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A Serine Protease-Producing *Bacillus pumilus* PUMMD23 Isolated from Agricultural Soil: Molecular Characterization and Biotechnological Potential.

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ABSTRACT

BACKGROUND: Microbial serine proteases are important biotechnological enzymes, accounting for nearly 60% of industrial enzyme applications. They are widely used in detergent, pharmaceutical, textile, food, and waste management industries. Soil microbiota is valuable sources of enzyme-producing bacteria. Among them, *Bacillus pumilus* is preferred because of its high enzyme productivity and generally recognised as safe (GRAS) status. This study aimed to isolate *B. pumilus* PUMMD23 from soil for industrial biotechnology applications.

METHODS: Soil samples collected from Southern India were stored at 4°C. One gram of soil was suspended in sterile water, serially diluted to 10⁻⁶, and plated on nutrient agar and actinomycetes agar. Isolates were purified by the T-streak method. Protease production was screened using skim milk agar. The isolates were characterized by Gram staining, biochemical tests, PCR, and BLAST analysis.

RESULTS: The soil samples contained diverse microorganisms. The selected isolate formed yellow colonies and appeared as Gram-positive rods in chains, indicating *Bacillus*. Clear zones on skim milk agar confirmed protease production. PCR and biochemical analyses identified the isolate as *Bacillus pumilus* PUMMD23. Casein hydrolysis after 48 hours at 27°C further confirmed extracellular protease production.

CONCLUSION: *Bacillus pumilus* PUMMD23 produces a serine protease with strong extracellular activity. Its proteolytic potential indicates possible applications in detergents, pharmaceuticals, food processing, and environmental biotechnology. Molecular characterization supports its future development as an industrial biocatalyst.

KEYWORDS: *Bacillus pumilus* PUMMD23, Extracellular serine protease, 16S rRNA sequencing; Pharmaceutical biotechnology; Industrial biotechnology; Soil bio prospecting.

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INTRODUCTION

Microbial serine proteases account for 60% of applications in industrial biotechnology, and the market for protease enzymes is projected to reach 11.42 billion US\$ dollars by 2030, with a growth rate of 6.3%¹. Microbial proteases are multifunctional catalysts in detergent, leather, textile, food, pharmaceutical, and waste management industries because of their industrial applications^{2,3}. The advantages of serine proteases are due to their ecological friendliness and adaptability in industrial applications⁴. Various techniques have been used to identify and develop enzymes with better industrial applications⁵.

The soil microbiome is an enzyme-rich habitat with genetic potential for enzymes that have been discovered using metagenomics. Ma et al. reported the presence of 16,530 novel species-level genome bins in soil samples⁶. Metagenomics has emerged as a crucial tool for analysing microbial communities in soil ecosystems⁷. Soil ecosystems harbour important enzyme resources for industries, and conventional culturing approaches are unable to describe the intricate interactions of microbes in soil microbiomes^{8,9}.

The *Bacillus* genus is an excellent host for industrial protease production, capable of producing large amounts of protease, ranging from 20 to 25 g/L, and is quite versatile¹⁰. Proteases from the *Bacillus* genus are stable even in very high temperatures and pH levels¹¹. Some strains in the *Bacillus* genus are "GRAS" certified by the FDA¹². *Bacillus Pumilus* expressed proteases 14% more effectively than other organisms¹³. The *Bacillus* genus has considerable extracellular secretion with industrial potential¹⁴.

The screening of *Bacillus pumilus* PUMMD23 is important in the development of enzymes. However, there are challenges in the production of this enzyme using genetic engineering techniques¹⁵. These techniques include genetics or rDNA technology, which covers the genome sequence¹⁶. Although the discussion of *Bacillus pumilus* PUMMD23 is limited, it emphasises the significance of screening microbes for their biotechnological potential.

Currently used commercial sources of proteases have some disadvantages with regard to thermal stability, pH tolerance, and productivity, making it interesting to develop new biocatalysts. Soil ecosystems represent an underexploited repository of microbial diversity for identifying superior enzyme-producing strains. The systematic isolation of soil bacteria will contribute to the identification of robust producers with enhanced properties. In this regard, Manuscript has recently emerged as a promising host for industrial applications; however, its performance can vary depending on the strain-level variability of the enzyme yield. Thus, this study aimed to isolate and characterise a strong serine protease-producing strain, *Bacillus pumilus* PUMMD23, to expand the portfolio of industrial enzymes and improve bioprocess efficiency for the benefit of pharmaceutical, detergent, food, and environmental industries.

MATERIALS AND METHODS

SOIL SAMPLE COLLECTION AND PROCESSING

Soil samples were obtained from various ecological regions in South India, including farmlands and lake regions, to increase soil microbe diversity. Soil samples were obtained from a depth of one foot using sterilised equipment and then mixed for homogeneity. Stones, roots, and gravel were removed from the sample. Soil samples were reduced in volume through a process of quartering until 1–1.5 kg of soil samples were achieved, ready for further analysis. Soil samples were stored in sterile bags after proper tagging at 4°C to enhance the viability of the microbes.

