St. Xavier's College
(Autonomous), Mumbai

Department of Life Science and Biochemistry

2019-2020

SLSC501PR

Internal Assessment Project Report

Isolation and identification of unknown protease producing bacteria from a suitable source

Name of the student: Muskan Misra

UID Number: 172028 **Date of Submission:**

INTRODUCTION

Protease is one of the most essential enzymes known and has proven to be of great significance. Proteases are a class of proteolytic enzymes that break peptide bonds between the amino acids. It is considered an important industrial enzyme, as alkaline proteases are used in a variety of industrial processes, like in case of detergents, food, silk and dairy products. Bacteria are considered one of the most efficient sources for extraction of protease as they can be genetically modified, have high proliferation rate and cultures can be maintained overtime. The main source chosen for the purpose of bacterial extraction and isolation was rotten meat.

One of the main purposes behind this experiment was to not only carry out isolation of protease producing bacteria for research or commercial purposes but to also understand techniques widely used in the field of microbiology like Gram staining, Streak plating, Genomic DNA extraction and Polymer chain-reaction , principles used in biochemical tests and as well as microbial classification.

MATERIAL AND METHODS

A. Collection of samples

↓

B. Gram staining of suspension

↓

C. Isolation and screening for protease producers

↓

D. Pure culture of the selected isolate

↓

E. Genomic DNA extraction from the isolate

↓

F. Polymer Chain Reaction(PCR)- 16s rRNA

↓

G. Biochemical characteristics of the isolate

A. **Collection of samples**:

I. Chicken meat was bought from a poultry shop, which was then minced and kept in a non-air tight container overnight to rot at room temperature.
II. One gram of the sample (rotten meat) was then measured and serially diluted with 9 ml of saline and cultured on Nutrient Agar plates.
III. Gram staining of the above mentioned suspension was done and observed under 100x microscope.

IV. The suspension was also streaked out using T streak methods on a Skimmed Milk Agar Plate (SMA1) to check the culture's protease producing activity. 0.1 ml of each aliquot was spread on SMA plate and incubated at temperature of 37· C for 24 hour[1].

V. The table below was followed for serial dilution and viable count of culture of the isolate.

Test tube / Solution	1	2	3
SALINE	9ml	9ml	9ml
SOURCE (raw meat)	1ml	1ml	1ml
DILUTION FACTOR	10^{-1}	10^{-2}	10^{-3}

B. **Isolation and screening for protease producers**:

I. Three colonies from the previously T streaked plate were then picked out and hexagonally spread out on separate Nutrient Agar plates namely- 6A, 6B and 6C to isolate each of the colonies.

II. Each of the isolated colonies from 6A, 6B and 6C obtained were then hexagonally streaked out on 3 different SMA plates for better evaluation of the protease producing activity of the isolates.

C. **Pure culture of the selected isolate**:

I. Culture 6B was spread out on a Nutrient Agar plate to be able to get better results in the form of fully isolated colony.[2]

II. Gram staining of the isolate obtained from the above plate was performed to categorize the Gram nature of the culture.

III. Capsule staining was used to check to further classify the bacteria (if capsules were present or not).

D. **Genomic DNA extraction from the isolate**:

I. Pure isolates from the SMA plate were inoculated in Super Optimal Broth medium to culture the bacteria for genomic DNA extraction.

II. Genomic DNA of the isolate was extracted using the following steps:

a) 1.5 ml of the culture was centrifuged at 14000 rpm/min for 20 mins till about a pellet was obtained.

b) The pellet was then suspended in 400 µl of Tris-EDTA buffer and 100µl of 10% Sodium Dodecyl sulphate. This was done for lysis of the cell membrane. The eppendorf tube was then incubated using dry heat block for 5-10 mins at 60 · C.
c) 100µl of 6M Sodium perchlorate was added to the mixture and again incubated at 60· C for 5-10 mins.
d) Now the solution was divided into two eppendorf tubes with equally and same volume of $CHCl_3$ is added. An emulsion was then obtained, centrifuged at 14000 rpm/min for about 20 minutes.
e) The aqueous layer formed was then separated and transferred into an eppendorf tube wherein double the volume 100% chilled ethanol was added.
f) DNA was observed forming the precipitate.

