St. Xavier's College
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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

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INTRODUCTION

Proteases (also known as peptidases or proteinases) are a class of enzymes that break peptide bonds between the amino acids of proteins, thus degrading them. They accomplish this by hydrolysis, and are therefore classified under the hydrolase domain. Along with their primary function as metabolic enzymes for several organisms, proteases are also important for industry. Proteases account for a large proportion of the enzymes used in various industries like dry cleaning, meat processing, dairy products, cosmetics, waste treatment, etc. [1]

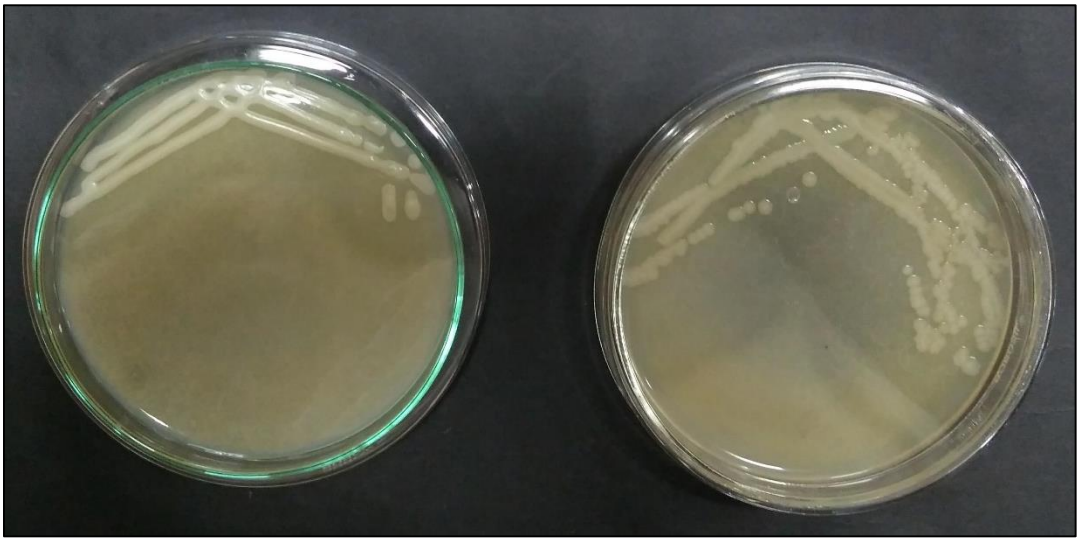
Thus, it is of both biological and economic interest to find novel and efficient methods of obtaining proteases. It is a very wide field of research, especially regarding using bacteria to produce proteases for commercial extraction. Bacteria are a preferred source of proteases; their low space requirement and rapid proliferation make them ideal for industrial scale production of these economically important enzymes. Bacteria are also relatively easy to genetically manipulate and optimize production. Therefore, there is an active search for new bacterial species from which proteases can be extracted.

In this project, we attempted to isolate and identify one species of protease producing bacteria found in local soil sources. We also used this project as an opportunity to familiarise ourselves with laboratory techniques like genomic DNA extraction, gel electrophoresis and PCR amplification.

MATERIALS AND METHODS

Soil sample was collected from a public garden in Sion, Mumbai. 1g soil sample was mixed with 9ml sterile saline, mixed and allowed to settle. The supernatant was used for preliminary Gram staining [2]. The supernatant was serially diluted to 10^{-3}, and the 10^{-1}, 10^{-2} and 10^{-3} dilutions were plated on nutrient agar (NA) and skim milk agar (SMA) plates to screen for protease producers. Colonies that showed zones of clearance after 24h incubation at room temperature were selected as isolates of interest and numbered 3(1), 3(2) and so on.

Clearance around colonies on SMA plates



Pure colonies of isolates 3(3) (left) and 3(4) (right) on NA plates

NA slants of the isolates were prepared for preserving the pure cultures.

Genomic DNA (gDNA) extraction was carried out. 3 ml overnight grown culture in SOB (super-optimal broth) was centrifuged (1.5 ml twice, 14,000 rpm, 5 min each time) on ice and the pellet obtained resuspended in 400 µl Tris-EDTA buffer and 100µl 10% SDS. After incubation at 60°C for 10 min, 100µl 6M sodium perchlorate was added and incubated at 60°C for 10 min. Equal volume of chloroform was added, centrifuged at 14,000 rpm for 30 min at 4°C. The aqueous phase was separated into a fresh sterile Eppendorf tube, double the volume of chilled 100% ethanol was added and mixed gently to precipitate the gDNA out.

