St. Xavier's College
(Autonomous), Mumbai

Department of Life Science and Biochemistry

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SLSC501PR

Internal Assessment Project Report

Isolation and identification of unknown protease producing bacteria from soil

Name of the student: Neha Nachanolkar

UID Number: 172059

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Introduction

Proteases are a class of enzymes that catalyze protein molecules by hydrolysis of peptide bonds which connects two amino acids. It is one of the largest group of enzymes. They are ubiquitous, i.e. they are present in all life forms such as bacteria virus, plants and animals. Hence, they play a major role in important physiological processes such as photosynthesis, digestion, regulation of protein function, etc. (1)

They can be classified into three major groups based on their acid behavior; acidic, neutral and alkaline. Acid proteases are commonly produced by fungi and their pH range is 2.0- 5.0. The neutral proteases are produced in plants and their pH range is 7.0- 8.0. Alkaline proteases have a pH range above 8. (2)

Due to their wide range substrate specificity, they have been extensively used in biotechnology industry and other industrial processes. (3) Proteases are most commonly used in laundry, textile and pharmacological industries. Due to the increased dependence on proteases, organisms from different sources are collected and protease producing organisms are isolated. This study analysis presents isolation and identification of protease producing bacteria from soil.

Materials and Methods

To isolate protease producing bacteria:

Serial dilution of sample, Spread Plate and Streak Plate

Materials required:

Culture: From Soil sample

Solutions: Saline- 1 flask of 100 ml

Glasswares: Sterile canister containing sterile 1.0 ml pipettes and 10.0 ml pipettes, sterile empty test tubes (5), sterile nutrient agar (NA) plates (4), Skimmed milk agar (SMA) plates (2), spreader, beaker with alcohol, turn table, test tube rack, incubator at 37°C

Procedure: (in aseptic conditions)

1. With a sterile 10ml pipette, aseptically 9ml of saline was transferred to each of sterile test tubes.
2. 1g of soil sample was weighed and transferred aseptically to the first test tube. It was vortexed and allowed to settle for 10 minutes (10^{-1} dilution).
3. Using a fresh sterile pipette, 1ml culture from 10^{-1} dilution was transferred aseptically to next tube (10^{-2} dilution).
4. Serial dilution of the culture was performed upto 10^{-5} dilution.
5. Spread plate technique was performed. Using fresh sterile 1 ml pipettes, 0.1ml from 10^{-3} dilution was transferred to a sterile nutrient agar plate. (Performed in duplicates).
6. Step 5 was repeated for 10^{-4} dilution.

7. The plates were incubated for 24 hours at 37°C.
8. Similarly, spread plate was performed for 10^{-1} and 10^{-2} dilutions on SMA plates and were incubated at 37°C for 24 hours.
9. A loopful of culture from 10^{-1} dilution was streak plated on SMA plate by hexagonal method and incubated for 24 hours at 37°C.

Streak plating of colonies on SMA plates

Materials required: Sterile SMA plates, nichrome loop and pure colonies from NA plates

Procedure:

1. Different colonies from NA plates (from spread plate method experiment) were streaked aseptically on SMA plates by hexagonal method.
2. SMA plates were incubated at 37°C for 24 hours
3. The pure colonies obtained from SMA plate [isolate 3(3)] was streaked on nutrient agar slants and was preserved for further tests.

To characterize the protease producing bacteria:

Gram Staining

Materials Required: Culture (from 10^{-1} dilution) and pure culture (from NA slants), clean and grease-free slides, nichrome loop, crystal violet stain, iodine solution, acetone- alcohol solution, safranin stain, compound microscope

Procedure:

1. A loopful of culture from 10^{-1} dilution and from pure culture were smeared on glass slides in aseptic conditions.
2. The smears were air dried and then heat fixed.
3. Few drops of crystal violet was added to it for 1 minute.
4. It was then drained off and iodine solution was added to it for 1 minute.
5. Iodine was washed off using acetone-alcohol solution for 15-20 seconds.
6. Safranin was added and was allowed to sit for 3-4 minutes.
7. The slide was washed with running water and was allowed to dry completely.
8. Using oil, the slide was observed under 100X in compound microscope.

Capsule staining

Materials required: Pure culture (isolate), clean and grease free slides, Maneval's stain A & B, nichrome loop, compound microscope

Procedure:

1. Few drops of Maneval's A solution was added on a slide.
2. Loopful of culture was then smeared aseptically on the slide and was allowed to air dry.