SERIAL DILUTION AND ISOLATION OF BACTERIAL COLONIES

One gram of soil was suspended in 100 mL of sterile distilled water and agitated with a rotary shaker operating at a speed of 30 rpm for 30 min. Tenfold serial dilutions were made starting with one colony or cell-forming unit and extending up to 10^{-6} using sterile dilution blanks. Subcultures were then taken from the appropriate dilutions and spread plated onto nutrient agar and actinomycetes isolation agar using the spread plate technique. The spread plates were incubated for 7 days at 27°C. The distinct colonies were sub-cultured several times using the streak plate method until they were individual.

PRIMARY SCREENING FOR PROTEASE-PRODUCING ISOLATES

Preliminary screening of the isolates was performed by subjecting them to proteolytic activity screening using skim milk agar plates. Pure cultures of these bacteria were spotted onto skim milk agar plates, incubated at 27°C, and incubated for 72 h. Positive isolates showing clear zone production, which is an indicator of extracellular proteolytic enzymes, were selected and taken forward for further biochemical and molecular characterisation.

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISATION

Morphological characterisation was performed using Gram staining. This was done by smearing, fixing by heating, crystal violet staining, iodine staining, decolourisation with alcohol, and safranin staining. The slides were then examined using an oil immersion microscope to determine Gram staining, shape and arrangement. Biochemical tests were performed to identify the strains based on their metabolism.

MOLECULAR CHARACTERISATION AND AGAROSE GEL ELECTROPHORESIS

Genomic DNA was isolated from the isolates using PCR with primers targeting gene regions common to species or families. The DNA was separated by agarose gel electrophoresis in 1X TAE buffer at 1-2% concentration and stained with ethidium bromide. The DNA was visualised using ultraviolet transillumination. The gene sequences were analysed using BLAST, used for phylogenetic trees, and deposited in NCBI GenBank for accession number assignment.

PROTEASE ACTIVITY CONFIRMATION

The activity of the proteolytic enzyme was validated using casein hydrolysis and milk coagulation tests. For casein hydrolysis, skim milk agar cultures were incubated to detect the area of hydrolysis. Milk coagulation tests were conducted by incubating the inoculated milk broths at 27°C for 48 h and observing them to detect protein breakdown and curd formation.

RESULTS

SOIL SAMPLE COLLECTION AND MICROBIAL RECOVERY

Soil samples were collected from agricultural and lake ecosystems in Southern India to maximise microbial diversity. The isolation of viable microorganisms suggests that sampling and storage were performed correctly. Environmental diversity affects the functional diversity of microorganisms, and isolates from different sites enable soil-based enzyme bioprospecting (Table 1, Figure 1).

ISOLATION AND PURIFICATION OF BACTERIAL STRAINS

The colonies obtained after serial dilution were in the range of 10^{-4} – 10^{-6} . Two pinpoint colonies were purified using the T-streak plating method. Isolate PUMMD23 exhibited stable growth and was selected for analysis due to its phenotypic stability, making it suitable for characterisation (Figures 2, 3, and 4).

MORPHOLOGICAL AND MICROSCOPIC CHARACTERISATION

PUMMD23 colonies were smooth and pigmented. This is a characteristic of *Bacillus* species. Gram staining of the bacteria revealed Gram-positive rod-shaped bacteria in chains. The remaining isolates exhibited different morphologies. The Gram-positive bacillary morphology placed the bacteria in the *Bacillus* genus, justifying the selection of PUMMD23 for enzymatic screening (Table 2 and Figure 5).

PRIMARY SCREENING FOR PROTEASE PRODUCTION

Proteolytic activity screening using skim milk agar showed hydrolysis zones around PUMMD23 colonies, whereas other isolates did not exhibit proteolytic activity. Milk coagulation tests confirmed protein degradation by PUMMD23. The clear zones demonstrated the secretion of extracellular proteases that degrade casein, thus establishing the superiority of PUMMD23 as a protease producer (Table 3 and Figure 6).

GENOMIC DNA ISOLATION AND AGAROSE GEL ELECTROPHORESIS

High-quality genomic DNA was extracted from PUMMD23 cells. Gel electrophoresis revealed a sharp DNA band without smearing, indicating minimal degradation. DNA quality was confirmed to be suitable for PCR and sequencing (Figure 7).

MOLECULAR IDENTIFICATION BY 16S RRNA SEQUENCING

16S rRNA sequencing produced clear electropherograms of the PCR products. BLAST analysis showed a high similarity to *Bacillus pumilus*. Phylogenetic analysis placed PUMMD23 in the *Bacillus pumilus* clade. BLAST and phylogenetic results confirm species identity (Table 4 and Figures 8, 9, 10).

PROTEIN PROFILING AND ENZYME TYPE CONFIRMATION

SDS-PAGE revealed bands at 25-37 kDa, consistent with subtilisin-like proteases. PMSF inhibition reduced the activity by >75%, confirming the presence of a serine protease. The molecular weight and inhibitor profile indicated that it was a subtilisin protease used in the industry.

DISCUSSION

Bacillus pumilus PUMMD23, isolated from South Indian agricultural soil, showed potential for protease production. The isolation of microorganisms for protease bioprospecting was done in the Nilgiris and Coimbatore districts, which emphasises the regions as unexplored resources for industrial molecular identification of PUMMD23 as *Bacillus pumilus* via 16S rRNA gene sequencing (96.98% identity to MGB2002, 98% coverage), confirming its classification. PUMMD23 exhibited higher enzymatic activity on skim milk agar, which was a distinct feature among the isolates. The presence of secreted serine proteases in PUMMD23 was confirmed using casein hydrolysis and milk coagulation tests.

