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 soil

Name of the student: SIENA DSOUZA

UID Number: 172047 **Date of Submission:** 12TH September,2019

Abstract : The objective of the study was to isolate protease producing bacteria from Soil samples that was collected from compost soil. a pigmented colony was found & isolated and this isolated organism was then characterized & identified by the Bergey's Manual of Determinative Bacteriology.

Introduction : living cells produce Enzymes which are biocatalysts. they bring about specific biochemical reaction generally forming parts of the metabolic processes of cell. Microbial enzymes are often more useful than enzymes derived from plants or animals because they are more stable than their corresponding plant and animal enzymes and their production is more convenient and safer . Only about 2% of the world's microorganisms have been tested as enzyme sources. Proteases is one among the three largest groups of industrial enzymes. Proteases are capable of hydrolyzing peptide bonds in proteins and they are also called peptidase or proteinase or proteolytic enzymes. Microbial proteases are degradative enzymes, which catalyze the total hydrolysis of proteins. Proteolytic enzymes are ubiquitous in occurrence which are found in all living organisms & are important for cell growth and differentiation. proteases are of great commercial value and find multiple applications in various industrial sectors 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.

Materials & methods:

- Sample collection & serial dilution: different compost soil samples were collected from garden & college .both samples that were collected were mixed .it was then diluted by taking 1gram of sample & diluting it will 9ml of saline. Serial dilutions were made from this solution. Dilutions of 10^{-3} , 10^{-4}, 10^{-5} were streak plated & 10^{-2}, 10^{-3} were streak plated on skimmed milk agar plates. These were then incubated at 37◦C for 24hours. Gram staining was performed using the supernatant solution to determine the gram nature of the sample collected.
- 7 isolated colonies were obtained from nutrient agar t-plate,skimmed milk agar plates that were streak plated on the 1st day & were labelled as 7A,7B,7C,7D,7E & 7F. these isolates were then streaked on nutrient agar using hexagonal streaking method. These were then incubated at 370C for 24hours.
- the isolates were then picked up from individual plates & they were streaked on skimmed milk agar. The nutrient agar plates were stored in the fridge
- : the plates were checked & isolates were observed in each plate
- isolates were then innoculated & grown to obtain pure cultures
- gram staining was done for the innoculated culture and DNA isolation was performed.

ISOLATION OF GENOMIC DNA FROM BACTERIA

30B MEDIA-Super optimal broth.innoculated 1ml culture in 10ml broth & kept this culture Overnight

↓

1.5ml culture-spun at 14000rpm for 5min.(repeated this once again)

↓

Resuspended the pellet in tris EDTA buffer(400microlitre) + SDS (10%)(100 microlitre) & gently overturn. Discarded the supernatant of the pellet

↓

Incubated at $60^{O}C$ (5-10MIN)

↓

Added 6M sodium perchlorate (100microlitre) & mixed vigorously

↓

Incubated at $60^{o}C$ (5-10min)

↓

Added cholorform (600microlitre)

↓

Centrifuged (20-30min) $4^{o}C$ at 14000rpm

↓

Separated the aqeous phase in fresh sterile eppendorf tube & added twice the volume of ethanol(cold)

↓

Spun & washed the pellet with 70% & dissolved it in T.E

- Extraction of genomic DNA – agarose gel electrophoresis

AGAROSE GEL ELECTROPHORESIS

a standard 0.8% agarose gel using 1g of agarose in 100ml TAE was poured into the gel tray

loading buffer was added to each DNA sample

the agarose gel was placed into the electrophoresis unit when solidified

TAE was filled until the gel was covered.

the gel was run at 120-150V until the dye reached 70-80% of the way down the gel

the power was turned OFF & the electrodes were disconnected from the power source

A device that had UV was used to visualize the DNA bands

using the DNA Ladder in the 1st lane , the size of the DNA sample in the lanes was infered.

- **Polymerase chain reaction** (**PCR**) was then performed to amplify the DNA sequences & generate multiple copies of the DNA sample.the genomic DNA culture of 7A was used for PCR. After this process the DNA sample was again allowed to run on agarose gel electrophoresis gel & the DNA bands were observed under UV.

In order to prepare the mixture for PCR the following were added → 1µL genomic DNA+ 1µL forward primer + 1µL reverse primer + 5µL TAG Master + 2µL water for injection.

