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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ABSTRACT: The aim of the experiment was to isolate and identify the protease from the given soil sample. The sample contained chopped intestine and chicken meat. The soil was kept like this for 2 days. Then it was isolated by first diluting the given soil sample and then plating it on the skimmed milk agar plate and nutrient agar plate, the colonies were isolated from there and again plated on the agar plate to get a pure colony. Then the colony was taken and many biochemical tests were done to identify the given protease.

INTRODUCTION:

Enzymes are catalyst biocatalyst which enhances metabolic rate of reactions. Protease is naturally present in all organism and it is an important enzyme produced in the industries.

It is an enzyme that catalyzes proteolysis which is the breakdown of proteins into smaller polypeptides or single amino acids. They do thid by cleaving the peptide bonds within proteins by hydrolysis. It is also called proteinase or peptidase or proteolytic enzyme.

There are three groups classified based on their acid-base behavior-
i) Acid protease- Have a pH range of 2.0-5.0 and produced only by fungi.
ii) Neutral protease- Have a pH range from 7.0-8.0 and are mainly found in plants.
iii)Alkaline protease- Have pH more than 8.

Proteolytic enzymes are ubiquitous in nature as they are found in all living organism. Protease enzyme can produce eco-friendly products and play a vital role in modern biotechnology industries. The microbial protease lacks pathogenicity and hence, can be grown easily culture medium and have wide applications in the industries.

Proteases are used in various industries like dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestives and for certain medical treatments of inflammation and virulent wounds.(1)

PROCEDURE:

- Aim

1. To prepare the saline suspensions of the soil sample.
2. To isolate the bacterial samples of concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} by spread plating it on nutrient agar plate and skimmed agar plate.
3. To perform gram staining of the sample with dilution factor 10^{-1} to check the gram nature of the bacteria present in the given sample.

   Materials required- Soil sample, 6 sterile nutrient agar plates, 3 sterile skim milk agar plates, saline, 7 sterile test tubes, crystal violet, gram's iodine, acetone alcohol, safranin, spreader, alcohol, vortex, slides

   Method-

1) 1g of the soil sample was mixed with 9mL of saline.
2) Then with a sterile 10mL pipette aseptically transferred 4.5mL of saline to 7 sterile test tubes.
3) Aseptically 0.5mL of the culture is transferred to the first dilution tube. It was then vortex. This is 10^{-1} dilution.
4) Using a new sterile pipette 1mL pipette 0.5mL of culture was transferred from10^{-1} dilution to the next tube. This is 10^{-2} dilution.
5) Rest dilutions were made according to the below serial dilution table.

Tube no.	1	2	3	4	5	6	7
Saline	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Culture	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cfu/mL	10^7	10^6	10^5	10^4	10^3	10^2	10^1
Cfu/0.1mL	10^6	10^5	10^4	10^3	10^2	10^1	0
Dilution factor	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}

6) The tubes with dilution factor 10^{-3}, 10^{-4}, 10^{-5} were spread plated on the nutrient agar and the test tubes with dilution factor 10^{-2}, 10^{-1} were streak plated on skim milk agar plate.

The first test tube contains when the sample is mixed with saline and the other three are the test tubes with different dilution factors.

Spread plate –

a) Using fresh sterile 1mL pipette, 0.1mL from 10^{-3} dilution was aspirated and added to a sterile nutrient agar plate. It was done one more time to make a duplicate of it.
b) The spreader was sterilized through flame sterilization using alcohol. The spreader was allowed to cool and aseptically surface spread the culture using this sterile spreader until the surface of plate is dry.
c) Above steps were repeated for the dilutions 10^{-4} and 10^{-5}.
d) The plates were kept for 24 hours at incubation at room temperature.

Streak plate-

i) A nichrome loop was taken and sterilized be flame sterilization. It was allowed to cool.
ii) This sterilized loop was then used to take a loopful of 10^{-2} dilution and then streak plate on the sterile skim milk agar plate.
iii) Above steps were repeated for the 10^{-1} dilution.
iv) The plates were incubates at room temperature for 24 hours.