III. To confirm the presence of extracted DNA, Agar Gel electrophoresis(AGE) was carried out. The following procedure was used:

a) 0.8% agarose gel in 1X TBE buffer was prepared. It was boiled till agarose had dissolved completely and a clear solution resulted.
b) Pipetted out 10-15µl of Ethidium bromide stock solution in the casting tray.
c) Poured agarose gel in center part of the tank. The gel was undisturbed. TBE buffer was poured into the gel tank and the combs were gently lifted.
d) Loading sample was prepared by adding 5µl of the gel loading buffer to 2µl of DNA sample obtained with the help of micropipettes.
e) The samples were loaded in the wells in desired order. Maximum current was set and it was run until the tracking dye reached $3/4^{th}$ of the gel.
f) The gel was then viewed on a UV trans-illuminator.

E. **PCR- 16s rRNA and AGE-** universal primer of 16s rRNA was used. In a sterile eppendorf tube, 1µl of genomic DNA, 1µl of forward and reverse primer and 5µl of Taq Master mix polymerase along with 2µl of the dNTPs were added and then placed in a Gradient PCR. The sampleDNA was then subjected to AGE wherein the gel was highlighted under UV trans-illuminator to observe if amplification took place or not.

F. Biochemical characteristics of the isolate

The following Biochemical tests were performed in order to determine the organism.

Sugar utilization: it is used determine whether a bacteria can utilize a particular carbohydrate or not and also the presence of gas and acid produced during carbohydrate fermentation.

The following sugars were pipetted out using a micropipette and transferred into a sterile test tube wherein each test tube was inoculated with the culture (preserved) with the help of a nichrome loop:

1. Maltose
2. Galactose
3. Sucrose
4. Lactose
5. Xylose
6. Mannitol

Positive end result- solution turns pink indicating fermentation. Presence or entrapment of gas bubbles inside the Durham's tube indicates production of gas.

IMViC test[3]:

INDOLE TEST: the tryptophan broth is inoculated with broth culture, Incubated at 37°C for 24-28 hours. 0.5 ml of Kovac's reagent is added to the broth culture. Observed for end result i.e. formation of red ring.

METHYL RED TEST: two tubes containing MR-VP Broth were inoculated with a pure culture of the microorganisms under investigation. Incubated at 35 °C for up to 4 days.5 drops of the methyl red indicator solution was added to the first tube. A positive test is indicated, if the colour of the medium changes to red within short time. Observed for red colour as the end result.

VOGES-PROSKAUER TEST: a tube of MR/VP broth was inoculated with a pure culture of the test organism. Incubated overnight. 1 mL aliquot of broth was added to a clean test tube. 0.6mL of 5% α-naphthol, followed by 0.2 mL of 40% KOH was added in order.

CITRATE UTILIZATION TEST: The medium most commonly used is the formula of Simmons. The medium was poured into a slant which was then inoculated with culture and kept for incubation. Colour change to blue shows alkalization.

Motility test: petroleum jelly was applied on the cavity slide on four corners. The broth was put at the center of the coverslip which is aligned with the slide. The slide is then inverted so that the drop hangs onto the cavity. Observed under microscope.

H_2O_2 Catalase test: bacterial culture was transferred onto a slide and drop of 3%H_2O_2 is mixed with it. Observed for rapid evolution of oxygen.

Nitrate test: Inoculate the nitrate broths with bacterial suspension. 6-8 drops of nitrite reagent A were added, followed by addition of 6-8 drops of nitrite reagent B.
Observed for at least 3 minutes for a red color to develop.[4]

7% NaCl test: An inoculum from a pure culture was transferred to a sterile tube of 7% NaCl broth. The tube is incubated for 24 hours. Observed for presence of turbidity. The medium used was 7% NaCl broth.

Urea test: The agar slant is streaked with the test organism with the help of a nichrome loop. Any urea medium, agar or broth can be used for this test. End result should be pink slant.

RESULTS

A. Collection of samples:

Image 1, 2 and 3 (from L-R):1 and 2-The plates of the dilution factors showed too numerous to count culture and hence was inconclusive. 3-The SMA plate did show clearances.

B. Gram staining of suspension:

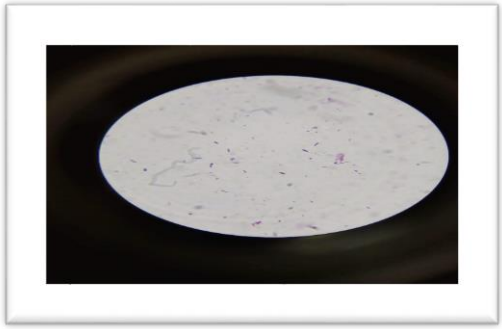


Image 4: The gram staining of the original suspension is shown.

