St. Xavier's College

(Autonomous), Mumbai

Department of Life Science and Biochemistry

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Internal Assessment Project Report

Isolation and identification of unknown protease producing bacteria from soil enriched with Chicken Intestines and Meat.

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ISOLATION AND IDENTIFICATION OF UNKNOWN PROTEASE PRODUCING BACTERIA FROM SOIL CONTAINING CHICKEN INTESTINES AND MEAT

INTRODUCTION

Enzymes are biocatalysts used for enhancing metabolic rate of reactions. A large number of enzymes are produced *in vivo* having great importance in industries. Proteases are enzymes that break proteins into smaller peptides or amino acids by a process known as proteolysis. As they hydrolyse peptide bonds in proteins they are also called peptidases or proteolytic enzymes.

Protease enzyme is naturally present in all organisms and it corresponds to 1-5% of total protein content [1]. Proteases are classified into three groups based on their acid base behaviour, that is, acid, neutral and alkaline proteases [2]. Acid proteases have a pH range of 2.0-5.0 and they are produced only by fungi. Neutral pH proteases range from 7.0-8.0 and they are mainly of plant origin and finally proteases with pH above 8.0 are said to be alkaline proteases [3] and mainly produced by microbes.

Protease enzymes can produce eco-friendly products and so they play a vital role in modern biotechnology industries. The major uses of free proteases occur in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic films, production of digestive and certain medical treatments of inflammation and virulent wounds [4]. In textile industries, proteases are used to remove the stiff and dull gum layer of sericin from raw silk to achieve better lustre and softness [5].

Bacterial Proteases are preferred as bacteria grow rapidly, need less space, are easy to maintain and are accessible for genetic manipulation [6]. Due to their multivariate uses, proteases are in huge demand. The aim of the experiment was to isolate and identify a protease producing bacteria from any particular source. Soil nourished with rotting chicken intestines and meat was chosen. Such a soil is available in abundance around slaughter houses and butcher houses. Meat and intestines consist of proteins which need to be acted upon by proteases in order to be degraded, thus, the surrounding soil is generally a rich source of protease producers.

MATERIALS AND METHODS

The research was conducted in the laboratory of Department of Life Science and Biochemistry at St. Xavier's College (Autonomous), Mumbai, Maharashtra.

Collection of sample

Pieces of chicken intestine and meat were taken from a local butcher and were introduced in a potful of soil and allowed to rot. After 2 days, the soil sample was collected and used for isolation and identification.

Isolation of Microorganism

1g of the soil sample was weighed and diluted with 9mL saline (0.85%) and shook vigorously. It was then allowed to settle and 0.1mL of the supernatant was taken and serially diluted till 10^{-5} dilution. 0.1 mL of dilutions 10^{-3}, 10^{-4} and 10^{-5} were plated in duplicates on Nutrient Agar (0.5% Peptone, 3% Beef/Yeast Extract, 1.5% Agar, 0.5% NaCl, Distilled Water)

plates by spread plate and streaked by T-plate method on one Skimmed milk agar for each dilution. The plates were then inverted and incubated at 37°C for 24 hours [7].

Skimmed Milk Agar plates were used for screening bacteria having proteolytic activity. The bacterial colonies that were isolated and showed a good clearance were selected for further identification tests. The bacteria were maintained by regularly transferring them to fresh, sterile nutrient agar plates/slants.

Table 1: Composition of Skimmed Milk Agar

Ingredients	Gm/litre
SM Powder	28.000
Tryptone	5.000
Yeast extract	2.500
Dextrose (Glucose)	1.000
Agar	15.000
Final pH at 25°C	7.0±0.2

Screening of the Microorganism

The colonies that were isolated and showed clearance on the SMA plates were picked up using a toothpick and further streaked to obtain a pure culture. The colony characteristics like shape, opacity, colour, margin, elevation, etc., were then observed. Morphological tests i.e. Gram staining, capsule staining and motility test were also performed.