This mixture was then added into an eppendorf tube followed by the thermal cycler for amplication process. This amplification in PCR is done in three main steps :

1) **Denaturing** → this process separated the two double stranded DNA strands into two single stranded DNA at 94º-95ºC
2) **Annealing** →in this stage the primers were attached to the template DNA AT around 50-56ºC.
3) **Extending** → in this stage the TAG polymerase enzyme had bound & a new DNA strand was made. This process took place at 72ºC .

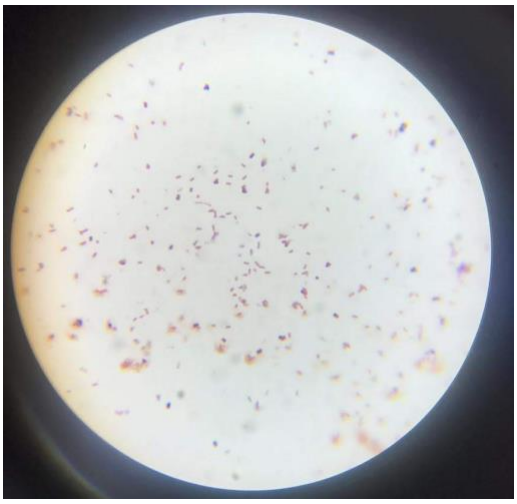
biochemical tests were performed for identification of bacteria based on the differences in their biochemical activities. Below are the bichemical tests performed.

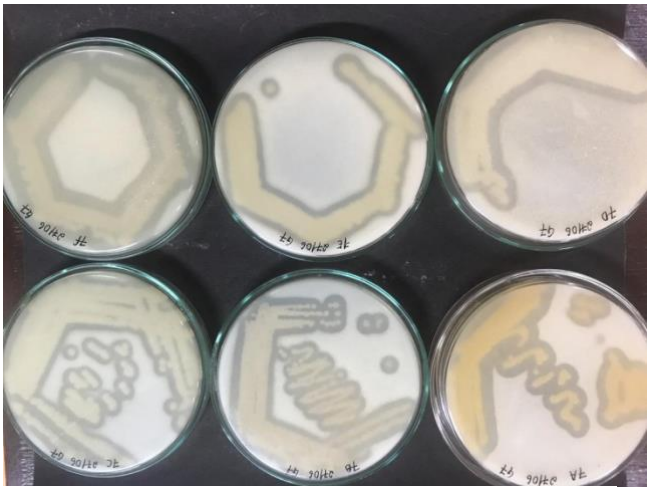
- Sugar utilization test → (glucose,Sucrose,maltose,galactose,lactose,xylose,mannitol): sugars are metabolized through different metabolic pathways depending on types of microbial species & aerobic or anaerobic environment.if fermenting bacteria are grown in a liquid culture medium containing the carbohydrate,they may produce organic acids as by-products of the fermentation.these acids lower the pH of the medium.if the PH indicator added to the medium,acid production will change the medium from its original color to yellow. Gases produced during the fermentation process can be detected by using Durham tube,within the liquid culture medium. If gas is produced,the liquid medium inside the tube will be replaced by the gas in the form of a bubble.
- Indole production test: This test Is performed to determine the ability of the organism to convert tryptophan into indole. Indole is generated by reductive deamination from tryptophan via the intermediate molecule indolepyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine ($-NH_2$) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonium (NH_4^+) and energy. Pyridoxal phosphate is required as a coenzyme.. The para-Dimethylaminobenzaldehyde reacts with indole present in the medium to form a red rosindole dye. The isoamyl alcohol forms a complex with rosindole dye, which causes it to precipitate. The remaining alcohol and the precipitate then rise to the surface of the medium.
- Urease : Urease catalyzes the breakdown of urea into ammonia and carbon dioxide. The test organism is cultured in a medium containing urea and the indicator phenol red. If the bacterial strain is urease-producing, the enzyme will hydrolyze the urea to give ammonia and carbon dioxide. With the release of ammonia, the medium will become alkaline & this is shown by change in color of indicator to reddish pink
- Methyl red: In the methyl red test (MR test), the test bacteria is grown in a broth medium containing glucose. If the bacteria has the ability to utilize glucose with production of a stable acid, the color of the methyl red changes from yellow to red, when added into the broth culture.
- Nitratase test : To determine the ability of an organism to reduce nitrate to nitrite which is then reduced to free nitrogen gas. The nitrogen in nitrate serves as an electron acceptor. The result of the denitrification process is the production of nitrite. Reduction of nitrate to nitrite is indicated if a red color develops quickly (within 1-2 minutes). If no color develops, add a very small amount of zinc powder (~20 mg) to the tube containing the reagents. If a pink to dark red color develops after adding the zinc powder within 5 min., the test is negative . If no color develops, the test is positive (the organism was able to reduce all the nitrate to nitrite and further to N2 which escaped from the tube
- Citrate utilization : The citrase enzyme hydrolyses the citrate to form oxaloacetic acid and acetic acid. When an organic acid such as citrate (remember Krebs cycle) is used as a