7) Gram staining-

Principle- The cell wall of the microorganism determines if it is gram positive bacteria or gram negative bacteria. When primary stain is added i.e. crystal violet, if the cell wall retains the stain even after adding decolorizer it is a gram positive microorganism or those that get decolorized and get colorized after adding counterstain i.e. safranin are gram negative microorganism(2).

Method-

a) A loopful of culture was taken in a sterile nichrome loop and was smeared on a clean slide.
b) The smear was allowed to dry.
c) Then the culture was heat fixed on the slide by passing it through the flame once.
d) It was kept on a tripod stand and then the slide was flooded with crystal violet and left for one minute.
e) The stain was removed by slightly tilting the slide in basin. Then Gram's iodine was flooded and left there for another one minute.
f) Then using acetone the slide was decolourised.
g) Then the slide was flooded with safranin and left there for 3 minutes.
h) The slide is then gently washed with water to remove excess of stain.
i) The slide was allowed to dry. After drying, a drop of oil was added at the point where the culture was and observed under 100X power of microscope.

Observations-

i) Both gram positive and gram negative cocci and coccobacilli were observed under the 100X microscope.
ii) After 24 hours the plates were checked. From the skim milk agar plate, isolate colonies were identified and those colonies or colony were again streak plated in hexagonal way on another nutrient agar plate(s) and skim milk agar plate.

Nutrient Agar plate

Skim milk Agar plate

- Isolation of Genomic DNA

1) 10mL of a culture grown overnight in a super optimal broth (SOB) was inoculated.
2) 1.5mL of the culture was spin at rate of 14000rpm for 5 minutes. This step was repeated again. The pellet obtained worth of 3mL of the culture supernatant was removed both the times.
3) The resuspended pellet was added in 400µL of TE buffer and 100µL of 10% SDS (detergent)
4) This was incubated for 10 minutes at 60°C.
5) 100µL of 6M sodium perchlorate was added and incubated for 5-10 minutes at 60°C.
6) 600µL of chloroform was added.
7) It was centrifuged at 14000rpm for 30 minutes at 4°C.
8) The aqueous phase was separated into fresh, sterile eppendorf tubes and double the amount of chilled 100% ethanol was added and mixed gently.

- Capsule staining-

a) A thin smear of culture is made on a clean grease free slide by using a sterile nichrome loop.
b) A drop of 1% congo red solution was added in the suspension and was allowed to air dry.
c) Maneval's stain was flooded on the slide and left there for 2 minutes.
d) Excess of stain was discarde at the end of 2 minutes.
e) The slide was left to air dry and then observed under 100x power microscope with a drop of oil in the middle.

- Extraction of gDNA from *E.coli*

1. PCR

i. Principle- PCR involves the primer mediated enzymatic amplificayion of DNA. It is based on using the ability of DNA polymerase to synthesize new strand complementary to the offered template strand(3).
ii. Materials- Master mix, isolated gDNA, thermal cycler
iii. There are three steps in this process- denaturation, annealing and elongation.
iv. Method- 1µL of gDNA isolated was mixed with 9 µL of the master mix. This mixture was kept in the thermal cycler.
v. Mastermix includes nuclease-free water and PCR master mix. This mix is a premixed ready to use solution containing Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR.

2. Gel electrophoresis

i. The fragments of gDNA obtained from PCR are used in the gel electrophoresis.

ii. Principle- It is a technique used to separate DNA fragments based on their charge and size(4).

iii. Materials- Fragments of gDNA, ethidium bromide, gel casting trays, sample combs, electrophoresis buffer, trans-illuminator, micropipettes

iv. 0.8% agarose gel is used for the gel electrophoresis.

v. This gel is made by using TAE IX buffer which contains tris base, EDTA and acetic acid.

vi. Less concentrated agarose i.e. 0.6-0.8% is used if the fragments of gDNA are large while more concentrated agarose i.e. 16% is used if the gDNA fragments are small.

vii. The loading sample was prepared by mixing the 2μL of loading dye i.e. ethidium bromide and 7μL of the gDNA.

viii. This sample was loaded in the well. It was run until it reached the $3/4^{th}$ of the gel.

ix. The gel was viewed on a UV trans-illuminator.