3. The slide was flooded with Maneval's B solution and kept for 20 minutes.
4. The slide was dried and then observed under 40X in compound microscope.

Isolation of Genomic DNA from bacteria

Materials required: Pure culture (culture from SMA plates- isolate from 3rd plate), sterile eppendorf tubes, super optimal broth, Tris-EDTA (TE) buffer, 10% SDS, 6M Sodium perchlorate, chloroform, chilled ethanol, micropipettes (1000 µl), sterile pipette tips, centrifuge machine

Procedure:

1. The culture was inoculated to super optimal broth (SOB) overnight.
2. 1.5ml of overnight culture was taken in an eppendorf tube and centrifuged at 14,000rpm for 5 minutes in ice. This was repeated till a pellet is formed.
3. The pellet was re suspended in 400 µl of TE buffer and 100 µl of 10% SDS. It was incubated at 60°C for 10 minutes.
4. Then 100 µl of 6M sodium perchlorate was added and incubated at 60°C for 10 minutes.
5. Equal volume of chloroform (600 µl) is added in eppendorf tube and centrifuged 40,000 rpm at 4°C for 30 minutes.
6. The aqueous phase was separated in a fresh sterile eppendorf and double volume of chilled ethanol was added to it.
7. DNA strands were observed in the eppendorf tube.

Agarose Gel Electrophoresis

Materials required: Extracted genomic DNA from pure culture (isolate), 0.8% Agarose gel, TAE buffer, Loading dye: Bromophenol blue and Ethidium bromide (10 µg/ml), horizontal gel electrophoresis apparatus with power pack, UV trans-illuminator, micro pipettes

Procedure:

1. Gel electrophoresis apparatus was set up with agarose solution in the central part of the tank.
2. 7 µl of genomic DNA was mixed with 2 µl of loading dye and was loaded in wells.
3. The gel is run at about 60-75 volts for 30 minutes.
4. The gel is observed on UV trans-illuminator.

16S rDNA PCR

Materials required: Extracted genomic DNA from pure culture (1 µl), 20 µM forward primer (1µl), 20 µM reverse primer (1 µl), Taq master mix (5 µl), water for injection (2 µl)

Procedure:

1. Genomic DNA (1µl) was mixed with Taq master mix and primers (9 µl) in eppendorf tube.
2. Eppendorf tubes are kept in thermal cycler.
3. Denaturation occurs at 94°C, annealing occurs at 55°C and extension occurs at 72°C. It is repeated 30 times.

4. Agarose gel electrophoresis was performed using PCR products and it was observed.

Catalase Test

Materials required: Pure culture (isolate), H_2O_2, clean grease free slides, micro pipettes

Procedure:

1. Small amount of bacterial colony was transferred on clean, grease free slides.
2. Small amount of H_2O_2 was added to it and was observed.

Motility Test

Materials required: Pure culture (isolate), clean grease free slides, coverslips, petroleum jelly, micro pipettes, compound microscope

Procedure:

1. Few drops of petroleum jelly was applied on the sides of the cavity of slide.
2. Drop of culture was added on the coverslip.
3. The glass slide was turned over the coverslip such that the coverslip sticks.
4. The prepared slide was observed under 40X in compound microscope.

Biochemical Tests

Materials required: Pure culture (isolate), nichrome loop, sterile test tubes, 1ml pipettes, test tube rack