carbon and energy source, alkaline carbonates and bicarbonates are produced ultimately. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source. Utilization of exogenous citrate requires the presence of citrate transport proteins (permeases). Upon uptake by the cell, citrate is cleaved by citrate lyase to oxaloacetate and acetate. The oxaloacetate is then metabolized to pyruvate and CO2. The carbon dioxide that is released will subsequently react with water and the sodium ion in the medium to produce sodium carbonate, an alkaline compound that will raise the pH. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source. Growth usually results in the bromothymol blue indicator, turning from green to blue. The bromothymol blue pH indicator is a deep forest green at neutral pH. With an increase in medium pH to above 7.6, bromothymol blue changes to blue

- Motility test : Motility by bacterium is demonstrated in semi solid agar medium. The medium mainly used for this purpose is SIM medium (Sulphide Indole Motility medium) which is a combination differential medium that tests three different parameters, Sulfur Reduction, Indole Production and Motility. This media has a very soft consistency that allows motile bacteria to migrate readily through them causing cloudiness. The inoculum is stabbed into the center of a semisolid agar deep using a sterile inoculating needle. Bacterial motility is evident by a diffuse zone of growth extending out from the line of inoculation. Some organisms grow throughout the entire medium, whereas others show small areas or nodules that grow out from the line of inoculation. The non-motile bacteria will only grow in the soft agar tube and only the area where they are inoculated.
- Catalase test: The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old
- NA+ 7% NaCl : This test determines whether the microbe can grow on a nutrient medium where the concentration of sodium chloride (NaCl) is 7%. If the microbe can grow in the presence of 7% NaCl, the broth will become turbid (cloudy) after incubation. If an agar plating medium is used, growth is indicated by appearance of bacterial colonies following incubation.

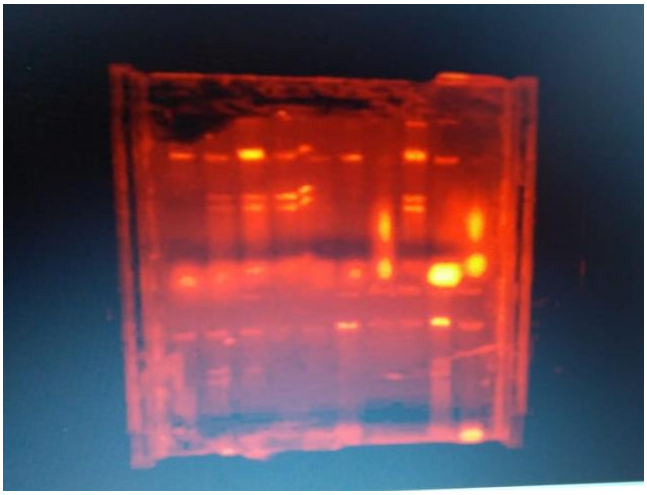
Results : the gram staining that was performed with the 10^{-1} dilution showed a mixed culture that contained both gram positive & gram negative short rods.

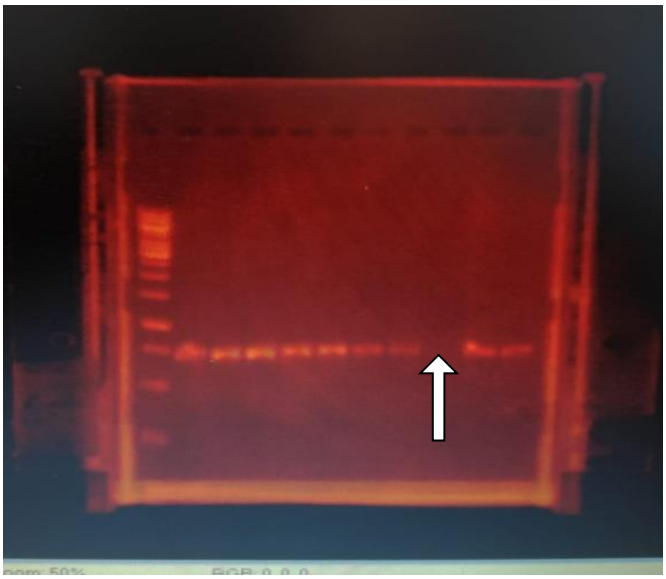




Streak plates of the 6 isolates from the compost soil sample.

