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

Food spoilage is a metabolic process that causes foods to be undesirable or unacceptable for human consumption due to changes in sensory characteristics. Some ecologists have suggested these noxious smells are produced by microbes to repulse large animals, thereby keeping the food resource for themselves. Spoiled food is a good source of protease producers because microorganisms break down proteolytic enzymes for their metabolic process. The project was mainly carried out to identify and isolate a protease producing organism form a particular substrate. The purpose of the project was to perform various techniques on the organism which help to determine the genus and species of the organism found. The substrate used was meat from a poultry, chopped and left for a day. Bergey's manual is used to identify the unknown organism isolated from the sample. Bergey's manual has a systematic identification of various gram positive and gram negative organisms with the help of various experiments conducted.

METHODS

DAY 1

1g of 1day old chopped chicken meat was mixed in 9ml of saline to make a suspension. Serial dilution was done. 10^{-1}, 10^{-2} and 10^{-3} dilutions were plated in duplicates on nutrient agar (NA) plates for viable count. Gram staining of the original suspension was done. The original suspension was streaked on a skim milk agar (SMA) plate by T shaped method to isolate protease producers. Both the NA plates and SMA plates were incubated for 24 hours at 37^0C.

DAY 2

The plates were observed. The viable count on the NA plates could not be determined as it was a mixed culture and the numbers were TNTC. Nothing was conclusive in this step. The gram stain showed various organisms of different gram nature and characteristics. For the SMA plate various colonies were grown which had some clearings around indicating break down of Caesin and presence of protease producers. Three such colonies namely 6A, 6B and 6C were picked and streaked by Hexagonal method on NA plates as well as SMA plates. Plates were incubated for 24 hours at 37^0C.

DAY 3

The plates were observed. Presence of similar organism on plate 6A and6C was seen. This was concluded by observing colony and plate characteristics. Also there was no clearance seen on the SMA plates. Hence these cultures were rejected. The SMA plate of 6B culture had a very predominant clearing indicating the presence of protease producers. But mixed nature of 6B culture was observed on the NA plate. He it was re-streaked again on a SMA plate and incubated.

DAY 4

Only one type of colony was observed on the re-streaked plate. Gram staining was performed with the isolate of 6B. The culture also seemed to be a pure culture indicating that may be the

re-streaking was successful. The pure colony was inoculated in a fresh broth and incubated for 24 hours at 37^0C.

DAY 5

Following are the tests performed from 6B culture:

1. CAPSULE STAINING

Capsule staining was performed for the culture.

Procedure: 1) Place a drop of Manewal's A solution on slide.

2) Mix it with a drop of suspension of the bacterial culture.

3) Spread it gently to form a thin film.

4) Allow it to partially dry.

5) When still moist add Manewal's B solution, allow it to react for 10mins.

6) Discard the excess strain air dry, put a drop of cedar wood oil and observe it under 100X oil emulsion lens.

2. SKIMMED MILK AGAR

Procedure: 1) Streak the test culture on a SMA plate (30ml of slimmed milk added to 20ml of distilled water containing 1.5g of agar. The mixture is sterilized and poured on a sterile petri plate and cooled) by hexagonal streaking method.

2) Incubate for 24 hours.

3. ISOLATION OF GENOMIC DNA & GEL ELECTROPHORESIS

Procedure: 1) Inoculate the pure culture from SMA plate in SOB.

2) Add small volume of SSC and pour the slurry in cold centrifuge tube, centrifuge at 4000 rpm for 20 mins.

3) Discard the supt and re-suspend the pellet in 4ml of 2.6M NaCl, gently stir with a glass rod. Centrifuge at 5000 rpm 30 mins.

4) Discard the pellet and decant the supt in a glass beaker.

5) Add double volume of chilled ethanol. Keep the beaker in freezer for 5 mins.

6) DNA fibers will be seen. Carefully spool the DNA using capillary tube. Dissolve the DNA in 1ml SSC.

7) Prepare 0.8% of agarose gel in 1X TBE Buffer. Boil till agarose dissolves completely and a clear solution results.

8) Seal the casting tray with cello tape and place the comb such that it is 2 cm away from the cathode.

9) Allow it to cool a little time and then pipette 10-15ul of ethidium bromide and gently swirl it to mix it.