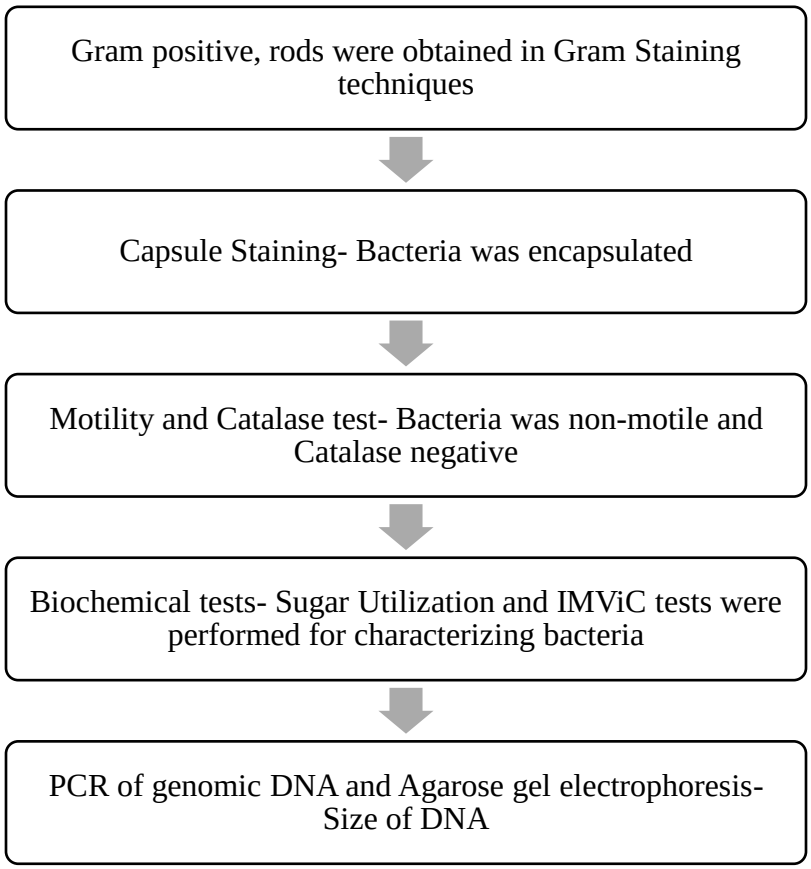
Reagents: Andrades broth with inverted Durhams tube in test tubes along with different sugars such as sucrose, lactose, maltose, xylose, galactose, mannitol, urea broth with phenol red indicator, nitrate broth, glyeraldehyde-6-phosphate (G6P) broth, tryptone water, citrate media, 7% NaCl,

Other reagents: Methyl red indicator, 40% KOH, α-naphthol, Kovac's reagent, sulphanilic acid (in acetic acid) and α-naphthylamine.

Procedure:

1. Loopful of culture was inoculated aseptically in each of the reagents.
2. All test tubes were incubated at 37°C for 24 hours.
3. After 24 hrs, G6P broth was divided into two test tubes. In the first test tube, methyl red test was performed by addition of methyl red indicator to the broth. In the second test tube, Voges- Proskauer test was performed by addition of 40% KOH and α-naphthol to the broth.
4. In tryptone water, indole test was performed. Kovac's reagent is added to the broth.
5. In nitratase broth, nitrate reductase test was performed by addition of sulphanilic acid (in acetic acid) and α-naphthylamine to the broth.
6. Observations for all tests was noted.

Results



1. Spread plate and streak plating was performed in NA and SMA plates. After 24 hours incubation at 37°C-

White, circular opaque colonies with whole margins were observed in NA plates.

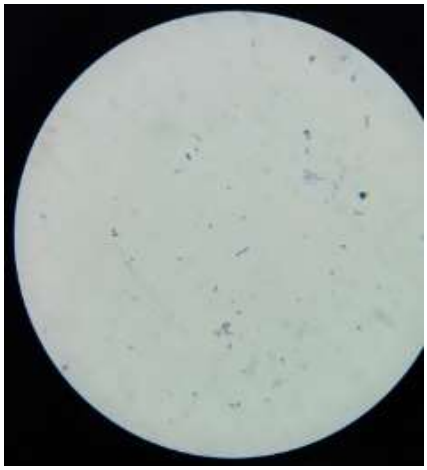
White, circular opaque colonies showing clearance on SMA plates were observed.

Figure 1: NA plates & SMA plates. Plate marked was used for isolate.

2. Preliminary Gram staining was performed using soil sample in saline (10^{-1} dilution).

Sr. no.	Gram Nature	Colour	Morphology	Arrangement
1	Gram Positive	Purple	Short rods	Chains
2	Gram Positive	Purple	Cocci	Clusters

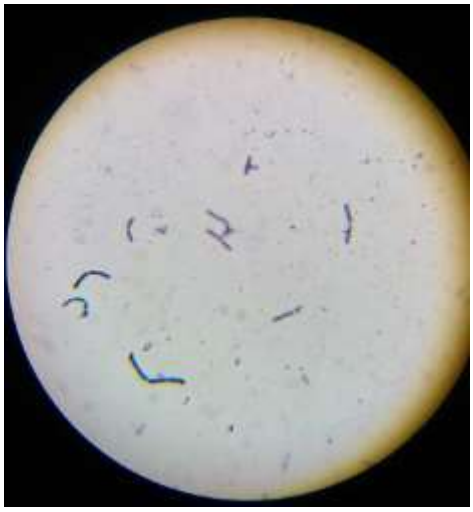
Figure 2: Gram Staining observed under 100X in compound microscope.