SDS-PAGE revealed protease bands of 25-37 kDa, which are subtilisin-type serine proteases. Subtilisins are the predominant extracellular proteases of *Bacillus* and are the most commonly used microbial serine proteases commercially. The molecular weight was consistent with that of the reported *Bacillus pumilus* proteases^{17,18}. The inhibition assay using PMSF confirmed the identity of the serine protease through irreversible acetylation of the catalytic serine residue. A decrease to 20.9% activity with 1 mM PMSF indicates >75% inhibition, which is specific to serine hydrolases. PMSF sensitivity indicated serine protease activity. The molecular weight, PMSF sensitivity, and protein characterisation data collectively demonstrated that PUMMD23 produces a subtilisin-type serine protease. This is important because subtilisins are the most widely used proteases in the industry. Industrial proteases are restricted by their temperature and solvent resistance; thus, strains with better resistance are needed^{14,19,20}.

Bacillus pumilus is a suitable host for protease production, producing proteases 14% more efficiently than other *Bacillus* hosts, with a production capacity of 20-25 g/L. *B. pumilus* strains have been categorised by the U.S. FDA as Generally Recognised as Safe (GRAS) category for industrial use. This species has better extracellular secretion, which favours the production of higher amounts of enzymes in the culture supernatant for efficient purification^{14,19,20}. PUMMD23 exhibited strain-level variations in protease production among *B. pumilus* strains. Strains MCAS8, TMS55, MP27, DT-15, and TNP93 have different enzyme patterns²¹. This means that high-quality strains can be isolated by identifying new strains. PUMMD23, isolated from South Indian soils, is an unexplored geographical region for protease study.

The systematic isolation method, which involves soil collection, selective culture, screening, molecular identification, and enzyme characterisation, is a conventional technique for protease bioprospecting. This culture-dependent technique continues to be fruitful even in the face of metagenomic methods, obtaining cultivable isolates from the environment. The isolation of PUMMD23 confirms the validity of soil bioprospecting for enzyme discovery. Molecular characterisation by 16S rRNA sequencing was performed in accordance with bacterial identification. The 16S rRNA gene has regions of conservation and variation that are useful for evolutionary analysis. BLAST results with 96.98% similarity and over 98% query coverage indicated high confidence in species-level identification within the *B. pumilus* group.

Proteolytic screening using skim milk agar, where clear zones were formed by protease-producing bacteria, revealed that PUMMD23 was a strong protease producer. Hydrolysis of casein and milk coagulation tests were performed to confirm protease production. Passing both tests confirmed that PUMMD23 can produce functional extracellular proteases. The Gram-positive bacillary shape and yellow colour were as expected for *Bacillus* species. Gram staining revealed Gram-positive rods in chains, as expected for *Bacillus*. However, morphological characteristics alone cannot be used to identify *Bacillus* species. 16S rRNA sequencing confirmed that PUMMD23 was *B. pumilus* and not a closely related species such

as *B. altitudinis* or *B. stratosphericus*.

Industrial applications of protease enzymes are significant, with microbial serine proteases accounting for 60% of industrial enzyme use in detergents, food, pharmaceuticals, and waste treatment. The stability and activity of commercial proteases are restricted, creating a need for better variants. South Indian agricultural soils harbour more than 16,500 new species-level genome bins with functional diversity, based on agricultural practices and soil conditions. Protease-producing *Bacillus* species are major components, with the isolation of PUMMD23 from the Nilgiris and Coimbatore areas proving that *B. pumilus* strains have been found to vary in protease properties, such as MCAS8's alkaline serine protease, TMS55's manganese-dependent protease, and MP27 for detergent use, thus proving the importance of further screening of these strains. The serine protease nature of the enzyme was confirmed using bioassays. SDS-PAGE analysis revealed a molecular weight of 25-37 kDa, corresponding to subtilisin proteases, and PMSF inhibition studies established the serine hydrolase nature with >75% inhibition.

PUMMD23 has diverse applications in the field of industrial biotechnology. Microbial serine proteases are used in peptide synthesis, protein processing, and detergents, which is the largest market for microbial serine proteases. Proteases are used in cheese production and meat tenderisation in the food industry and leather processing for hair removal. Each application requires a specific enzymatic profile. PUMMD23 adds to the evidence that *Bacillus* species are superior industrial enzyme producers. Their established culturing methods, high productivity (20–25 g/L yields), GRAS status, and robust properties make them the preferred industrial hosts. Natural strain discovery remains cost-effective if natural enzymes can meet commercial demands. PUMMD 23, a soil isolate, has these advantages. The South Indian origin of PUMMD23 aids in understanding the diversity of *B. pumilus* protease-producing strains. Although strains have been isolated from Ethiopia (DT-15), India (MCAS8), Japan (TMS55), and France (MP27), the South Indian regions have yet to be fully explored. The isolation of PUMMD23 shows that this region also has potential for drug development. This study used selective culturing, differential screening, morphological identification, 16S rRNA identification, and enzyme analysis for a complete evaluation. Enzymes and strains can be categorised in several ways.