1. **Gram Staining**: A thin smear of bacterial culture is made on a slide and heat fixed. A few drops of 1% Crystal Violet are added and left for 1 minute. The stain is gentle washed off and Iodine is added, this serves as the mordant and forms a complex with Crystal Violet. This step is followed by washing with a decolouriser (Acetone-Alcohol) which removes the stain from gram negative bacteria while gram positive bacteria retain the stain. A counter stain (safranin) is added and kept for 3 minutes, this stains the gram negative bacteria. The slide is then observed under 100X with a drop of oil and the bacteria are observed. Purple coloured bacteria are gram positive in nature while pink/red bacteria are gram negative.
2. **Capsule Staining:** A small drop of negative stain like Eosin/ Congo Red is taken on a slide and loopful of bacterial culture is mixed with it under sterile conditions and a thin smear is made. The smear is then allowed to air dry, heat fixing is not done as this leads to shrinking of the capsule. 1% Crystal Violet is added and allowed to remain in the slide for 1 minute. The stain is then drained off and the slide is allowed to dry, once the slide is dry it is observed by oil immersion method under 100X of a compound microscope. Clear zones or Halo around the bacterial cultures indicate the presence of encapsulated bacteria.
3. **Motility Test [18]:** Hanging drop preparation is a special type of wet mount (in which a drop of medium containing the organisms is placed on a microscope slide). In this method a drop of culture is placed on a coverslip that is encircled with petroleum jelly (or any other sticky material). The coverslip and drop are then inverted over the well of a depression slide. The drop hangs from the coverslip, and

the petroleum jelly forms a seal that prevents evaporation. This preparation gives good views of microbial motility.

The following Biochemical Tests were performed as well:

1. **Carbohydrate Utilisation and gas production:** The required sugar is dissolved in Andrade's Peptone water and the bacteria is inoculated into it and kept for incubation. If the microorganism utilises the particular sugar, it results in the formation of CO_2 gas which is trapped in the Durham's Tube (a bubble in the Durham's Tube is taken as a positive test for gas production) and also produces acids which cause a change in the colour of the indicator from light yellow to pink due to change in pH.
    a) Sucrose: Consists of α-D-Glucose and β-D-Fructose subunits.
    b) Maltose: Consists of two α-D-Glucose subunits.
    c) Galactose
    d) Lactose: Consists of β-D-Galactose and β-D-Glucose subunits.
    e) Xylulose
    f) Mannitol
2. **Urease Test [13]:** If the microorganism produces the enzyme urease it leads to the breakdown of urea to carbon dioxide, water and ammonia. The increasing concentration of ammonia in the medium causes an increase in alkalinity and cause the indicator i.e. Phenol Red to change its colour from orange yellow to bright pink.

$$(NH_2)_2CO_2 + 2H_2O \xrightarrow{\text{Urease}} CO_2 + H_2O + NH_3$$

3. **Nitratase test**: This test is used to differentiate between organisms based on their ability to produce Nitrate reductase enzyme which can hydrolyse Nitrate to Nitrite which can then further be degraded to give gas. A loop full of the isolate is inoculated in nitrate broth, which is then tested for the reduction of nitrate by the addition of reagents like sulfanilic acid and α- naphthylamine. If reduction has taken place, the nitrous acid formed will react with sulfanilic acid to form diazotized sulfanilic acid which in turn reacts with α- naphthylamine to form a red coloured compound. Therefore, a red medium is an indicative of a positive test. In case there is no red coloured medium, a small amount of powdered Zinc is to be added. If the medium now turns red, it indicates that there was some amount of unreduced nitrate and therefore a red colour in this step is an indication of a negative result.
4. **Indole Test [16]:** Pure bacterial culture must be grown in sterile tryptophan or peptone broth for 24–48 hours before performing the test. Following incubation, five drops of Kovac's reagent (isoamyl alcohol, para-Dimethylaminobenzaldehyde, concentrated hydrochloric acid) are added to the culture broth. If the bacteria produce tryptophanase enzyme a red or red-violet colour is seen in the alcohol layer on top else the layer remains yellow.
5. **Methyl Red Test [15]:** Some bacteria have ability to perform mixed acid fermentation of glucose in MR-VP medium. The products of mixed-acid

fermentation are a complex mixture of acids, particularly lactate, acetate, succinate and formate as well as ethanol and equal amounts of H2 and CO2. This causes the medium to acquire an acidic pH. Methyl Red is a pH indicator, which remains red in colour at a pH of 4.4 or less.

