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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A. Introduction

Protease is a type of enzyme that catalyses proteolysis, i.e., the breakdown of proteins into smaller amino acids. They break the peptide bonds within proteins by hydrolysis with the help of water. One can find proteases in all forms of organisms, from a single cell organism to a multicellular one. They are a part of almost every biological function including digestion, catabolism and cellular signalling. It can perform various activities like deactivating a protein by digestion, by activating the function of another, or it can be a part of signalling pathway.

With the ever growing population in the world, the use of chemicals in various industries has tremendously increased, adding to adverse environmental conditions. In the last few decades, a lot of research is being carried out on what is known as "Green Chemistry" to replace these detrimental chemicals with environmental friendly products to help reduce the adversity of the planet. To replace the chemical processes with the enzymatic ones has been the prime objective of the modern day scientists.

Enzymes like microbial proteases are the most significant one as it is used essentially in various corporate sectors such as detergent, leather, textile, waste and others. One of the advantages of using proteases is that they are found ubiquitously in plants and animals, as well as microbes. Among various producers of proteases, microbes are the most commercially exploited source for protease because of the ease of production. Protease being a natural compound is undoubtedly considered as a successful eco-friendly alternative to chemicals. Also, since microbes can survive in extreme conditions, the protease produced by them can tolerate a wide range of harsh conditions like high temperature and pH.

B. Materials and Methods

- **Source of sample collection:**

The source of sample was prepared by mixing 10 grams of whey protein extract and 10 grams of spoilt cheese in 50 ml of warm water and keeping it in the open for 24 hours at room temperature. The sample was used to carry out gram staining and the microbial population in the sample was observed.

- **Isolation of the microorganisms:**

The techniques used for isolation of bacteria were serial dilution and spread plate method. 1 ml of the sample was pipetted out aseptically and diluted with 9 ml of saline. The mixture was mixed well using a vortex and the supernatant was taken. The supernatant solution was serially diluted to obtain 10^{-1}, 10^{-2} and 10^{-3} dilutions. 0.1 ml of each aliquot was spread on nutrient agar plates in duplicates and 0.1 ml of $10^{-1}$and 10^{-2} was spread on skim milk agar plates at temperature 37 C for 48 hour.

The zone of hydrolysis was noted for each sample. The colony showing highest zone of inhibition was selected for further study. The colony was grown on skim milk agar plate repeatedly and preserved on nutrient agar slant at 4 °C. Based on the morphological and biochemical tests the bacterial isolate was identified (Sneath et al, 1986).

- **<u>Identification of microorganism</u>:**

The identification of bacteria was carried out by morphological studies i.e. staining including gram staining , capsule staining and motility test. Cultural characterisation on agar plates like colony morphology i.e. shape, size, margin, elevation, opacity, texture and growth in broth was also observed.

Various biochemical test were performed which includes:

- **Catalase test**: Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralise toxic forms of oxygen metabolites; H_2O_2. The catalase enzyme neutralises the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme.

- **Sugar utilisation test**: It tests for the presence of acid and/or gas produced from carbohydrate fermentation. Basal medium containing a single carbohydrate source such as Glucose, Lactose, Sucrose or any other carbohydrate is used for this purpose. A pH indicator (such as Phenol red) is also present in the medium; which will detect the lowering of the pH of the medium due to acid production. Small inverted tubes called Durham tube is also immersed in the medium to test for the production of the gas (hydrogen or carbon dioxide).

- **Indole test**: Indole test is used to determine the ability of an organism to split amino acid tryptophan to form the compound indole. Tryptophan is hydrolysed by tryptophanase to produce three possible end products, one of which is indole. Indole production is detected by Kovac's reagent which contains 4 (p)-dimethylamino benzaldehyde, this reacts with indole to produce a red coloured compound.