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- Biochemical test- The culture was grown in nutrient broth and this broth was taken to the following biochemical tests.

  Materials- Sucrose. Maltose, galactose, lactose, xylose, mannitol, trypton water, Kovacs reagent, glucose-6-phosphate, methyl red, 5% α-naphthol, 40% KOH, agar slant tube, urea, sulphanilic acid, α-naphthylamine, 3% H_2O_2, Andrade's reagent, culture broth, sterile test tubes, nichrome loop

1. Loopful of the culture was taken for the following biochemical tests

i. Sugar utilization- Loopful of the culture was taken and put in 6 different test tubes which contained sucrose, maltose, galactose, lactose, xylose and mannitol with Andrade's reagents. 200μL of these sugars were taken in sterile test tubes. The tubes were then incubated for 24 hours at room temperature(5).

Principle-when microorganism ferment carbohydrate an acid or acid with gas are produced. The end product varies depending on the organism and the substrate fermented. The production of the acid lower than the test medium is detected by the color change of the pH indicator. Color changes if sufficient amount of acid is produced.

ii. IMViC test-

a) Indole test- Loopful of culture was taken and put in trypton water and was incubated for 24 hours at room temperature. This broth was taken and Kovacs reagent was added.

Principle- It is a qualitative test for determining the ability of bacteria to produce indole by deamination of tryptophan(6).

b) Methyl red test- Loopful of the culture was taken and put in glucose-6-phosphate solution and incubated for 24 hours. Little bit of this culture broth was taken and the experiment was performed. In this methyl red indicator is added.

Principle-it determines whether the microbes perform mixed acids fermentation when supplied glucose. In mixed acid fermentation, three acids i.e. acetic, lactic, succinic are formed. The large amounts of these acids result significant decrease in the pH of medium below 4.4. This causes the change in the color of the medium(7).

c) Voges-Proskauer test- The culture grown in glucode-6-phosphate was taken for this test too. In this 5% α-naphthol and 40% KOH.
Principle- It is used to determine if an organism produces acetylmethyl carbinol from glucose fermentation. If present is converted to diacetyl in the presence of the above given reagent and atmospheric oxygen which cause formation of pinkish red polymer(8).

d) Citrate utilization test- in this the loopful of culture is taken and streaked in an agar slant tube and incubated for 24 hours at room temperature.
Principle- It is tested to see an organism's ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salts as sole source of nitrogen(9).

iii. Urease test- Loopful of culture is taken and put in the test tube containing urea. It was kept for incubation for 24 hours.
Principle- Hydrolysis of urea produces ammonia and CO_2 and formation of ammonia makes the medium alkaline which causes the color change of phenol red from light orange at 6.8 to pink at pH 8.1(10).

iv. Nitrate test- Loopful of the culture was taken and put in the test tube containing sulphanilic acid and α-naphthylamine. It was kept for incubation at room temperature.
Principle- It detects the formation of nitrite and its ability to form red compound when it reacts with sulphanilic acid to form a complex of nitrite-sulfanilic acid which then reacts with α-naphthylamine to give red precipitate i.e. prontosil, which is a water-soluble azo dye.(11)

v. Nutrient agar slant containing 7% NaCl- Loopful of the culture was taken and streaked on the nutrient agar slant which contains 7% NaCl.

vi. Catalase test- In a test tube containing 3% H_2O_2 is taken and in it the culture broth is added.
Principle- Catalase is produced by microorganisms that live in oxygenated environment to neutralize toxic forms of oxygen metabolites; H_2O_2.(12)

vii. Gram staining-
The cell wall of the microorganism determines if it is gram positive bacteria or gram negative bacteria. When primary stain is added i.e. crystal violet, if the cell wall retains the stain even after adding decolorizer it is a gram positive microorganism or those that get decolorized and get colorized after adding counterstain i.e. safranin are gram negative microorganism.(1)

viii. Motility test- Few drops from the nutrient broth was taken and was observed under the microscope to check whether the microorganism was motile or not.

