further identification of bacterial strain was done by Sanger sequencing method.
- The microbial strain was identified as *Bacillus subtilis*.

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