C. Isolation and screening for protease producers:

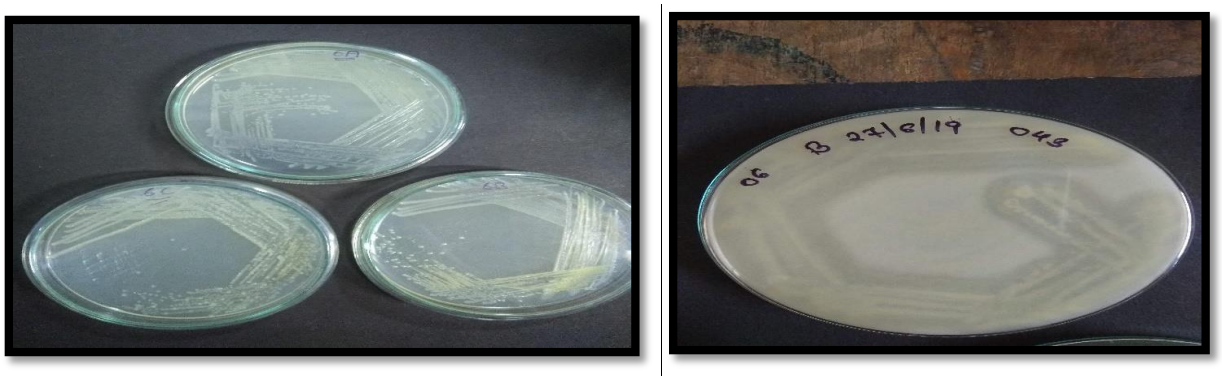


Image 5 and 6 (from L-R): 5 shows NA plates of three colonies that were previously isolated namely 6A, 6B and 6C. 6 shows the SMA plate of culture 6B.

D Pure culture of the selected isolate:

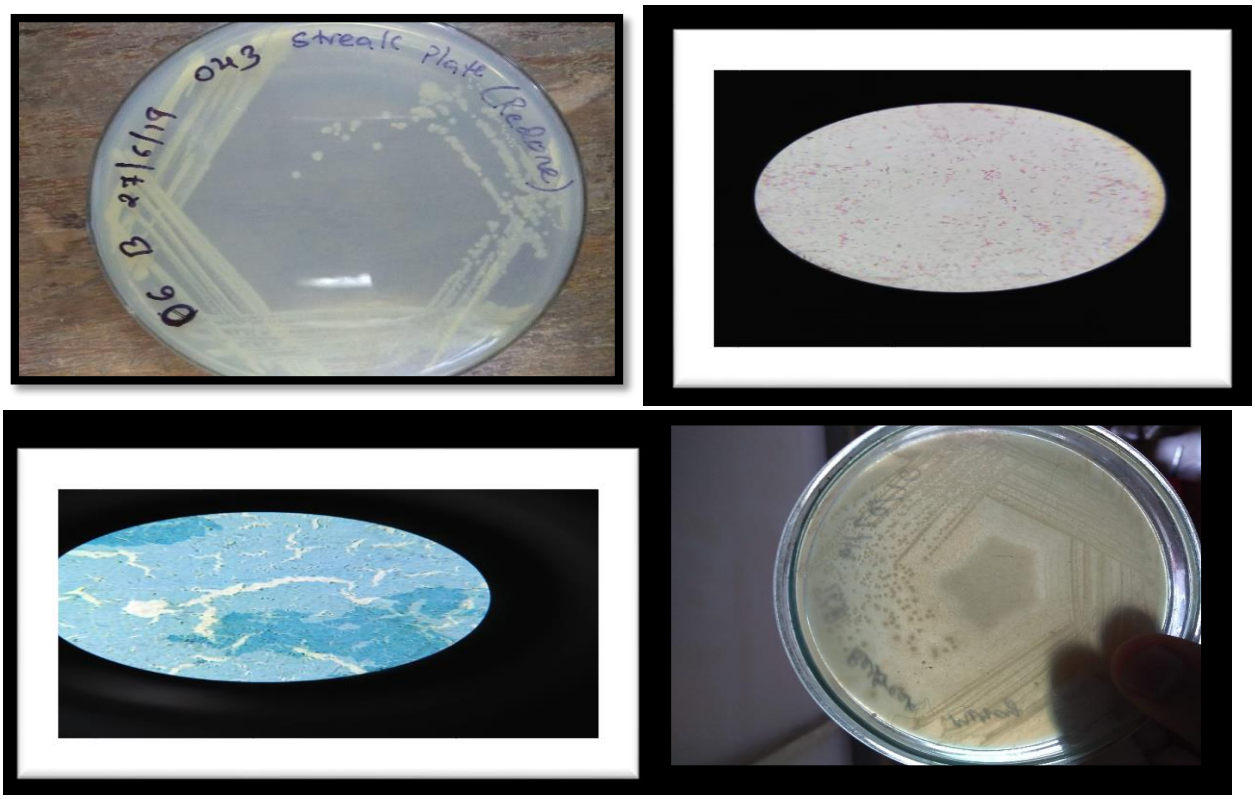


Image 7, 8, 9 and 10 (from L-R): 7 shows us the re-streaked culture of 6B in order to isolate the colonies which at first seemed to be of mixed type. 8 displays the Gram negative nature of the isolated culture 6B. 9 is the field observed after performing capsule staining for the culture 6B and 10 shows the SMA plate of the culture isolated from the sample and referred to as 6B (clearance is observed.).

E. Genomic DNA extraction from the isolate:

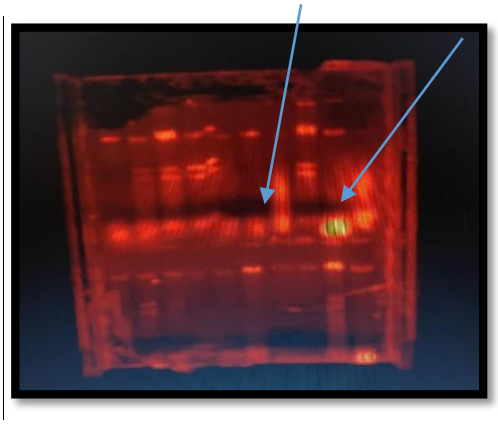


Image 11 – After performing Agar Gel electrophoresis of the extracted genomic DNA, when it was run under UV Trans-illuminator showed no bands were formed for 6B. The two arrows point out the wells containing 6B gDNA.

F. PCR- 16s rRNA and AGE:

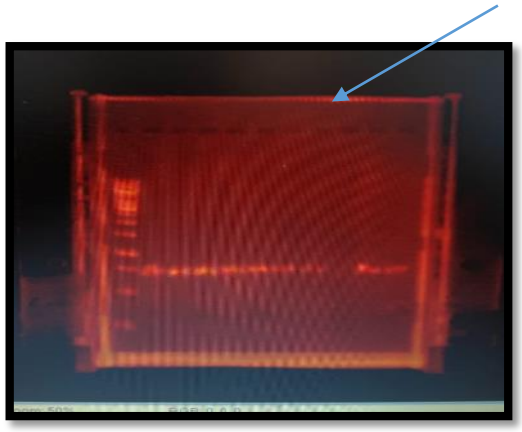


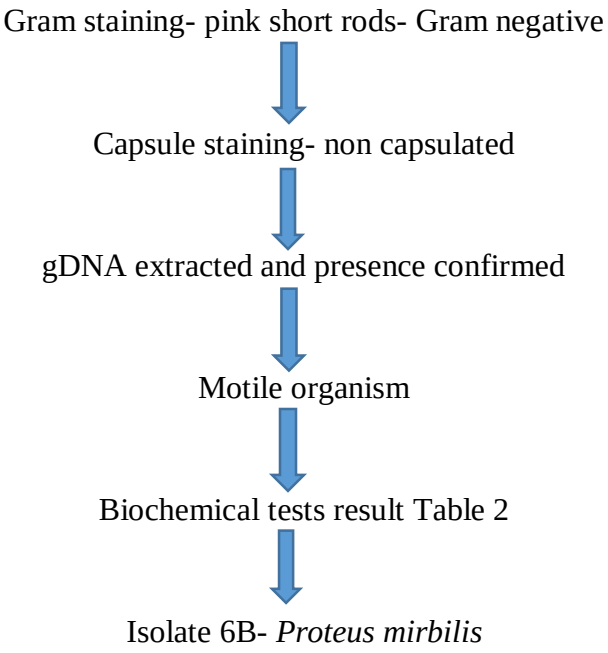
Image 12- The PCR of gDNA was carried out and observed under UV trans-illuminator. 6B well is highlighted by the arrow. Bands
Between 750 and 1000 bp are observed under UV illumination.

