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

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Date of Submission: 9th September 2019

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1) INTRODUCTION:-

Various types of bacteria are known to inhabit different eco-systems. These micro organisms coexist or compete with other micro organisms to create a distintive micro flora in that environment.

This study aims to isolate a protease producing bacteria from a sample of homemade compost mainly made of used tea leaves and vegetable peals. Bacterial isolation, purification and identification are the first steps of bacteriological studies. Isolation is done to obtain pure bacterial cultures. Pure culture is essential in the study of the morphology, physiology, biochemical characteristics, and susceptibility to antimicrobial agents of a particular bacterial strain.[1] Bacterial species differ in morphological, physiological and biochemical characteristics and those can be used when coding or labelling them. Therefore, identification is accomplished by performing several morphological, physiological and biochemical tests. Results of these tests are compared to established taxa or identification schemes using the Bergey's manual as a guide.

Proteases is a common family of enzymes that are involved in most of the processes in an organism's physiology. Peptide bonds in proteins are hydrolysed by proteases and they are also called as peptidases or proteinases or proteolytic enzyme.[2] Due to their wide range substrate specificity, proteases are used in many industrial applications such as leather processing, detergent formulations, baking, brewing, meat tenderization, peptide synthesis, cheese manufacture, soy sauce production, protein hydrolysate, pharmaceutical industry, waste treatment, silk industry, organic synthesis, and silver recovery from waste photographic film.[3]

Soil microbial populations play crucial role in soil properties and influence below-ground ecosystem processes. Microbial composition and functioning changes the soil quality through decomposition of organic matter, recycling of nutrients, and biological control of parasites of plants. Moreover, the discovery that soil microbes may translate into being benificial for biotechnology, management of agricultural, forest, and natural ecosystems, biodegradation of pollutants, and waste treatment systems maximized the need of scientists for the isolation and their characterization. The goal of measuring the soil microbial diversity is difficult because of the limited knowledge about bacterial species and classification through families and orders.[4]

2) MATERIALS AND METHODS:-

2.i) BASIC REQUIREMENTS:-

Burner, nichrome loop, tripod stand, glass slides, cavity slides, test tubes, cover slips, canister, pipette, cotton, filter paper, incubator, microscope.

2.ii) PREPARATION OF SERIAL DILUTION:-

A sample of homemade compost basically made up of used tea leaves, raw vegetables peels, spoilt vegetables and food was brought to the lab on 24th June 2019 and was kept at room temperature until used for the experiment. Serial dilution of the sample was conducted in the following manner.

1g of the compost sample was mixed in 9ml of saline solution (9gms/L NaCl) in a test tube at left to settle for 10mins, making it a 10^{-1} dilution.

Test Tubes	1	2	3	4	5
Saline solution (ml)	9	9	9	9	9
Sample	1g	1ml	1ml	1ml	1ml
Dilution factor	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Plating method	T-streak & Spread plate	Spread plate	Spread plate	Spread plate	Spread plate
Plate used	Skimmed milk agar	Skimmed milk agar	Neutrient agar	Neutrient agar	Neutrient agar
Incubation	All the plates were incubated at 37^{O}C for 24 hours.				

Table 1: The dilution table representing the dilution factor(df) and wich df tubes were used for plating and the types of plates used.

2.iii) MAINTAIENCE OF PURE CULTURE:-

In order to maintain the culture, a colony was picked up under sterile conditions and was streak plated on a sterile neutrient agar plate or slant. These were then labelled and then incubated at 37 ^{O}C . After 24-hour incubation, it was preserved by refrigeration at 2-8^{O}C. To check if the culture is a pure culture without any contaminations perform a Gram Stain.

2.iv) ISOLATION OF ORGANISM:-

A protease producing bacterial colony was picked up with a sterile nichrome loop under sterle conditions from a skimmed milk agar plate and then inoculated into a sterile saline solution containing neutrient broth and streaked on a neutrient agar plate. The neutrient broth and plate was then incubated at 37 ^{O}C. This broth containing a pure culture of the organism was maintained and used to perform various biochemical and biological tests on the organism.

2.v) ISOLATION OF GENOMIC DNA (gDNA) FROM THE ORGANISM:-

Requirements:- Sterile Optimal Broth (SOB), TE buffer, 10% SDS, 6 M sodium perchlorate, $CHCl_3$, chilled ethanol, centrifuge machine, heat block.