- **Methyl red test:** It determines whether the microbe performs mixed acids fermentation when supplied glucose. Types and proportion of fermentation products produced by anaerobic fermentation of glucose is one of the key taxonomic characteristics which help to differentiate various genera of enteric bacteria. In mixed acid fermentation, three acids (acetic, lactic and succinic) are formed in significant amounts. These large amounts of acid results significant decrease in the pH of the medium below 4.4. This is visualised by using pH indicator, methyl red, which is yellow above pH 5.1 and red at pH 4.4. The pH at which methyl red detects acid is considerably lower than the pH for other indicators used in bacteriologic culture media. Thus, to produce a colour change, the test organism must produce large quantities of acid from carbohydrate substrate being used.

- **Citrate utilisation test**: Citrate utlilization test is used to determine the ability of bacteria to utilize sodium citrate as it's only carbon source and inorganic ammonium dihydrogen phosphate (NH4H2PO4) as the sole fixed nitrogen source.

- **Voges Proskauer test**: this test detects if the microorganism produces acetyl methyl carbinol, a product of the butylenes glycol pathway. Pyruvic acid, the pivotal compound in the fermentative degradation of glucose, is further metabolised through various metabolic pathways, depending on the enzyme systems possessed by different bacteria. One such pathway results in the production of acetion (acetyl methyl carbinol), a neutral-reacting end product. In the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and alpha-naphthol serves as a catalyst to bring out a red complex.

- **Urease production test**: Urea is a diamide of carbonic acid. It is hydrolyzed with the release of ammonia and carbon dioxide. Many organisms have an urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.

- **Nitrate reduction test**: Heavy inoculum of test organism is incubated in nitrate broth. After 4 hrs incubation, the broth is tested for reduction of nitrate (NO_3^-) to nitrite (NO_2^-) by adding sulfanilic acid reagent and α-naphthylamine. If the organism has reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. When sulfanilic acid is added, it will react with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α-naphthylamine to form a red-colored compound. Therefore, if the medium turns red after the addition of the nitrate reagents, it is considered a positive result for nitrate reduction. If the medium does not turn red after the addition of the reagents, it can mean that the organism was unable to reduce the nitrate, or the organism was able to denitrify the nitrate or nitrite to produce ammonia or molecular nitrogen. Therefore, a small amount of powdered zinc is added. If the tube turns red after the addition of the zinc, it means that unreduced nitrate was present. Therefore, a red color on the second step is a negative result.

- **NaCl tolerance test**: it is used to test ability of the bacteria to grow in the presence of variable amount of Sodium Chloride (NaCl). This test is particularly useful for presumptive identification of the enterococcal group D organisms, which have the specific ability to grow in the presence of 6.5% NaCl incorporated into either a broth or an agar medium.

- **Isolation of genomic DNA from microorganism:**

Protocol

Inoculate the sample in sterile standard SOB (Super Optimal Broth) media.
↓(overnight)
Take 1.5 ml of culture; spin at 14,000 rpm for 5 minutes
↓
Dissolve the pellet in Tris-EDTA buffer (400 µl)
+
10% SDS (100 µl) [mix softly]
↓
60 °C for 5-10 mins
↓
6 Molar Na per chlorate (100 µl) [Mix vigorously]
↓
60 °C for 5-10 mins
↓
add equals volume of $CHCl_3$ (600 µl)
↓
Centrifuge for 20 mins at 4 °C; 14,000 rpm
↓
Separate the aqueous phase (500 µl) in a fresh sterile eppendorf tube
+
Add double the volume of chilled ethanol slowly along the wall of the tube

- **Extraction of genomic DNA from the microorganism:**

Gel electrophoresis: it is a technique used to separate DNA fragments (or other macromolecules, such as RNA and proteins) based on their size and charge. Electrophoresis involves running a current through a gel containing the molecules of interest. Based on their size and charge, the molecules will travel through the gel in different directions or at different speeds, allowing them to be separated from one another. All DNA molecules have the same amount of charge per mass. Because of this, gel electrophoresis of DNA fragments separates them based on size only.
It was carried out to check if the isolation of the genomic DNA was successful or not. And later to check if the gDNA was successfully amplified using PCR or not. (Aaij C, 1972)

The following steps were carried out:

1 g of agarose powder was mixed with 100 mL TAE in a flask and microwaved for 2-3 mins until completely dissolved

↓

Ethidium bromide (EtBr) was added, it binds to the DNA and allows the DNA to be visualised under ultraviolet (UV) light

↓

Agarose Gell was poured into a gel tray with the well comb in place and was let to sit at room temperature for 20-30 mins, until it had completely solidified

↓

Once solidified, the agarose gel tray was put into the electrophoresis unit and filled with TAE until the gel was submerged completely

↓

A molecular weight ladder was loaded carefully into the first lane of the gel and 2 µl DNA samples were mixed with 7µl Bromophenol (loading dye) and loaded subsequently in each well

↓

The gel was run at 80-150V until the dye was approximately 75-80% of the way down the gel

↓

The electrophoresis unit was disconnected and the gel was removed and visualised under UV light

↓

Using the DNA ladder in the first lane as a guide, the size of the DNA in the sample lanes was determined

Polymerase chain reaction (**PCR**): it is a common laboratory technique used to make many copies of a particular region of DNA. Typically, the goal of PCR is to make enough of the target DNA region that it can be analysed or used in some other way. For instance, DNA amplified by PCR may be sent for sequencing, visualised by gel electrophoresis, or cloned into a plasmid for further experiments. (Mullis KB, 1990)

Taq polymerase: Like DNA replication in an organism, PCR requires a DNA polymerase enzyme that makes new strands of DNA, using existing strands as templates. The DNA polymerase typically used in PCR is called Taq polymerase, after the heat-tolerant bacterium from which it was isolated (*Thermus aquaticus*). Its DNA polymerase is very heat-stable and is most active around 70°C. This heat-stability makes Taq polymerase ideal for PCR.

PCR primers: They are short pieces of single-stranded DNA, usually around 20 nucleotides in length. Two primers are used in each PCR reaction, and they are designed so that they flank the target region. That is, they are given sequences that will make them bind to opposite strands of the template DNA, just at the edges of the region to be copied. The primers bind to the template by

complementary base pairing. When the primers are bound to the template, they can be extended by the polymerase, and the region that lies between them will get copied.

The PCR recipe used was: 1µl gDNA + 1µl forward primer + 1µl reverse primer + 5µl Taq polymerase enzyme + 2µl water for injection.

The mixture was put in an eppendorf tube which was introduced in a thermal cycler for amplification of the DNA.

The amplification in PCR takes place by three main steps:

Denaturing: it is carried out at high temperatures like 94-95°C causing the double stranded DNA to get separated into two single stranded DNA for the polymerase to come bind to them.

Annealing: it is carried out at temperatures 50-56°C, which is ideal for the primers to attach to the template strand.

Extending: it is carried out at 72°C, causing the Taq polymerase to bind to the template strand and start polymerisation to give a double stranded DNA.

These steps are repeated in the same order for the amplification of the DNA, which is directly proportional to the number of cycles it undergoes. The gDNA that was amplified was used for gel electrophoresis and the size of the amplified region was determined by comparing it with the DNA ladder.

C. **Results**

Gram staining

The gram nature was found to be gram positive rods.

↓

Capsule staining

The microorganism was found to be capsulated.

↓

Motility test

The microorganism was found to be highly motile.

↓

Gel electrophoresis and PCR

The gDNA was isolated and after PCR the genomic DNA corresponds to the band that is having 750-1000 bp on the standard DNA ladder.