G. Biochemical characteristics of the isolate : Table. 1

Biochemical characteristics of the isolated strain: + = positive result , - = negative result

BIOCHEMICAL TEST	RESULTS OBTAINED
Maltose utilization	-
Galactose utilization	- (late fermenter)
Sucrose utilization	- (late fermenter)
Lactose utilization	-
Xylose utilization	-
Mannitol utilization	-
Indole test	-
Methyl red test	+
Voges Proskauer test	-
Citrate utilization	+
Motility test	Motile organisms
H_2O_2 catalase test	+
Nitrate test	+
Urea test	+
7% NaCl test	+

Image 13, 14, 15, 16,17and18 (from L-R): 13 and 14 display the end result of sugar utilization of Xylose and Galactose. 15 shows us the end result of citrate test (blue slant). 16 displays positive end result for urea test wherein the solution turned pink. The nitrate test performed is shown in 17, the solution turns red. A positive end result for methyl red is observed in 18.



Flowchart summarizing the results

DISCUSSION

Collection of samples: Image 3- the SMA plate which was T streaked using the original suspension had clearances, which indicates the presence of protease producing bacteria (or organisms) and that degradation of casein took place (which was incorporated in the agar plate).

Gram staining of suspension: Image 4- the Gram staining of the original substance displayed the presence of numerous organisms and mostly that of Gram negative short pink rods. The staining wasn't indicative of the culture's nature at large and was carried out to understand the technique of Gram staining.

Isolation and screening for protease producers: Image 5. 6B plate seemed to have two different colonies present and therefore re-streaking was to be done, for proper isolated colonies to be obtained. Image 6-The SMA plates of 6A and 6C showed presence of motile organism indicating that maybe the streaking wasn't carried out properly and no clearance was observed. The SMA plate of 6B showed proper clearance.

Pure culture of the selected isolate: Image 7- NA plate of restreaked culture of 6B showed the presence of only one type of colony. Hence, 6B was the isolate used properly for further experiments. Image 9 capsule staining of the culture was carried out and no capsules were found.

Genomic DNA extraction from the isolate- The gDNA extraction was carried out and AGE was performed. Image 11-When the gel was highlighted under UV trans-illuminator, 6B culture showed no banding which indicates the gDNA wasn't extracted.

Biochemical tests helped in characterization of the isolate. *Proteus mirabilis* [5] is known to utilize sugars such as galactose and xylose but during the experiment, end result for both the sugars wasn't pink in colour but a lighter shade it, which could also indicate late fermentation. Few aspects such the one mentioned led to uncertainty about the isolate's characterization.

During the month of experimentation, a lot hardships were faced. One of the main ones being students working in groups of 4 wherein each person performed a particular task and missed out on certain techniques and even coordination proved to be an issue at times. Maintainenace of the culture was the prime focus but due to restricted practical timings, was mostly done in dispersed intervals.

CONCLUSION

Protease occurs naturally in organisms and is considered an essential constituent for all the existing forms. Based on the experiment conducted and using Bergey's Manual of determinative bacteriology, Isolate 6B was identified as ***Proteus mirabilis.***

REFERENCES

1. Rupali, D. (2015). Screening and isolation of protease producing bacteria from soil collected from different areas of Burhanpur Region (MP) India. Int. J. Curr. Microbiol. App. Sci, 4(8), 597-606.
2. Ponnuswamy Vijayaraghavan , Samuel Gnana Prakash Vincent (2013) A simple method for the detection of protease activity on agar plates using bromocresolgreen dye, J Biochem Tech (2013) 4(3): 628-630
3. Microbeonline (2017). IMViC Tests: Principle, Procedure and results. Retrieved 07-09-2019 from https://microbeonline.com/imvic-tests-principle-procedure-and-results/
4. Microbilogy info.com(2019) Nitrate Reduction Test – Principle, Procedure, Uses and Interpretation retrieved 05-09-2019 from https://microbiologyinfo.com/nitrate-reduction-test/
5. Buchanan, R. E., Bergey, D. H., Gibbons, N. E., & Breed, R. S. (1974). Bergeys Manual of determinative bacteriology. R.E. Buchanan & N.E. Gibbons, co-editors. Baltimore:Williams & Wilkins.