10) Pour the agarose gel in central part. Keep the thickness of gel to 0.5 to 0.9cm. Keep the gel undisturbed at room temperature for agarose to solidify.

11) Pour 1X TBE buffer into the gel tank till the buffer level stands at 0.5 to 0.8cm above the gel surface. Gently lifts the combs, ensuring that wells remain intact.

12) Prepare the loading sample by adding 5ul of the gel loading buffer to the DNA sample and mix it properly by tapping it gently.

13) Load about 20ul of samples in desired wells.

14) Set it at maximum current and a constant voltage. Let it run till the tracking dye from the well reaches $3/4^{th}$ of the gel.

15) View the gel on UV trans-illuminator.

4. MOTILITY

2) Hold a clean coverslip by its edges and carefully dab Vaseline on its corners.

3) Place a loopful of the broth culture in the center of the prepared coverslip.

4) Turn the prepared slide on the coverslip so that it is sealed.

5) Turn the slide over so that the coverslip is on the top and the drop of the culture can be observed hanging from the coverslip.

6) Observe it under the microscope.

5. INDOLE TEST

Procedure: 1) Take a sterilized test tube containing 4ml of tryptophan broth.

2) Inoculate the tube aseptically by taking a loop full of test culture.

3) Incubate at 37^0C for 24 hours.

4) Add 0.5ml of Kovac's reagent to the broth and observe.

6. METHYL RED TEST & VOGES- PROSKAUER TEST

Procedure; 1) Inoculate two sterile test tube containing MR-VP broth with the test culture

2) Incubate the test tubes for 24 hours at 37^0C

3) Add about 5 drops of methyl red indicator (0.5g of Methyl Red dissolve in 300ml of 95% ethanol with 200ml of distilled water) to one test tube for MR test and add about 5 drops of Barrit's reagent (Reagent A: 5% alpha-naphthol about 50g in 1000ml of

absolute ethanol. Reagent B: 400g of potassium hydroxide in 1000ml of deionized water. Both are mixed together) is added to another test tube for VP test.

4) Observe for a colour change.

7. SIMMON'S CITRATE TEST

Ammonium Dihydrogen Phosphate is the sole source of nitrogen. Dipotassium Phosphate acts as a buffer. Sodium Chloride maintains the osmotic balance of the medium. Sodium Citrate is the sole source of carbon in this medium. Magnesium Sulfate is the cofactor for a variety of metabolic reactions.

Procedure: 1) Dissolve the above salts in 1000ml of deionized water and adjust the pH to 6.9.

2) Add agar and Bromothymol blue and heat the mixture gently till the agar is dissolved. The medium is poured in a slant test tube.

3) Autoclave at 121^0C under 15 pis pressure for 15 mins.

4) Cool the slant and streak the culture on the slant.

5) Incubate at 37^0C for 24 hours.

8. UREA TEST

Procedure: 1) Inoculate a loopful of culture in the Urea test broth (20g of urea, 9.5g of Na_2HPO_4, 9.1g of KH_2PO_4, 0.1g of yeast extract and 0.01g of phenol red. pH is 6.8).

2) Incubate at 37^0C for 24 hours.

9. NITRATE TEST

Procedure: 1) Inoculate the culture in nitrate test broth (Mix Peptic digest of animal tissue 5.0g, meat extract 3.0g, 1.0g of potassium nitrate, 30.0g of NaCl in 1000ml of distilled water).

2) Incubate and observe the next day.

10. SUGAR TESTS

The sugar tests included tests for Maltose, Sucrose, Galactose, Lactose, Xylose, Mannitol.

Procedure: 1) The above sugars available in the market were diluted in distilled water.

2) 3ml of each diluted solution were taken in sterile test tubes. Inoculate a loopful of culture in each test tubes.

3) Incubate it for 24 hours.

11. 7% NaCl TEST

Procedure: 1) Inoculate test culture in 7%NaCl solution.

2) Incubate aerobically for 24 hours at 37^0C.

12. H_2O_2 CATALASE TEST

Procedure: 1) Transfer a small of test culture on a clean and dry slide.

2) Place a drop of 3% H_2O_2 on the glass slide.

3) Observe for the evolution of oxygen bubbles.

DAY 6

The results of the above tests were observed.