↓

Biochemical test

Positive: sucrose, maltose, galactose, methyl red, nitratase and 7% NaCl

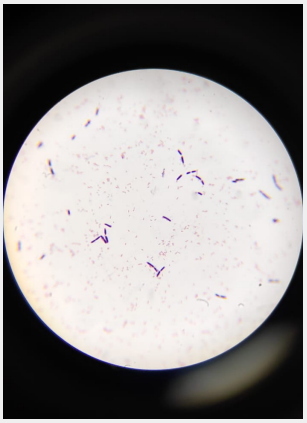
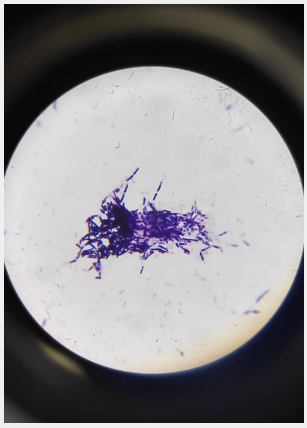
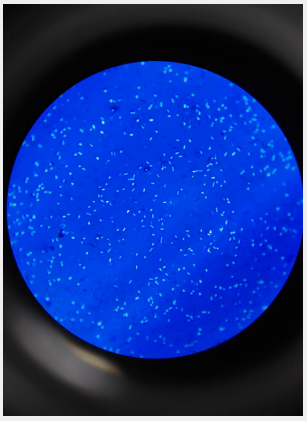
Negative: lactose, xylose, mannitol, indole, Voges Proskauer, citrate, urease.

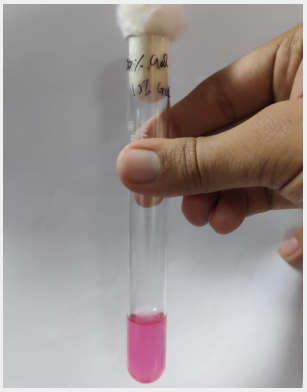
↓

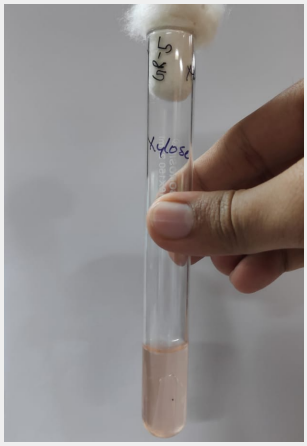
Identification of the microorganism

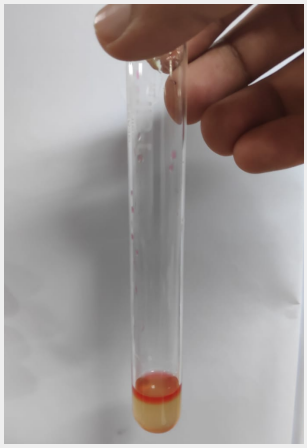
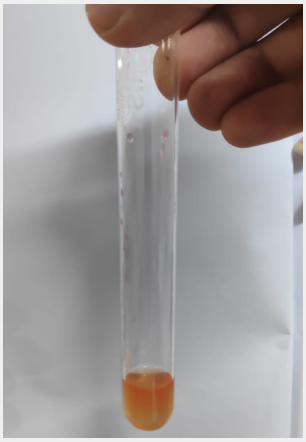
The microorganism was identified as a *Bacillus* species.

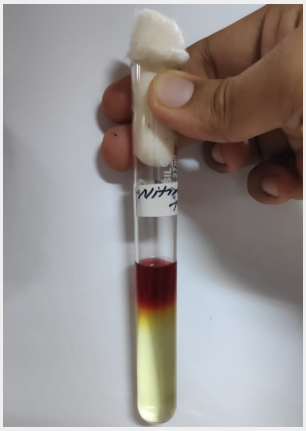
D. **Following is the result of the various tests conducted on the isolate:**

SERIAL NO.	1	2	3
CHEMICAL TEST	Gram staining (sample)	Gram staining (isolate)	Capsule staining
RESULT	Mixed Culture	Positive	Positive
PICTURE			