3. Gram Staining was performed from the pure colonies of isolate obtained from SMA plates after streak plating

Isolate	Gram Nature	Colour	Morphology	Arrangement
3(3)	Gram positive	Purple	Short rods	Chains

Figure 3: Gram staining observed under 100X in compound microscope

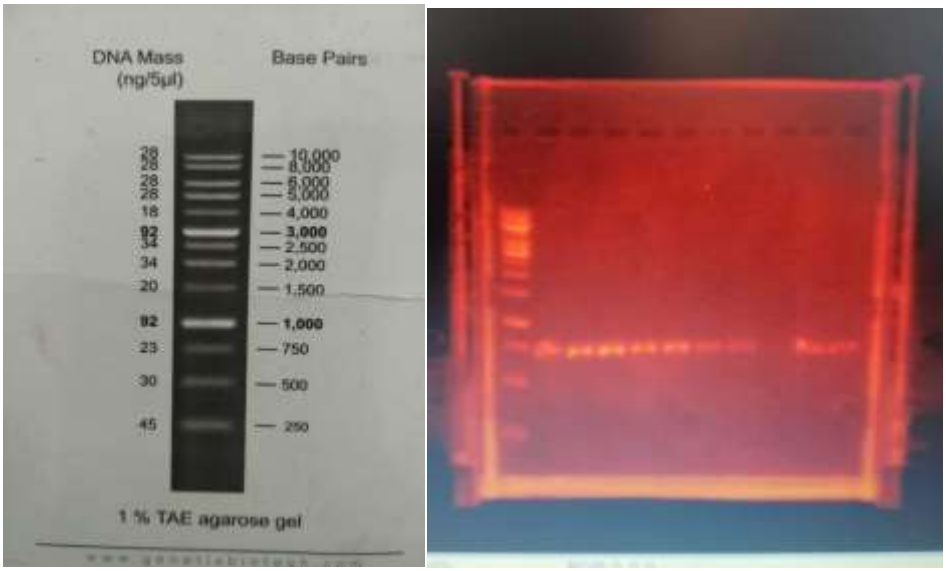


4. Maneval's capsule staining was performed to determine the presence of capsule in bacteria. The bacteria observed were capsulated under 40X of compound microscope.

Figure 4: Capsule staining observed under 40X of compound microscope.

5. Genomic DNA was extracted from bacterial isolates. White strands were observed on addition of ethanol in the eppendorf tube. PCR was performed and the 16S rDNA obtained (PCR products) was run on agarose gel electrophoresis. The size of the genomic DNA of the PCR products was approximately 750 base pairs.

Figure 5: The first well represents the DNA ladder consisting of 13 bands. The fifth well consists of PCR products of genomic DNA of isolate obtained.



6. Catalase test and Motility of the isolates was tested by Hanging Drop method.

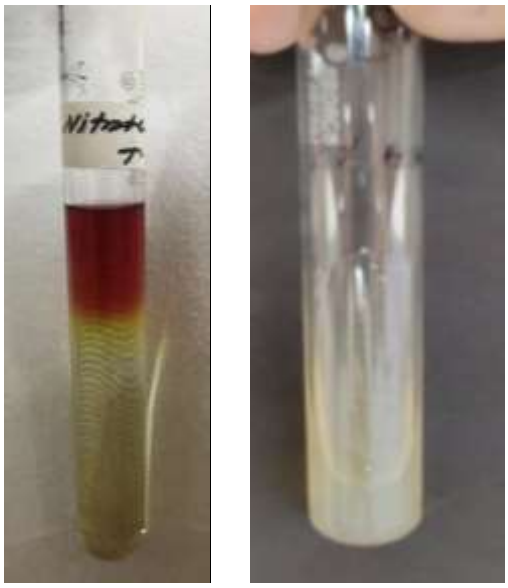
Catalase test was negative for obtained isolate and the bacteria were non-motile.

7. Biochemical tests of the isolates yielded the following results:

TEST	OBSERVATION	INFERENCE
Sucrose utilization	-	Culture does not utilize sucrose.
Maltose utilization	-	Culture does not utilize maltose.
Galactose utilization	-	Culture does not utilize galactose.
Lactose utilization	-	Culture does not utilize lactose.
Xylose utilization	-	Culture does not utilize xylose.
Mannitol utilization	-	Culture does not utilize mannitol.
Urease test	No colour change	Urea is not hydrolysed.
Nitrate reduction test	Red colouration	Nitrate is reduced to nitrite.
Methyl red test	No red layer formation	Methyl red negative.
Vogues-Proskauer test	No pink colouration	V-P negative.
Tryptone water test	No cherry red coloration	Indole negative.
Citrate test	No turbidity	Citrate negative.
7% NaCl tolerance test	+	Culture is 7% NaCl tolerant.