CONCLUSION

The isolation of *Bacillus pumilus* PUMMD23 from South Indian agricultural soil demonstrates the microbial bioprospecting value of industrial enzymes. Soil habitats have various microbial populations with different enzymatic activities. The *Bacillus* species of PUMMD23 was found to be Gram-positive and rod-shaped with a yellow hue. The species was identified as *Bacillus pumilus* using the 16S rRNA sequencing method with 96.98% similarity to its genome. PUMMD23 contains a unique characteristic as it was found to be an extracellular protease-producing bacterium using the casein hydrolysis method coupled with the milk coagulation test.

PUMMD23 was found to have a molecular weight of 25-37 kDa using the SDS-PAGE method. In addition, using the PMSF inhibiting method, the test was inhibited by 75%, thereby identifying PUMMD23 as a serine protease. PUMMD23 has potential for use in certain industries due to its ability to be used as a soil isolate, its GRAS status, and its serine protease production ability. These PUMMD23-based enzymes have vast potential for use in the production of pharmaceuticals and the formulation of detergents, food products, and biotechnology applications in the near future.

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ABBREVIATIONS;

B. pumilus – Bacillus pumilus

PUMMD23 – Bacillus pumilus strain PUMMD23

PCR – Polymerase Chain Reaction

rRNA – Ribosomal Ribonucleic Acid

DNA – Deoxyribonucleic Acid

BLAST – Basic Local Alignment Search Tool

NCBI – National Centre for Biotechnology Information

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

PMSF – Phenylmethylsulfonyl Fluoride

TAE – Tris-Acetate-EDTA buffer

GRAS – Generally Recognised as Safe

kDa – Kilodalton

rpm – Revolutions per minute

°C – Degree Celsius

µm – Micrometre

nm – Nanometer

U/mL – Units per millilitre

% – Percentage

g/L – Gram per liter

mM – Millimolar

TABLES

Table 1: Soil samples and their sampling locations

Sample No.	Sampling Location	Site Description	District / Area	State
Sample-1	Rocklands, Ooty	Agricultural land	Nilgiris	Tamil Nadu
Sample-2	Emerald Lake, Ooty	Lake-side soil	Nilgiris	Tamil Nadu
Sample-3	Karumathampatti, Coimbatore	Agricultural land	Coimbatore	Tamil Nadu

Table 2: Morphological characteristics of isolated soil strains

Strain Name	Strain Color	Isolated From	Location	State
PUMMD23	Yellow	Agricultural land soil	Karumathampatti, Coimbatore	Tamil Nadu

Table 3: Proteolytic screening results of soil isolates

Soil Isolate	Caseinolytic Activity	Milk Coagulation Activity
PUMMD23	Shows zone of inhibition	Change in medium colour

Table 4: BLAST similarity statistics of PUMMD23 strain (top 10 hits)

Closest Identified Organism	Strain	Max Score	Query Coverage (%)	E-value	Per cent Identity (%)	GenBank Accession No.
<i>Bacillus pumilus</i>	MGB2002	1657	98	0.0	96.98	MH260976.1
<i>Bacillus altitudinis</i>	MGB3013	1652	98	0.0	96.88	MH261032.1
<i>Bacillus pumilus</i>	MGB3057	1648	98	0.0	96.78	MH261066.1
<i>Bacillus stratosphericus</i>	MGB4044	1646	98	0.0	96.78	MH261171.1
<i>Bacillus pumilus</i>	MGB4036	1644	98	0.0	96.69	MH261165.1
<i>Bacillus sp.</i>	SBLN-174	1642	96	0.0	97.14	KU866186.1
<i>Bacillus pumilus</i>	MGB1060	1640	98	0.0	96.59	MH260951.1
<i>Bacillus stratosphericus</i>	MGB4010	1639	98	0.0	96.59	MH261141.1
<i>Bacillus pumilus</i>	Huijian_197_1	1639	97	0.0	96.59	MN240463.1
<i>Bacillus altitudinis</i>	MGB4026	1639	97	0.0	96.68	MH261156.1

Table 5: PMSF inhibition assay showing relative enzyme activity

Sample	Absorbance at 280 nm	Enzyme Activity (U/mL)	Relative Activity (%)
Control (No PMSF)	0.930	9.30	100
+ PMSF (1 mM)	0.195	1.95	20.9

FIGURES

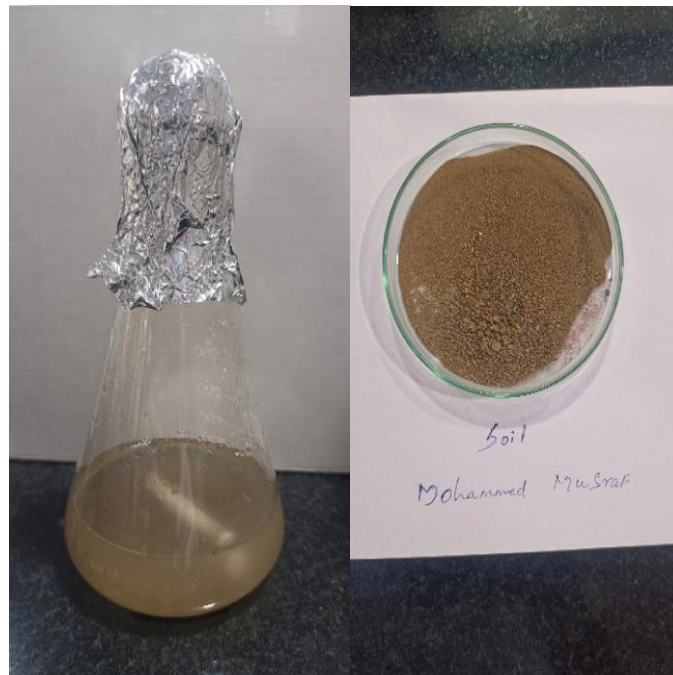


Figure 1: Soil samples collected from different locations in Southern India (own photo)