OBSERVATIONS-

1. Gram staining-
   Color- Purple
   Morphology- Rod shaped
   Arrangement- Chain

2. Capsule staining-
   Encapsulated organism was presnt.

3. Biochemical tests

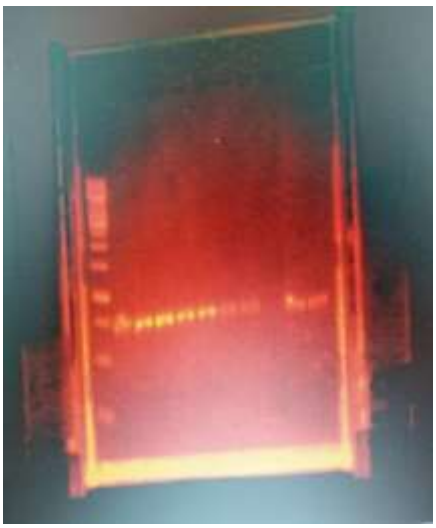
TESTS	OBSERVATIONS
a) Sugar utilization test	
Maltose	Pink color
Sucrose	No pink color and bubble formation
Mannitol	No pink color and bubble formation
Galactose	No pink color and bubble formation
Lactose	No pink color and bubble formation
Xylulose	No pink color and bubble formation
b) IMViC test	
Indole test	No red ring formation
Methyl red test	No change in the color of the solution to red
Voges- Proskauer test	No change in the color of the solution to red
Citrate utilization test	No growth of the culture
c) Urease test	No change in the color of the solution to pink
d) Nitrate test	Red ring formation
e) Nutrient agar slant containing 7% NaCl	Growth of the culture observed
f) Catalase test	Effervescence observed

4. Motility test- The microorganism seen under the microscope was motile.

Maltose test

7% NaCl nutrient slant agar

5. Gel Electrophoresis-
   After observing it in the UV- illuminator itwas observed that the gDNA we got after running it through the PCR is of the size750 bp.



FLOW CHART

Isolation of the microorganism from the source i.e. soil by spread plate technique

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Gram staining gave the result that the organism present is rod shaped, gram positive organism is present

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Capsule staining was done to confirm if it was gram positive bacteria was present

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The next test done was catalase test in which slight effervescence was observed
Then the sugar utilization test was done and it was observed that it could breakdown maltose

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Nitrate test was done which gave the red ring

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Motility test of the organism is checked

RESULT

The microorganism present in the given source is a rod shaped motile gram positive bacteria *Bacillus firmus.*

DISCUSSION

The organism isolated and identified is a gram positive, rod shaped bacteria, *Bacillus firmus*, is mostly found in the soil. From the above tests that were done, it was observed that the organism was able to ferment maltose and not the other sugars. Since, it is a gram positive organism it did not give any IMViC test because these tests are for gram negative organism. The positive catalase test tells that the organism is able to neutralize toxic forms of oxygen metabolites. The organism is also able to grow in a high salt concentration and is motile.

But these are tests are not enough to confirm it the organism that was isolated from the source. More tests need to be done to get the confirmation. Other tests that could have been done were starch hydrolysis, mannitol salt agar, blood agar, novobiocin susceptibility test, coagulase test, tyrosine agar and also for sugar fermentation test more sugars could have been used. And also to know the specific bacteria it's better to send the gDNA for sequencing.

The mistakes that could have happened during the experiment is that the amount of soil that was taken and mixed in the saline was not enough and the concentration of the bacteria are not enough to give a proper result for some of the experiments like the sugar fermentation test. Even in the catalase test the effervescence seen was not that rapid and due to the less concentration of the bacteria.

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REFRENCE

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