6. **Voges-Proskauer Test[14]:** VP is a test used to detect Acetoin in a bacterial broth culture. The test is performed by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth which has been inoculated with bacteria. A cherry red colour indicates a positive result, while a yellow-brown colour indicates a negative result.
7. **Citrate Test[17]:** Bacteria are inoculated on a medium containing sodium citrate and a pH indicator such as bromothymol blue. The medium also contains inorganic ammonium salts, which are utilized as sole source of nitrogen. Use of citrate involves the enzyme citrase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and carbon dioxide (CO2). Production of sodium bicarbonate (NaHCO3) as well as ammonia (NH3) from the use of sodium citrate and ammonium salts results in alkaline pH. This results in a change of the medium's colour from green to blue.
8. **Catalase Test [12]:** This test is used to identify organisms that produce the enzyme, catalase. This enzyme detoxifies hydrogen peroxide by breaking it down to H_2O and O_2. This results in effervescence. This test is mostly shown by gram positive bacterial strains.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

9. **High salt concentration tolerance (7% NaCl):** The bacterial strain is streaked on a slant with Nutrient Agar enriched with high concentration of NaCl. If bacteria have tolerance for high concentration of NaCl, the bacteria grow on the medium on incubation.

Isolation of Genomic DNA from the isolated microorganism

Overnight grown culture was inoculated in 10mL of Super Optimal Broth. 1.5mL of the culture was then taken in a micro-centrifuge tube and centrifuged at 14,000 rpm for a minute. The pellet obtained was suspended in T.E. (Tris- EDTA) buffer which acts as a chelating agent and chelates metallic ions. This was followed by addition of 100µL of 10% SDS (Sodium Dodecyl Sulphate) which lyses the DNA. Lysozyme is also added if the culture consists of Gram Positive bacteria. This solution was then incubated at 60°C for 5-10 minutes. Following the incubation 100µL of Sodium perchlorate was added and the contents were mixed vigorously. The solution was again incubated at 60°C for 5-10 minutes. Equal amounts of chloroform was then added till a stable emulsion formed. The solution was then subjected to centrifugation for 20-30 minutes at 14,000rpm. Centrifugation resulted in the formation of three distinct layers- aqueous phase-precipitate- organic phase. The aqueous phase consists of the DNA so was removed in a fresh sterile micro-centrifuge tube and double the amount of chilled ethanol was added. The genomic DNA was then seen precipitating. The obtained DNA was mixed with Tracking dye (Bromophenol Blue) and loaded onto a double decker 0.8%

Agarose gel along with many other samples from different sources. The results were visualised under a UV Transilluminator and images were taken.

Polymerase Chain Reaction (PCR) Analysis

16S rDNA PCR was performed using universal primers. In broad-range 16S rDNA PCR, primers are used that target the highly conserved regions of the 16S ribosomal subunit, present in all bacterial ribosomal genes (rDNA). These subunits also consist of variable regions that differ between bacterial genera and species. Using broad-range 16S rDNA PCR, followed by DNA sequencing, allows the amplification and identification of any bacterial DNA present in a clinical sample. After sequencing, the sequences are compared with known nucleotide sequences on databases such as GenBank to identify the bacteria. The PCR reaction mixture consisted of the following:

Table 2: Components of PCR Reaction Mixture

Component	Volume
gDNA	1µL
Forward Primer	1µL
Reverse Primer	1µL
Taq Master Mix (Buffer, salts, taq polymerase, dNTPs, etc.)	5µL
Water for injection	2µL

The PCR Reaction Mixture was then placed in a PCR Thermal Cycler using the following setting:

Table 3: Setting for PCR Thermal Cycler

94°C (05:00')	94°C (00:30')	55°C (00:15')	72°C (00:45')	72°C (03:00')	10°C
	I----------30X----------I				------II------

The contents were then mixed with Tracking dye (2µL of dye + 7µL of PCR mixture) and gel electrophoresis was performed. A DNA ladder was loaded in one of the wells to compare the results obtained. The results were viewed under a UV Transilluminator and photographs were taken.