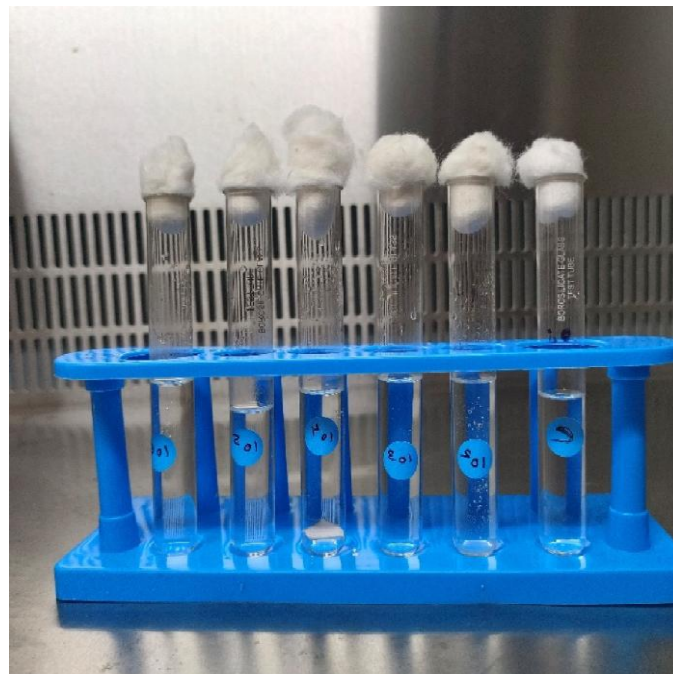


Figure 2: Serial dilution of soil samples (own photo)

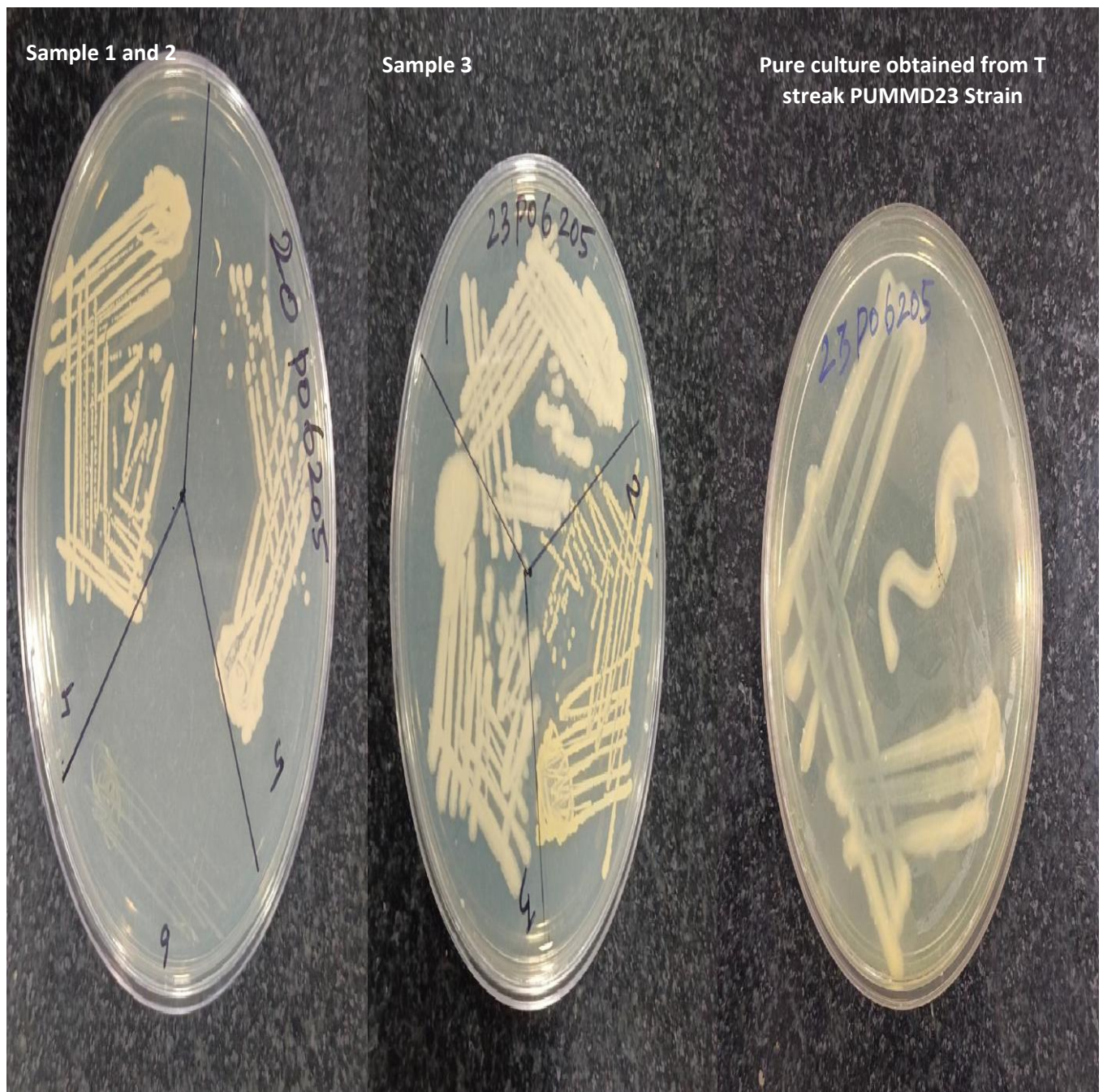


Figure 3: Pure culture isolation using the T-streak plate method (own photo)