RESULTS

TESTS	OBSERVATION	PRINCIPLE
1. GRAM NATURE	Gram negative	
2. GRAM STAINING	Pink, short rods	
3. PIGMENTATION	Yellow	
4. SIZE	0.3mm	
5. MARGIN	Smooth	
6. ELEVATION	Raised	
7. CAPSULE STAINING	No capsule was observe	Bacterial capsules are non-ionic, so neither acidic nor basic stains will adhere to their surfaces. Therefore, the best way to visualize them is to **stain the background using an acidic stain** *(e.g., Nigrosine, congo red)* and to **stain the cell itself using a basic stain** *(e.g.,crystal violet, safranin, basic fuchsin and methylene blue).*
8. SMA PLATE	Clearance was observed	Proteolytic bacteriawill be surrounded by a clear zone, due to the conversion of casein into soluble nitrogenous compounds.

9. ISOLATION OF GENOMIC DNA AND GEL ELECTROPHORESIS	Observation was not clear. This can be either due to shredding of DNA during extraction or error while loading the sample.	Citrate in SSC buffer is used s a chelating agent to chelate Mg and Ca ions, which are required as co-factors for DNAses. High concentration of NaCl is used to dissociate protein DNA complexes and to remove cationic complexes. It also neutralizes charge on sugar phosphate back bone. Ethanol is used to precipitate DNA by reducing dielectric constant of water. Electrophoresis is a technique used to separate charged molecules. As DNA is negatively charged it migrates towards anode when electric field is applied. Migration can be observed by a visible dye bromophenol blue.
10. MOTILITY	The organism was observed to be motile	
11. INDOLE TEST	Negative	When **indole** is combined with **Kovac's Reagent** (which contains hydrochloric acid and p-dimethylaminobenzaldehyde in amyl alcohol) the solution turns from yellow to **cherry red**. Because amyl alcohol is not water soluble, the red coloration will form in an **oily layer at the top of the broth.**
12. MR TEST	Positive	Methyl Red (MR) test determines whether the microbe performs mixed acids fermentation when supplied glucose
13. VP TEST	Negative	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.

14. SIMMON'S CITRATE TEST	Positive	Bacteria that can grow on this medium produce an enzyme, **citrate-permease**, capable of converting **citrate to pyruvate**
15. UREA TEST	Positive	Ability to hydrolyze urea with the enzyme urease.
16. NITRATE TEST	Positive	. Put few drops of each reagent into the tube containing culture to be tested. A distinct red or pink colour indicates nitrate reduction. The results should be recorded within 5 - 10 seconds as the colour fades on standing. A control (uninoculated) tube should also be tested. If there is no pink colour formation, add pinch of zinc dust to confirm the absence of nitrate in the medium (5). Nitrate reduction is not a confirmatory test. Complete identification should include the morphology, gram reaction, biochemical and serological tests. Addition of excess zinc may result in false-negative reaction
17. MALTOSE	Negative	
18. SUCROSE	Negative	
19. GALACTOSE	Positive	
20. LACTOSE	Negative	
21. XYLOSE	Positive	
22. MANNITOL	Negative	
23. 7% NaCl	Positive	The salt acts as a selective agent for bacteria and interferes with membrane permeability and osmotic equilibrium high salt concentration thus inhibits a range of bacteria but allows salt-tolerant organisms to grow.
24. H_2O_2 CATALASE TEST	Positive	The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them.

		Anaerobes generally lack the catalase enzyme

As per the expected results, the organism is *Proteus mirabilis*. It can use urea. It is commonly found in the human digestive system. The test which should have been negative is positive due to human error. Test for galactose and xylose is positive with slight pink color indicating the organism to be a late fermenter. Since the characteristic tests which were done resembled the characteristics of the organism, hence the organism is concluded to be *P. mirabilis*. The species were identified with the help of Bergey's manual.