Identification of the Microorganism

Morphological, Biological and PCR Analysis tests were used for the identification of bacteria and all results were compared with Bergey's Manual of Determinative Bacteriology [8]

RESULTS

The plates with dilutions 10^{-3} and 10^{-4} showed excessive growth and a spread was obtained thereby individual colonies could not be seen for both Nutrient Agar and Skimmed Milk Agar plates. The plates with dilution 10^{-5} showed individual colonies in the both types of plates and two individual colonies (labelled as 2A and 2B) from SMA were picked up using a toothpick and plated onto another Sterile Nutrient Agar Plate using the T plate and Hexagonal plating method and incubated for 24 hours to obtain a pure culture. The colony characteristics were then studied and were as follows:

Table 4: Colony Characteristics of Strain 2B

Characteristic	Results
Shape	Round
Opacity	White, opaque
Margin	Entire
Elevation	Slightly raised

The following results were obtained from morphological tests:

Table 5: Morphological Results of Strain 2B

Test	Result
Gram Nature	Gram Positive
Shape and Arrangement	Rods existing mostly in chains.
Capsule Staining	Capsulated
Motility	Motile

Fig 1: SMA plate of 10^{-5}

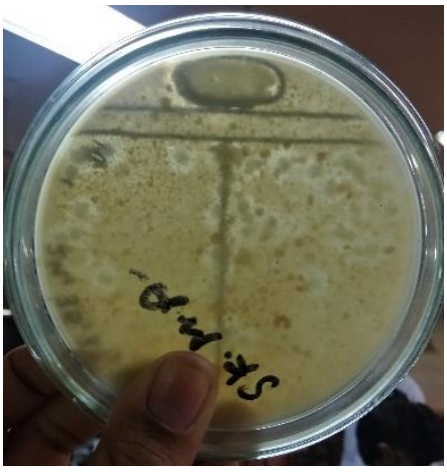


Fig 2: Gram Staining of 2B

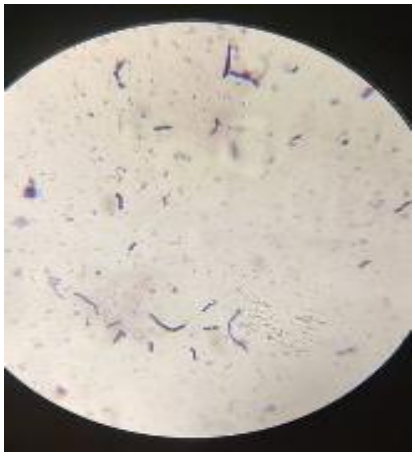


Fig 3: Capsule Staining of 2B

Biochemical Tests yielded the following results:

Table 6: Results of Biochemical Tests for Strain 2B

Test	Result
Carbohydrate Utilization and Gas Production a. Sucrose b. Maltose c. Galactose d. Lactose e. Xylulose f. Mannitol	Negative. No gas bubble. Positive. No gas bubble. Negative. No gas bubble. Negative. No gas bubble. Negative. No gas bubble. Negative. No gas bubble.
Urease Test	Negative
Nitratase Test	Positive. No gas bubble.
Indole Test	Negative
Methyl Red Test	Negative
Voges- Proskauer Test	Negative
Citrate Utilisation Test	No growth
Catalase Test	Slight Effervescence
High Salt concentration tolerance	Growth seen

Fig 4: Growth of 2B on 7% NaCl

Fig 5: Maltose Utilisation, colour changes to pink

A thick band of Genomic DNA along with two thin strands (Figure 6) were obtained post gel electrophoresis and observing under UV Transilluminator. DNA was amplified in the highly conserved 16S ribosomal subunit coding gene sequence regions using universal primers and the amplicon size was found to be nearly 750 base pairs.