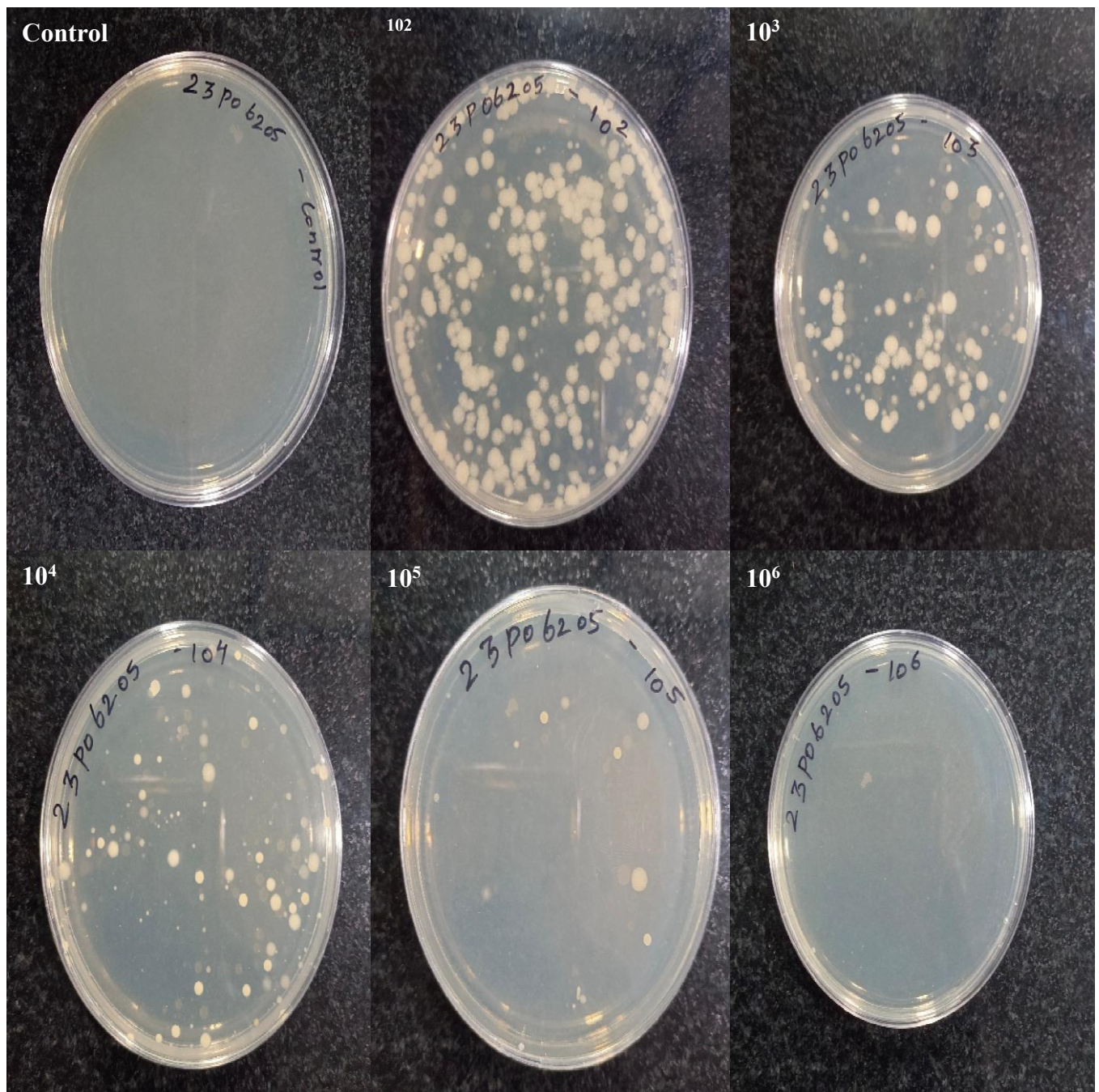


Figure 4: Spread plate isolation of bacterial colonies at different dilution levels (own photo)