The 9th well from above & 10th well from below were loaded with the bacterial DNA sample (7A). The 9TH well showed two bands. The band closer to the well indicates longer DNA molecule. The DNA molecule ran till the bottom of the gel from the 10th well. This showed that less amount of DNA sample was loaded into the well which then lead the shorter fragments run faster.





After performing the PCR, the DNA(7A) was then run on agarose gel electrophoresis to check the presence of DNA as well its amplification. When observed under UV Light, DNA from the isolate didn't run on the gel & hence showed no migration.

Tests for sugar	glucose	maltose	galactose	lactose	xylose	mannose
positive				+		
negative	-	-	-		-	-

tests	urease	nitratase	Motility	CU	VP	MR	Indole	catalase
positive	+	+	+					
negative				-	-	-	-	-

In the biochemical tests performed urease , nitratase reduction & lactose showed positive results.

In the urease test (fig1) ⟶ the color of the solution changed from yellow to red indicating that the ph indicator in the solution has changed and has made the solution alkaline due to the formation of ammonium carbonate.

Figure 1

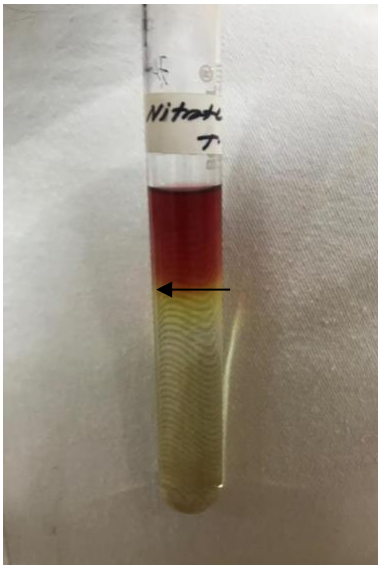


Figure 2

In the nitratase reduction test(fig2), the solution changed from yellow to red in color which indicates the reduction of nitrate to nitrile.

Lactose test(fig3) there was a slight change in color of the solution as compared to that of the control.