Gel electrophoresis of the gDNA was carried out using bromophenol blue as loading dye. PCR amplification of the gDNA was carried out. 10 µl PCR mixture (1 µl gDNA, 1 µl forward 16S rRNA primer, 1 µl reverse 16S rRNA primer**,** 5 µl Taq master mix, 2 µl WFI) was run through 30 cycles of amplification over 10 min followed by gel electrophoresis of the PCR products.

The following biochemical tests were conducted to identify the isolates:

1. Utilization of sucrose/maltose/galactose/lactose/xylose/mannitol using Andrade's indicator (acid fuchsin in NaOH). If sugars are utilized, medium becomes acidic and colour changes pink.
2. IMViC assay [6]:
   a. Indole test: A red ring is formed if the tryptophanase enzyme is active and produces indole, detected by Kovac's reagent.
   b. Methyl red test: Determines whether the microbe performs mixed acids fermentation when supplied with glucose. Positive result is the medium turning red after addition of methyl red indicator
   c. Voges-Proskauer test: A positive test the development of a red colour after the addition of the reagents indicating the presence of diacetyl, the oxidation product of acetoin.

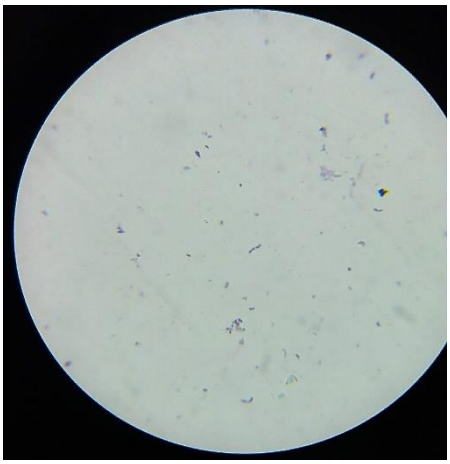
d. Citrate utilization test: Determines the ability of bacteria to utilize citrate as its only carbon source. Bromothymol blue indicator changing from green to blue is a positive result.

3. Urease test: Decarboxylation of amino acids leads to the production of urea, which produces ammonia and carbon dioxide on hydrolysis. The formation of ammonia turns the medium alkaline, indicated by the change in colour from light orange to magenta. An organism that tests positive for urease makes the medium pink. [7]
4. Nitratase test: To determine the ability of an organism to reduce nitrate to nitrite, indicated if a red colour develops quickly (within 1-2 minutes). [8]
5. NaCl tolerance test: 7% sodium chloride and bromocresol purple as a pH indicator is used. The fermentation of dextrose results in the production of acid. This changes the pH of the media causing the media to turn from purple to yellow. [9]

RESULTS

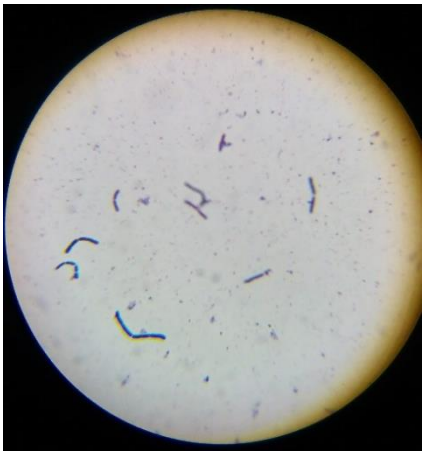
Preliminary Gram staining of the supernatant obtained after diluting the soil sample yielded the following observations:

Sr. no.	Gram nature	Morphology	Arrangement
1	positive	short rods	chains
2	positive	Cocci	clusters



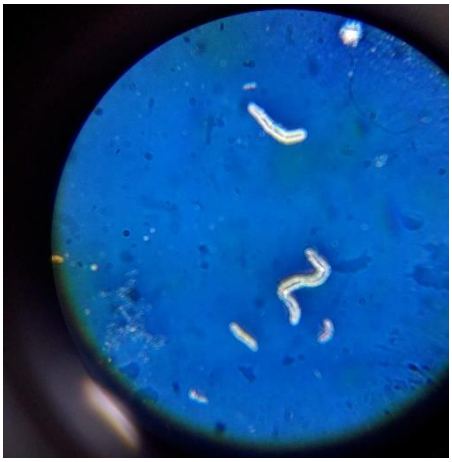
Dissimilar colonies were selected and the isolates grown on sterile NA plates at 37°C for 24h. Gram staining of the isolates was carried out:

Isolate	Gram nature	Morphology	Arrangement
3(3)	positive	rods	chains

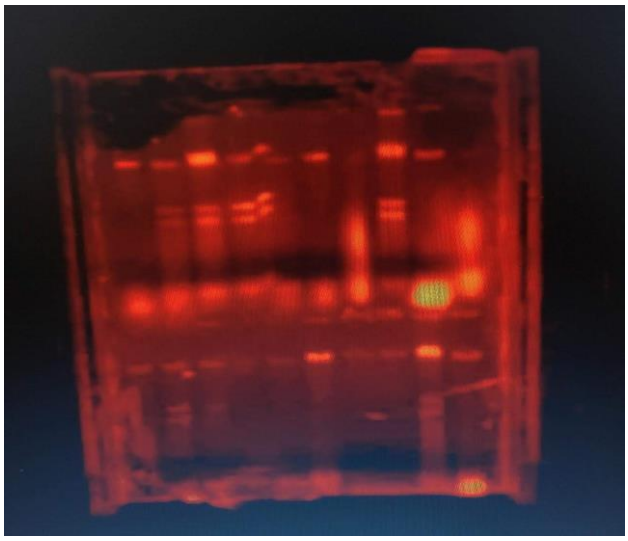


Manewal's capsule staining was carried out

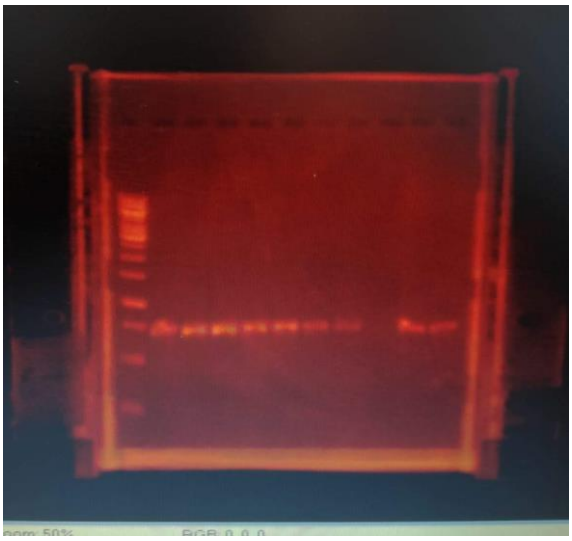
Isolate	Nature
3(3)	Capsulated



gDNA extracted from the bacterial isolates was subjected to gel electrophoresis. Fragments of varying molecular weight were obtained, hence multiple bands are observed under UV illumination (*left*). The products of PCR, subjected to gel electrophoresis. Bands between 750 and 1000 bp are observed under UV illumination. (*right*)



3(3) gDNA in well number 5 of both rows.



Well number 6 contains PCR products of 3(3)

Motility of the isolates was tested

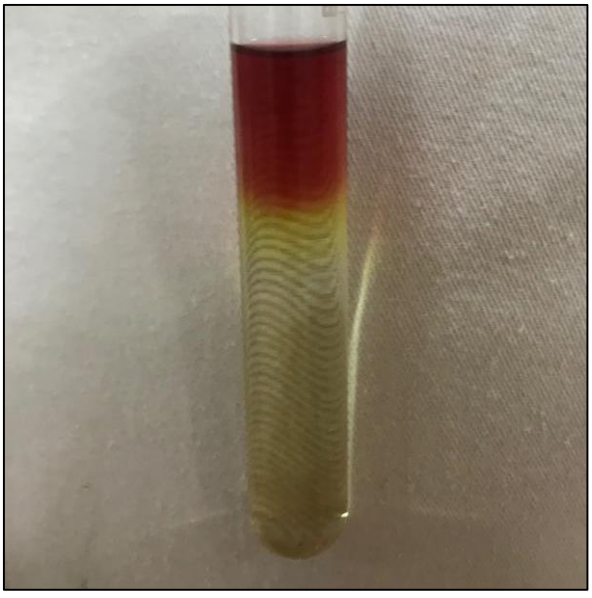
Isolate	Nature
3(3)	Non-motile

Biochemical tests of the isolate yielded the following results:

Key: + positive result - negative result

TEST	OBSERVATION	INFERENCE
Sucrose utilization	-	Does not utilize sucrose
Maltose utilization	-	Does not utilize maltose
Galactose utilization	-	Does not utilize galactose
Lactose utilization	-	Does not utilize lactose
Xylose utilization	-	Does not utilize xylose
Mannitol utilization	-	Does not utilize mannitol
Urease test	-	Does not exhibit urease activity
Nitratase test	+	Exhibits nitratase activity
Methyl red	-	Does not perform fermentation of sugars
Voges-Proskauer test	-	Does not produce acetoin
Indole test	-	Does not exhibit trytophanase activity
Citrate test	-	Does not utilize citrate as carbon source
7% NaCl resistance	+	7% NaCl resistant

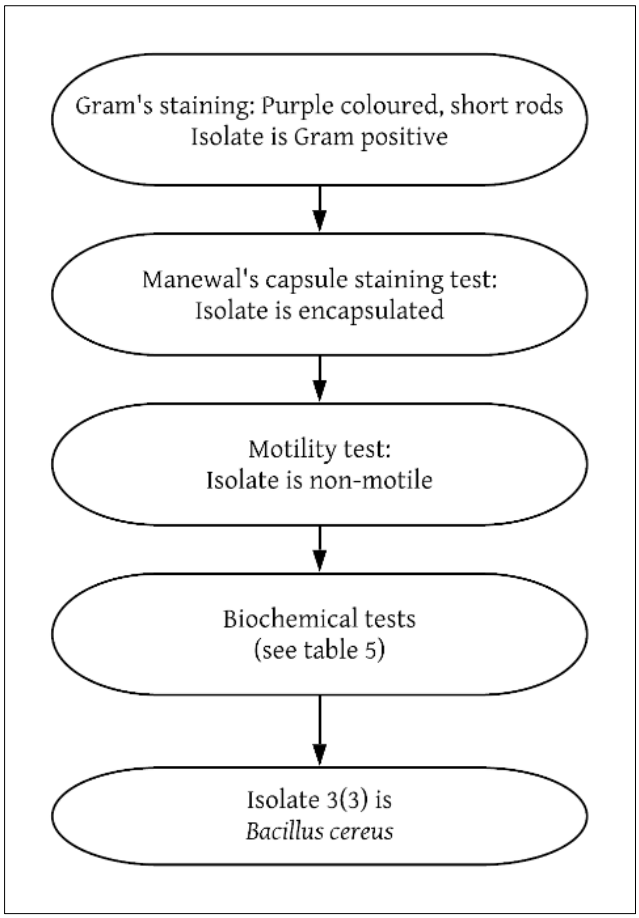
Table 5: Results of biochemical tests



Positive nitratase test

7% NaCl resistance (colonies growing on agar)

The results can be summarised in the following flowchart:



CONCLUSION

Based on the tests conducted and using Bergey's Manual of determinative bacteriology [11], Isolate 3(3) was identified as *Bacillus cereus.*

DISCUSSION

The relative simplicity of the procedure of isolating protease producing bacteria from environmental samples makes it an ideal experiment for students studying genetics and microbiology to perform. Obtaining a pure isolate at the end of an experiment running over several weeks has its challenges however, some of the important ones of which are discussed below.

The first problem we encountered was that there always seemed to be heterogeneity in our isolate whenever it was tested by Gram's staining. This could mean contamination, but that was not always reflected when we cultured our isolate on NA plates. Thus it could also mean that our bacterial species displayed varying morphologies, as some species are known to do. *Bacillus cereus* itself has both motile and non-motile, encapsulated and unencapsulated variants. This added an element of difficulty and uncertainty to our process of isolating and identifying the bacterium of our interest.

Secondly, our group did not only isolate one bacterial species. We had in fact performed all the tests until the end on two isolates: 3(3) and 3(4). However, we have only mentioned 3(3) in our reports because the results with 3(4) were too inconsistent to ascertain the species to which it belonged.

Lastly, a more practical and real-world challenge is conducting a project like this with a class of 28 students, with 4 students per group. It involves having to perform aspects of the experiment in dispersed intervals over several weeks, which can lead to difficulties in coordination. It also leaves the project vulnerable to external factors like weather and lab availability, which can hamper its progress.

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