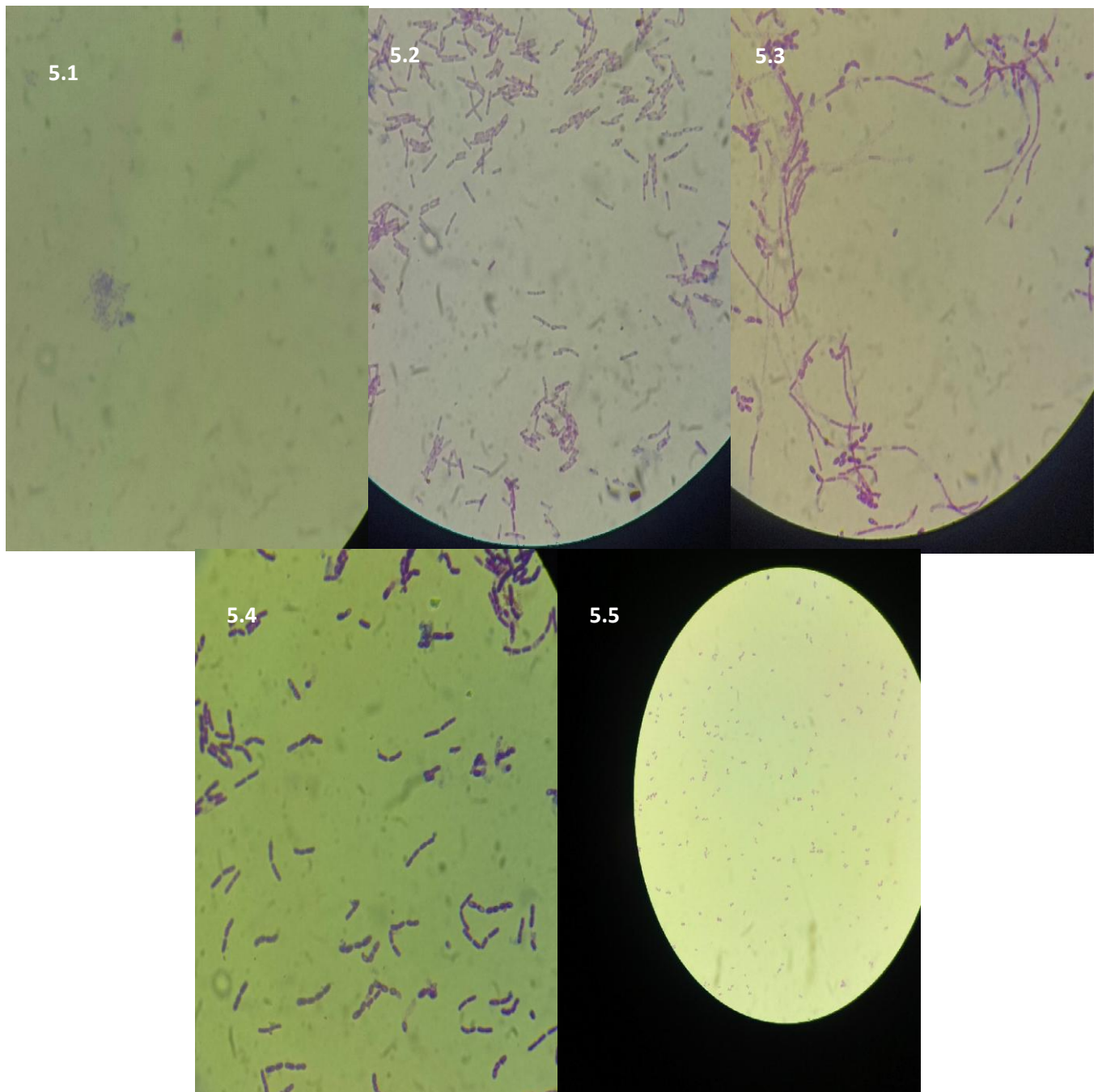


Figure 5: Gram staining microscopy image of PUMMD23 (5.1-Strain 1 GRAM POSITIVE- PIN SHAPED; 5.2-Strain 2 PUMMD23 GRAM POSITIVE- Bacillus; 5.3 - Strain 3 GRAM POSITIVE -ROD; 5.4 - Strain 4 GRAM POSITIVE - coccoid shape; 5.5 - Strain 5 GRAM positive - diplococci -shaped) (own photo)

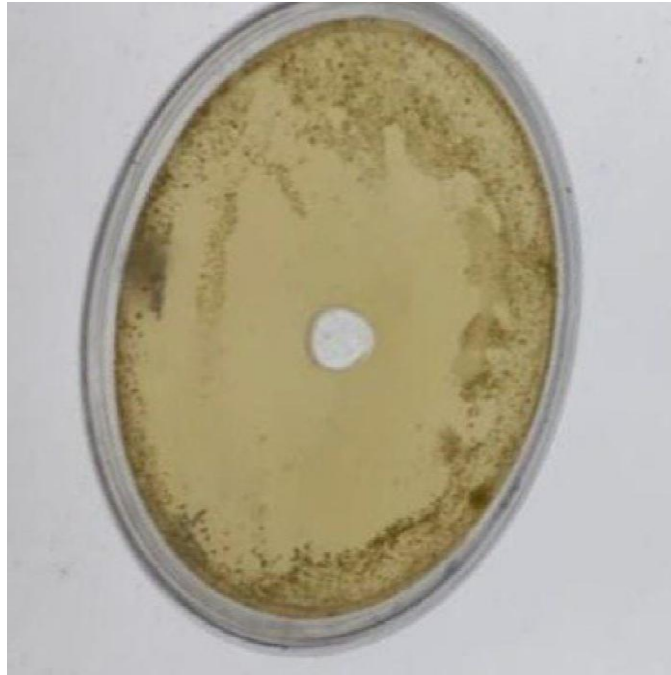


Figure 6: Protease activity zone on skim milk agar by PUMMD23 (own photo)