Fig 6: UV Trans illumination post Gel Electrophoresis of Genomic DNA

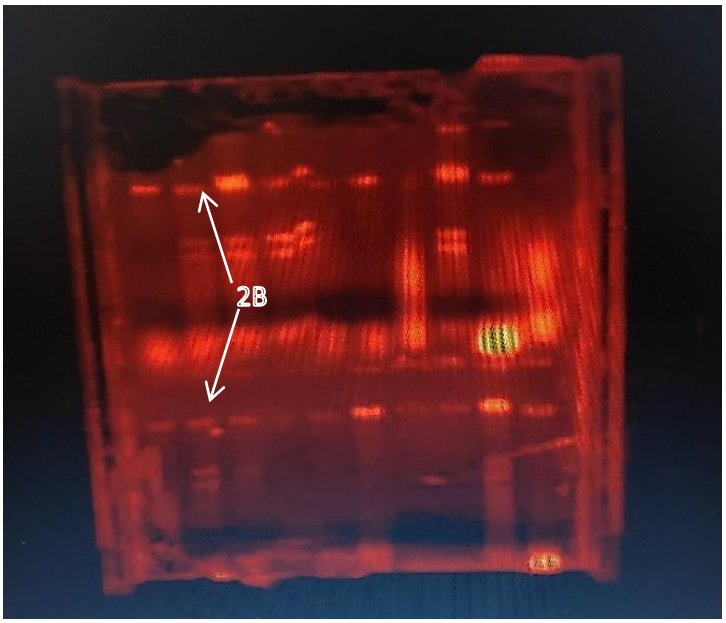


Fig 7: UV Transillumination post PCR