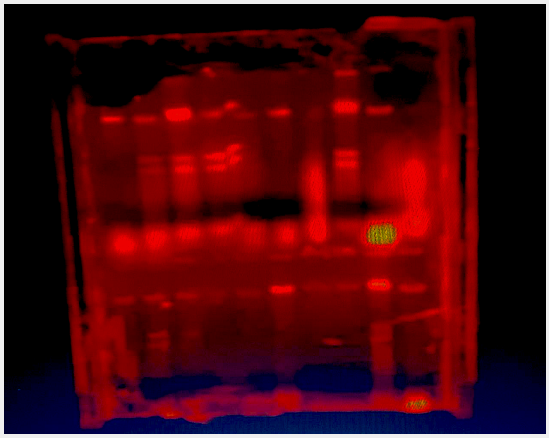
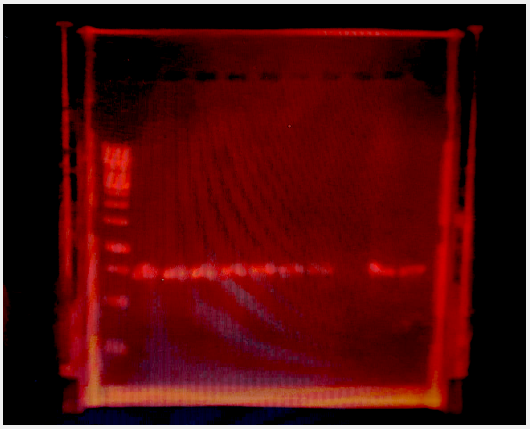
SERIAL NO.	4	5	6
CHEMICAL TEST	Sucrose utilisation test	Maltose utilisation test	Galactose utilisation test
RESULT	Positive	Positive	Positive
PICTURE			

SERIAL NO.	7	8	9
CHEMICAL TEST	Lactose utilisation test	Xylose utilisation test	Mannitol utilisation test
RESULT	Negative	Negative	Negative
PICTURE			

SERIAL NO.	10	11	12
CHEMICAL TEST	Indole test	Methyl red test	Voges Proskauer test
RESULT	Negative	Positive	Negative
PICTURE			

SERIAL NO.	13	14	15
CHEMICAL TEST	Citrate test	Salt tolerance test	Nitratase test
RESULT	Negative	Positive	Positive
PICTURE			

SERIAL NO.	16	17	18
CHEMICAL TEST	Urease test	Casein test	Extraction of gDNA
RESULT	Negative	Positive	Positive
PICTURE			

SERIAL NO.	19	20
TECHNIQUE	Gel electrophoresis (after extraction of gDNA)	Gel electrophoresis (followed by PCR)
RESULT	gDNA successfully extracted	PCR successful at amplification of gDNA
PICTURE		

E. **Discussion**:

The microorganism was found to be gram positive rods, occurring either single or in pairs, highly motile, aerobic and catalase positive thus it was considered to belong to the genus *bacillus,* since it is in line with the key features of the genus according to the Bergey's manual. It was difficult to identify the species since it doesn't completely fit into any of the species mentioned in the manual. The closest species it resembles to are *Bacillus pantothenticus, Bacillus soli* and *Bacillus galactosidilyticus.*

Further biochemical tests are required to completely identify the species of the organism. The gDNA that was isolated and amplified should be sent for sequencing and then bioinformatic tools can also be used to identify the organism's species.

F. **Acknowledgement**:

I would like to express my sincere gratitude to my supervisors Dr. Binoj Kutty and Dr. Sangeeta Shetty who supervised the whole experiment providing their invaluable guidance, comments and suggestions throughout the course of the project. Without them the project could not have been possible.

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At last I would also like to thank the Life Science Department for providing the lab and the equipments required for the project work and all the lab staff for all their help.

G. **References**:

1. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G. 1986. Volume 2. *Bergey's Manual of Systematic Bacteriology*. The Williams & Wilkins Co., Baltimore and London.
2. Aaij C, Borst P. The gel electrophoresis of DNA. Biochim Biophys Acta. 1972 May. DOI-10;269(2):192–200.
3. Mullis KB. The unusual origin of the polymerase chain reaction. Scientific American. 1990;262(4):56–61. 64–5.
4. Khan M.A., Ahmad N., Zafar A.U., Nasir I.A., Qadir M.A. Isolation and screening of alkaline protease producing bacteria and physio-chemical characterization of the enzyme. Afr J Biotech. 2011; DOI-10:6203–6212.