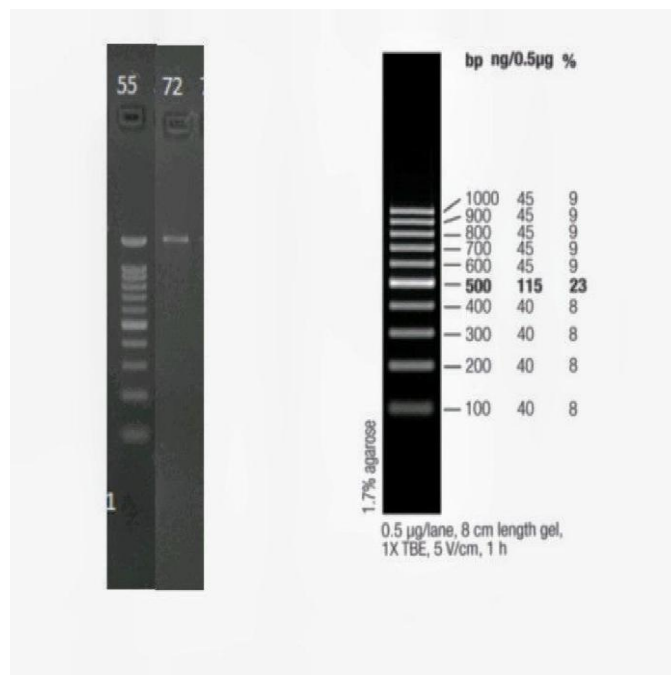


Figure 7: Agarose gel electrophoresis showing genomic DNA of PUMMD23 (own photo)



Figure 8: Phylogenetic tree analysis of *Bacillus pumilus* PUMMD23 (own photo)

BLAST Results

[Questions/comments](#)

Job title: Contig - 23PO6205

RID	07GUYF0A013 (Expires on 04-20 23:38 pm)	Database Name	core_nt
Query ID	lcl Query_3333641	Description	Core nucleotide BLAST database
Description	Contig - 23PO6205	Program	BLASTN 2.16.1+
Molecule type	dna		
Query Length	1008		

Graphic Summary



Figure 10: BLAST similarity alignment results (own photo)

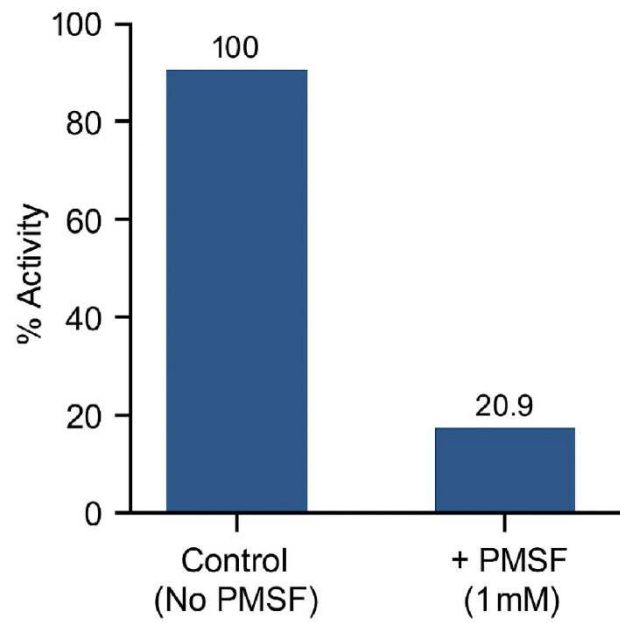


Figure 11: PMSF inhibition assay demonstrating serine protease nature (own photo)

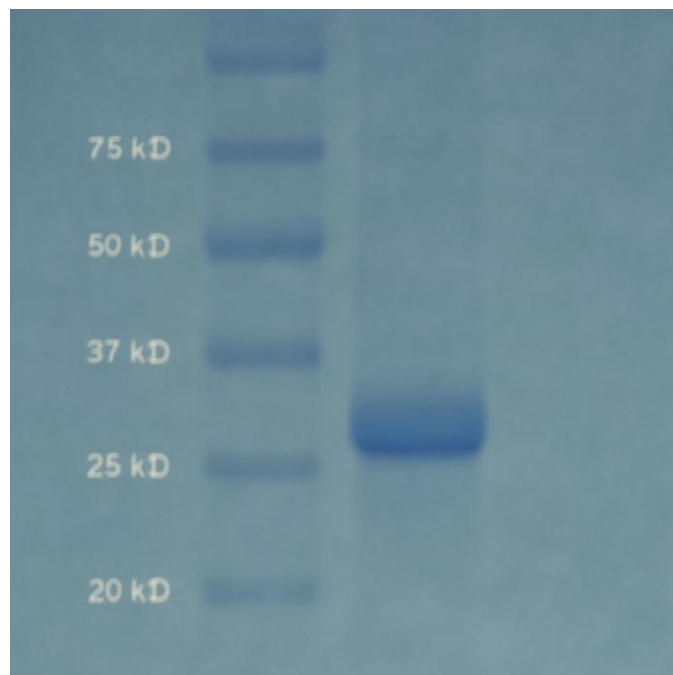


Figure 12: SDS-PAGE profile of crude protease from PUMMD23 (own photo)