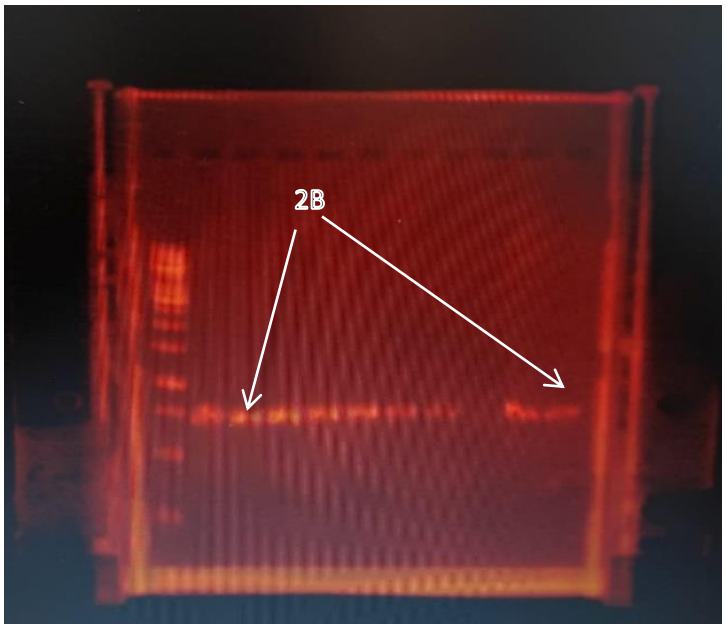
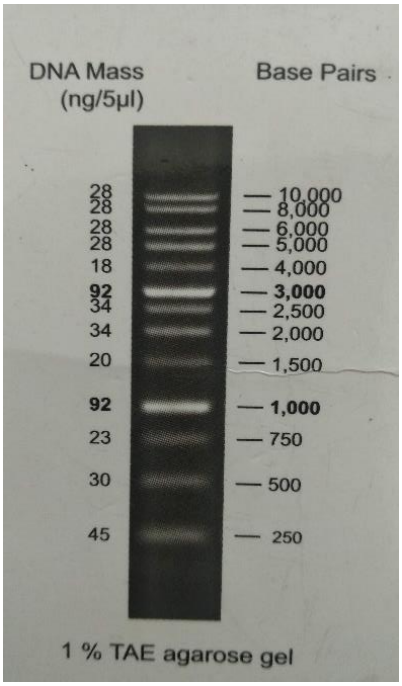
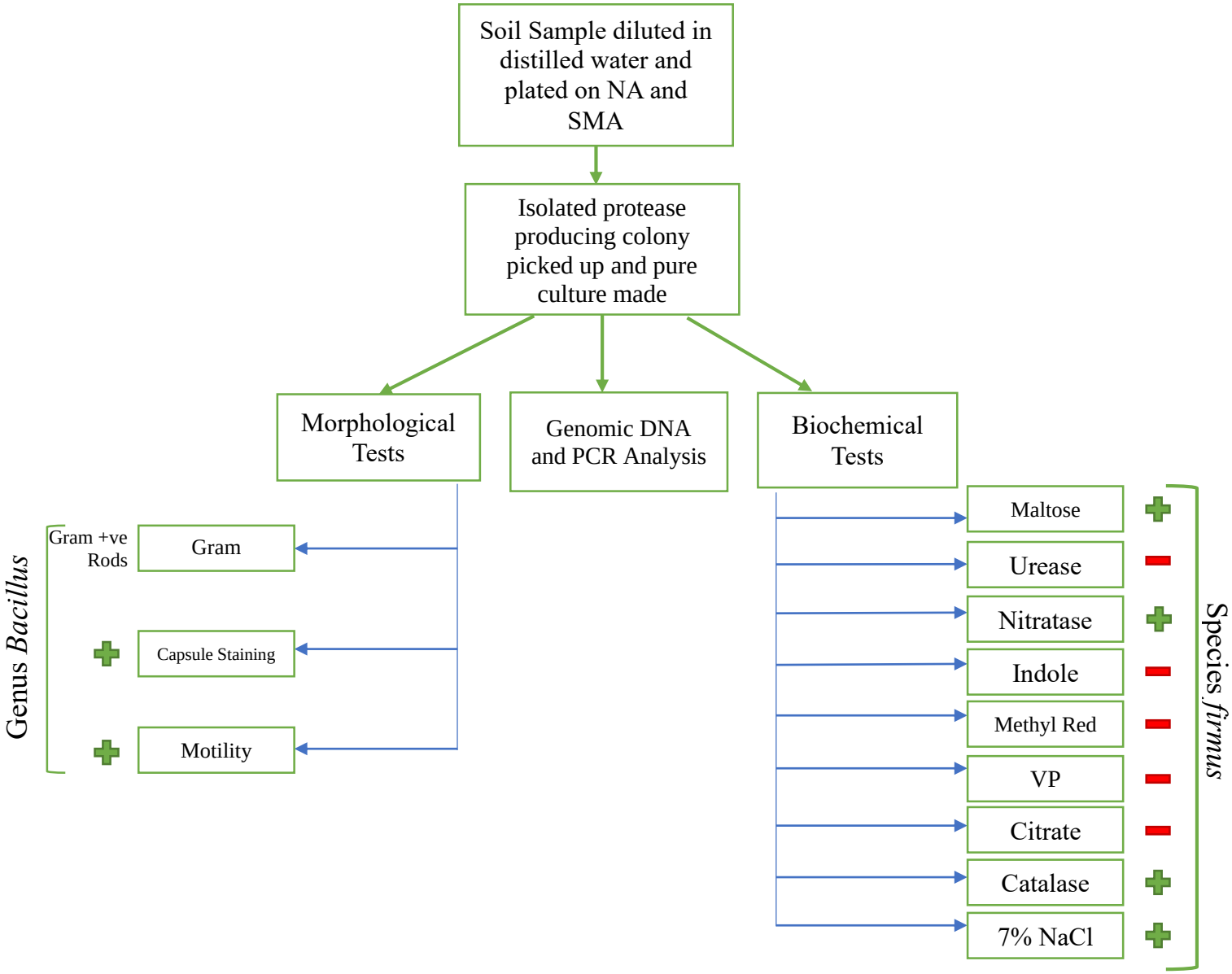