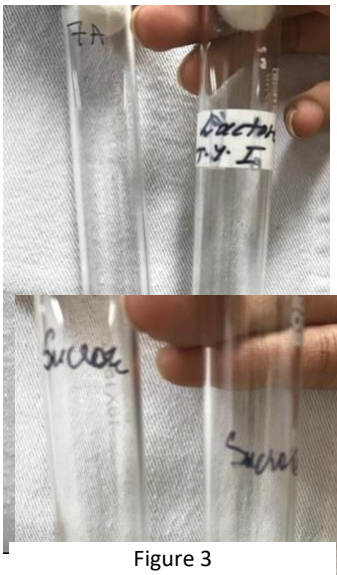


Figure 3

Figure 4 → maltose

Figure 5 → galactose

Figure 6 → VP

Figure 7 → sucrose

Figure 8 → mannitol

Figure 9

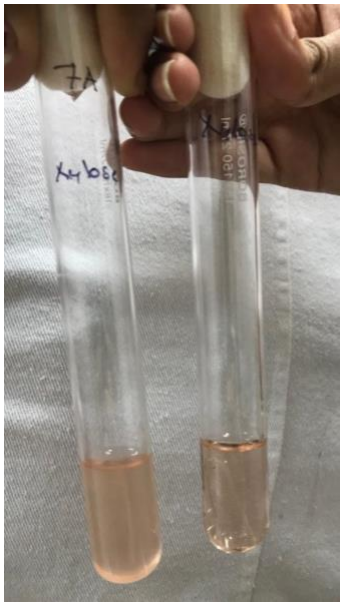


Figure 10 → xylose

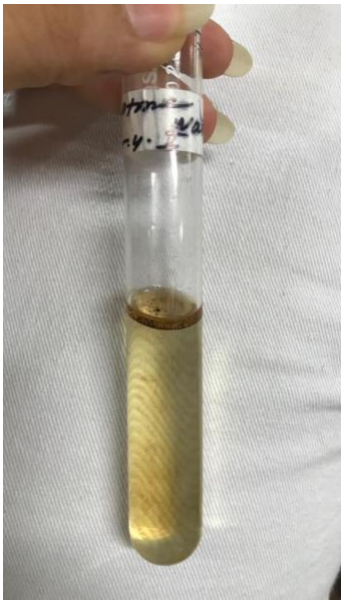
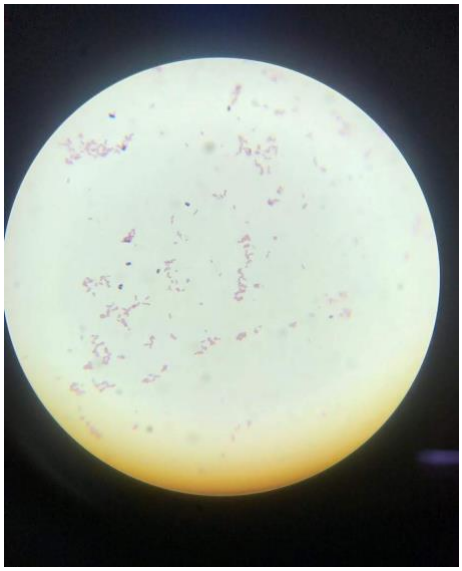


Figure 11 → indole

Gram staining (fig 12) ⟶ was performed for the isolated colony and was viewed under 100X on the microscope.The organism was stained purple & hence was found to be **gram positive** in nature. the colonies was were in the form of **short-rods**.



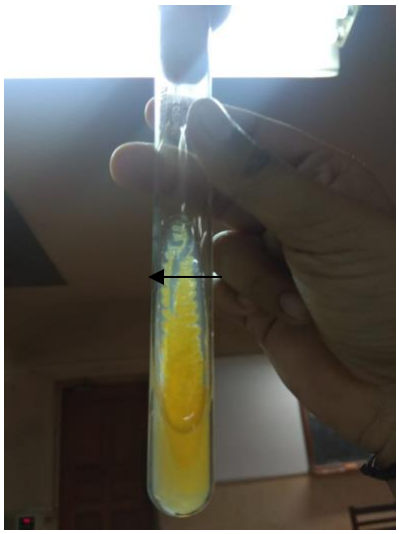


Figure 13

An agar slant (fig 13) was made to culture & grow the isolate for identification. After iccubation for 24hours at 37^{o}C **yellow pigmented colonies** were grown.

Discussion : the isolate(7A)that was obtained from the compost soil was streak plated & the culture grown had yellow colonies. Through gram staining it was found that the culture was gram positive in nature with short rods.the organism showed slight motility in the motility test. in order to identify the organism various biochemical tests were performed. In the sugar utilization test only lactose showed slight positive results but didn't show any production of gas.the other tests done for sugars that included glucose,maltose,xylose,galactose,mannitol showed negative results. The result of the Urea hydrolysis test was positive & a distinct color change was observed.from the agarose gel electrophoresis that was performed, results showed that there was a difference in the DNA migration between the two same samples (7A) that were loaded on the top & bottom wells. One of the possible reasons for this could be unequal amounts of DNA Samples were loaded into the wells or due to human error while loading the samples.due to a large migration in the bottom well , it could be possible that it contained a large amount of genomic DNA as compared to that of the top well whose migration was less. The DNA obtained after its amplification from the PCR showed no migration. This could be due to loading less amount of DNA sample.the universal primer was selected on the basis that all the strains that were used were conserved .since our DNA sample didn't show any migration,it could be possible that this universal primer wasn't suitable for the 7A Culture. Improper DNA isolation could also be one of the limitations but its gets ruled out because the DNA showed migration in the agarose gel electrophoresis. DNA sequencing wasn't carried out after the PCR & hence the isolate (7A) was identified based on the results obtained from the biochemical tests.

Conclusion : an unknown protease producing bacteria from compost soil was isolated & identified. With the help of bergey's manual of determinative bacteriology ,the organism was identified at the genus level & was found to be Nocardia based on the gram nature,colony characertistics & the biochemical tests. *Nocardia* species are mainly found in the soil that is rich in organic matter . Some species of *Nocardia* are pathogenic & cause Nocardiosis. *Nocardia* species are gram positive in nature having short rods. A few more biochemical tests like the acid fast test, test for growth on blood agar,antibiotic suceptibilty tests could help enhance our study to identify the organism. On the basis of the data & results obtained, *Nocardia flavescens* could possibly be the 7A isolate taken from the

compost soil sample. This isolate (7A) also shows close resemblence to few other species like *N.fordii & N.maculata*.

KINGDOM	Bacteria
PHYLUM	Actinobacteria
CLASS	Actinobacteria
ORDER	Actinomycetales
SUBORDER	Corynebacterineae
FAMILY	Nocardiaceae
GENUS	*Nocardia*
SPECIES	*flavescens*