6A Isolate Streaked on NA

6B Isolate Streaked on NA

6C Isolate Streaked on NA

6A Isolate Streaked on SMA

No clearance

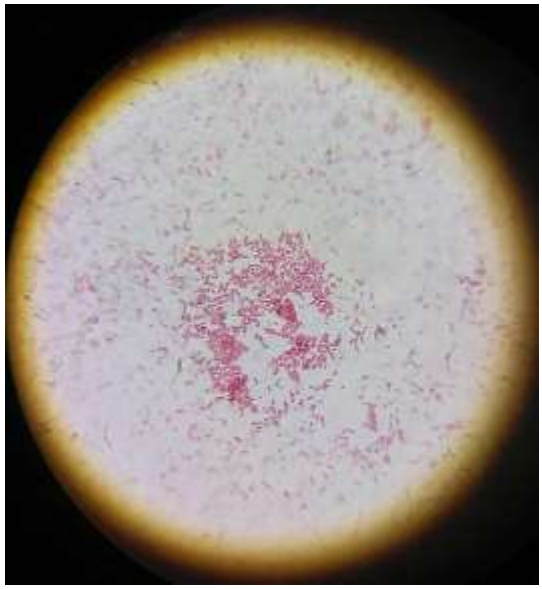
6B Isolate Streaked on SMA

Clearance observed

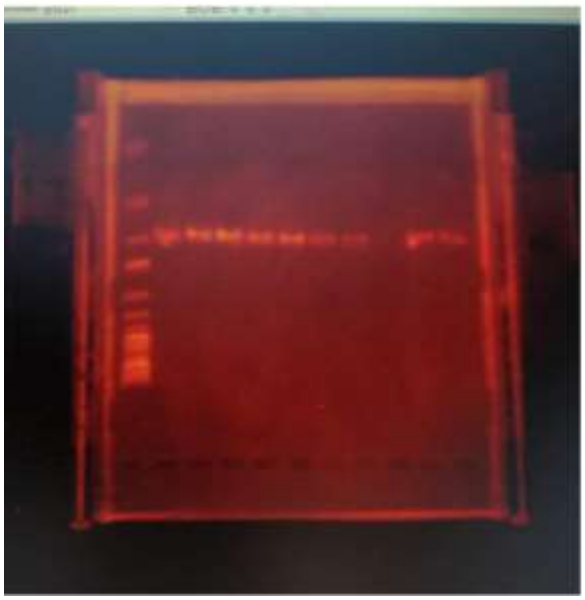
6C Isolate Streaked on SMA

No clearance

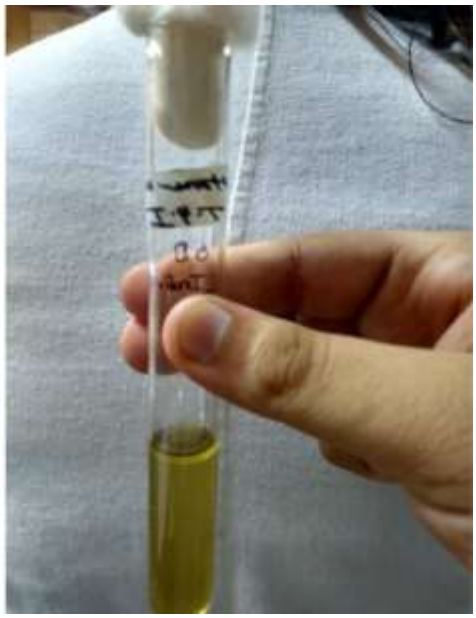
Re-streaked 6B culture to Obtain a pure colony.