Key: + Growth
- No growth observed

Figure 6: Nitratase test and 7% NaCl resistance test



Conclusion

Isolation of protease producing bacteria was performed by spread plating and streak plating on NA and SMA plates. According to the results obtained from several tests performed and referring to Bergey's Manual of Determinative Bacteriology 7th Edition, the protease producing bacteria could be *Bacillus cereus.*

Discussion

The soil culture consisted of several micro-organisms. By methods such as serial dilution and streak plating technique, the bacteria that were protease-producers, aerobic and those that could sustain in the nutrient medium at incubation of 37°C were obtained. These protease producers were then characterized by several methods. It could be concluded that the protease producing bacteria was gram positive, rods that are non-motile and encapsulated.

In the sugar utilization tests, it could be inferred that the sugar was not utilized completely and hence there was less acid formation leading to no colouration or gas formation. Due to production of less acids, methyl red test also showed negative results. Similarly, due to production of less non-acidic or neutral products VP test is negative. Therefore, the products formed were not highly acidic or neutral to be detected. The culture did not utilize citrate as a source for energy giving citrate test negative. In the urease test, there was no change in colour. This could be due to no formation of urea or due to absence of urease enzyme that converts urea to ammonia. In the nitrate reduction test, red colouration was obsered signifying the presence of nitratase enzyme which converts nitrate to nitrite. In the indole test, no colouration was observed, showing that tryptophan was not hydrolyzed. The 16S rDNA obtained from PCR was used to determine the size of DNA and sequencing was not performed.

The biochemical tests gave inadequate details for the identification of the organism. This could be due to fewer colonies present in the culture or due to insufficient time for the organisms to grow and utilize the sugars or other substrates provided in the medium. Therefore, tests should be performed in duplicates and time of incubation could be increased to enhance the experiment. The catalase test was also shown negative due to insufficient colonies in the isolate. The reagents used should be freshly prepared to avoid false results. Repetitive use of pure culture for various tests could lead to contamination. Hence, the pure colonies should be checked by streak plating for contaminations and a nutrient agar slant should be maintained. Aseptic conditions should be maintained throughout to prevent foreign organisms interfering with the test. Other tests such as Bacara agar tests, hemolytic test and gelatin hydrolysis should also be performed to enable better identification of bacteria.

Acknowledgement

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Grading Rubric:

	GRADED ITEM	Max. Score	Obtained Score
1.	Format Guidelines followed? (margins, font, spacing, title pg, etc.), Neatness, spelling, grammar, nomenclature • Report written in professional, objective manner--no personal pronouns • Have all scientific names been written using proper nomenclature? • Is grammar and spelling proper and accurate? Is report prepared in a tidy fashion according to requirements?	**2**	
2.	Introduction Purpose of study clearly stated	**2**	
3.	Materials and Methods • Was each technique used described in appropriate detail? • Was streak plate isolation described and any problems discussed? • Were isolation and biochemical testing procedures performed correctly? • Was isolation accomplished and pure cultures maintained regularly.	**4**	
4.	Results Tables - • Flow Chart: Is path of action indicated for the unknown, including any extra work? • Is there a complete table of test results showing visual AND interpretive information? • Were results correctly interpreted (info in notebook and in discussion section)	**4**	
5.	Correct ID • Was the unknown correctly identified? • If unknown ID incorrect, was it the result of student error (technique or judgement) or a factor out of the student's control?	**1**	
6.	Discussion • Has student avoided repetition of methods and results in this section? • Have rationales for identifications been adequately described? • Has student demonstrated he/she can make appropriate conclusions? • Has students identified any errors in technique or judgment and suggested alternatives for future work? • Is discussion written in a professional manner? • Have all changes in original plan been discussed?	**3**	
7.	References • Have all required components been included in proper scientific format? • Have all authors been cited, in the order in which they appear?	**1**	
9.	Laboratory working and discussions • Participation in discussions, following proper techniques, following up with results and suggesting logical steps for further tests.	**3**	
10.	Report submitted late (-10% per day)		
	TOTAL	**20**	

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