Fig 8: DNA Ladder used for comparison



Flowchart 1: Identification of the Organism



DISCUSSION

Bacteria are the most abundant microorganisms in soil with a population of 10^{10}-10^{11} individuals and 6,000-50,000 species per gram of soil [9]. It is because of this the Nutrient Agar and SMA plates for the first two dilutions plated (i.e., 10^{-3} and 10^{-4}) were overcrowded and isolated colonies could not be obtained. Only the plates with 10^{-5} dilution showed individual colonies which were selected for further screening. Out of the two colonies chosen (2A and 2B) **strain 2B** was chosen arbitrarily for further investigation.

Once pure culture was obtained, gram staining showed gram positive rods arranged in chains i.e. the bacteria retained crystal violet indicating the presence of a thick layer of peptidoglycan in the cell wall. Capsule staining showed the presence of capsule which indicates that the bacteria may have virulent properties. This was an unexpected finding because generally gram positive bacteria do not show a capsule, but it greatly helped narrow down the

choices for identification of the genus and the bacterial species. The bacteria also showed motility.

Results from the Biochemical Tests performed also indicated towards the presence of gram positive bacteria as all the tests performed for gram negative bacteria were found to be negative i.e., IMViC- Indole, Methyl Red, Voges-Proskauer and Citrate. The bacteria were found to be positive for Maltose utilization i.e. it utilised maltose as a source of carbohydrate and cleaved it to give two units of glucose which then enter into glycolysis pathway and help in the production of energy [10]. The bacteria were also found to be Nitratase positive (A cherry red colour was observed after addition of Reagent A) which means that the bacteria reduce Nitrate to Nitrite, there is no production of gas.

The strain showed tolerance for high concentration of salt (i.e. 7% NaCl) as well which means that the bacteria has adapted [11] to survive in high concentration of salt as the area from where the soil was collected was non-saline (fertile soil from St. Xavier's College's flower garden). High salinity is soil would impede with proper growth of the plants. It also showed a weak coagulase test, the weak result could be due to the presence of less bacteria in the culture used for the test as the culture was a fresh one and had not been incubated for too long.

PCR Analysis showed an amplicon of nearly 750 bp. The amplified genes were not sequenced thus phylogenetic analysis could not be done. All of these results obtained were compared with Bergey's Manual of Determinative Bacteriology [8] and the bacteria was found to be ***Bacillus firmus.***

Table 7: Classification of *Bacillus firmus*

Domain	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Bacillus*
Species	firmus

UV Transillumination post Gel Electrophoresis of the genomic DNA isolated showed a thick band and two thin bands indicating that genomic DNA isolation was successfully performed.

CONCLUSION

The isolated bacteria were found to be *Bacillus firmus*. The entire research happened smoothly except for a few instances. It was a little difficult to extract the genomic DNA as the bacteria were encapsulated but even though the genomic DNA could not be seen precipitating post addition of chilled ethanol, gel electrophoresis and UV Transillumination showed three bands thus indicating that genomic DNA had been successfully isolated. While performing the catalase test, the culture taken had not been incubated for a long period of time and as the number of bacteria per volume of the culture was low, weak effervescence was seen on addition of Hydrogen Peroxide. Most of the Biochemical Tests performed were helpful in identification of bacteria by comparing the results to Bergey's Manual of Determinative Bacteriology. The

results could have been more accurate had sequencing of the genomic DNA been performed post PCR. The sequence obtained could have been compared to the data available online and the exact species could have been identified.

The Research Project has been a very good learning experience and has added to the knowledge of the students. The students are now more clear regarding a lot of concepts and the techniques learnt will surely help them in carrying out similar researches in future more smoothly, taking into account the difficulties encountered this time and rectifying them.

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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**	

Teacher-in-charge