Gram staining in 6B



Gel Electrophoresis in 6B

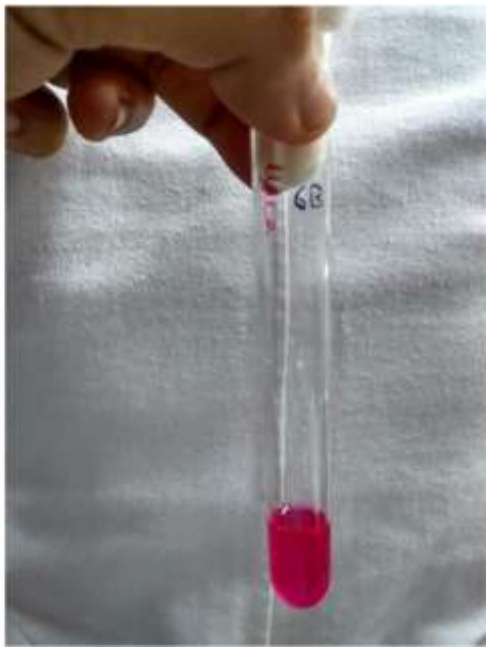


Negative Indole test in 6B

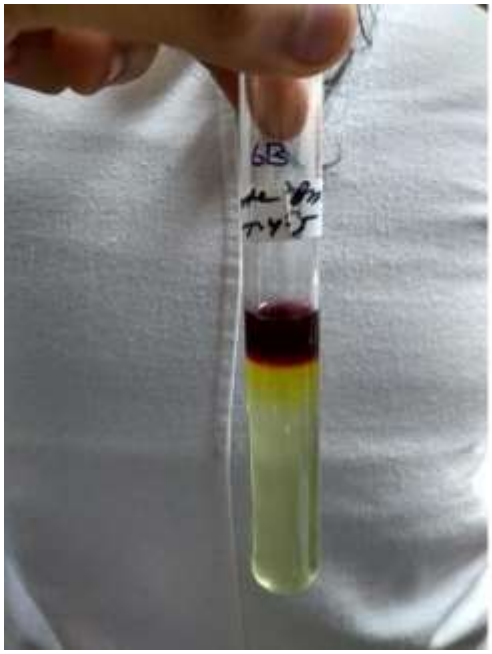
Positive MR test in 6B

Negative VP test in 6B

Positive Citrate test in 6B



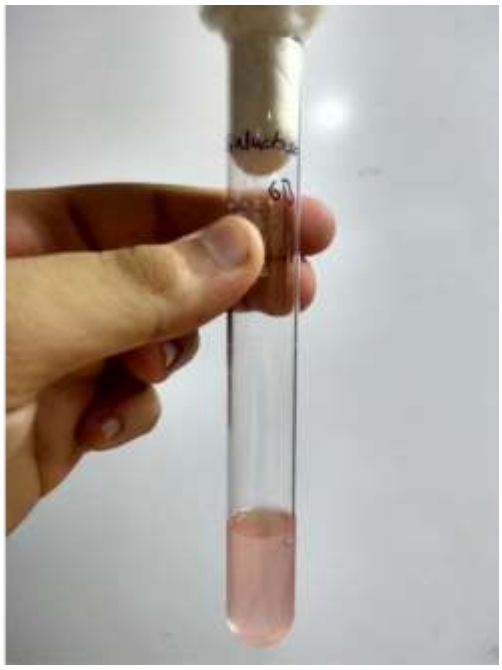
Positive Urea test in 6B



Positive Nitrate test in 6B

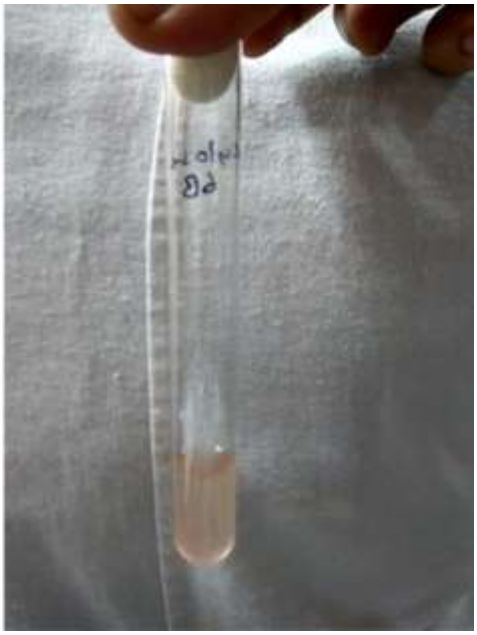
Negative Maltose test

Negative Sucrose test



Positive Galactose test

Negative Lactose test



Positive Xylose test

Negative Mannitol test Positive NaCl test

DISCUSSION

Various new techniques were learnt, methods of using them, handling the culture was done with great care. *P. mirabilis* is the organism found in meat and it has been studied in great detail. As the culture had to be preserved for further analysis it was made sure that the culture should not be contaminated, everything should have been done in sterile environments. The bacteria is a slow fermenter. Due to many tests done simultaneously, contamination would have taken place. Nichrome loop through which inoculations were made was not properly sterilized or not cooled. Hence for next projects or tests care should be taken that the area or work place where experiments are conducted should be properly sterilized, the inoculations should be done with a lot of care.

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