Procedure:-
a) Inoculate 10ml of SOB with the isolated organism and incubate it at 37 ^{O}C overnight.
b) Take 1.5ml of this culture in an eppendorf tube and spin at 14,000 rpm for 5 minutes. c) The supernatant is discarded and the pellet obtained is resuspended in 400µl TE buffer mix and add 100µl 10% SDS (these additions should be done in a cold environment containing ice) and incubate the eppendorf in a heat block at 60 ^{O}C for 5 – 10 minutes.
d) To this add 100µl 6M sodium per chlorate (pH 4.8) and incubate at 60 ^{O}C for 5 – 10 minutes.
e) Then add 600µl of CHCl and spin at 14,000 rpm for 20 minutes at 4 ^{O}C.
f) Carefully separate the supernatant in a new sterile eppendorf tube and discard the pellet.
g) Then add double the volume of chilled ethanol slowly and carefully from the wall of the eppendorf. Keep it the freezer for 5 minutes. DNA fibres will be seen in the interface between alcohol and water.

2.vi) AGAROSE GEL ELECTROPHORESIS (AGE):-

Requirements:- Tris Borate EDTA (TBE) buffer, Ethidium bromide, Gel loading buffer (0.25% Bromophenol blue + 0.25% xylene cyanol in 30% glycerol), horizontol gel electrophoresis apparatus with power source, UV – transilluminator.

Procedure:-

a) A horizontal gel electroporesis apparatus containing a 0.8% agarose gel with 20 wells was prepared and given to carry out the gel electrophoresis.
b) A loading sample was prepared on a wax paper with 7 µl of gel loading buffer and 2 µl genomic DNA, this was then loaded into the wells.
c) The power source was set at maximun current and a constant voltage of about 60 – 75 volts.
d) The tracking dye was let to run till it reached 3/4th of the gel. Then view the gel in a UV transilluminator.

2.vii) POLYMERASE CHAIN RREACTION (PCR):-

The 16S rRNA gene was selectively amplified using PCR.

Requirements:- Gradient PCR machine, Universal forward and reverse primers, TaqMM polymerase (master mix), WFI, gDNA of the isolated organism.

Procedure:-

Based on the composition and length of the primer used the aneeling temperature is decided.
In a sterile eppendorf tube 1µl of genomic DNA, 1µl of forward primer, 1µl of reverse primer, 5 µl of TaqMM polymerase and 2µl of WFI were added. To get a total volume of 10 µl.
This eppendorf tube was then placed in a Gradient PCR machine.
Agarose Gel Electrophoresis was then performed on the DNA product of thr PCR.

Process	Initial	Denaturation	Aneeling	Polymerization	Extension	End of PCR
Temperature	94 ^{O}C	94 ^{O}C	55 ^{O}C	72 ^{O}C	72 ^{O}C	10 ^{O}C
Time taken	5.0 minutes	0.30 seconds	0.15 seconds	0.45 seconds	3.0 minutes	———
No of cycles		<——— 30 cycles ———>				

Table 2: Settings at wich the Gradient PCR was functioning for amplification of 16S rRNA gene.

2.viii) BIOLOGICAL AND BIOCHEMICAL TESTS:-

The identification of the bacteria were carried out by morphological and biochemical tests. The methods used are given below.

Sr. No.	Name of Test	Chemicals required and Composition	Procedure
1	Gram Stain	Crystal violet stain, grams iodine, acetone alcohol, saffranine stain.	Flood an air dried and heat fixed smear of the culture on a slide with crystal violet and keep for 1 minutes, rinse. Repeat the same procedure for grams iodine. After rinsing off the iodine rinse the slide with acetone alcohol for 10 seconds. After this, flood the slide with Safranin and rinse off after 3 minutes and observe the slide under 100 x magnification. [13]
2	Capsule staining	Manewal's A (1% congo red), Manewal's B (acid fuchsin, FeCl3, phenol, acetic acid).[5]	Add a drop of Manewals solution A (Congo red) to a clean slide. Then smear a loopful of culture in this drop. After 10 minutes this was rinsed off under running tap water and Manewals solution B (methylene blue) was added and kept for 10 minutes. After rinsing off, the slide was observed under 100 x magnification.[14]
3	Motility test	Petroleum jelly (or Vaseline)	Using a capillary tube, vaseline was applied on the four corners of a cover slide. Then a tiny drop of culture was added onto this cover slip. This cover slip was then overturned over the well of a cavity slide and the edge of the hanging drop was studied under the microscope, first under 10x and then 45x.[15]
4	Skimmed milk agar	SM powder, tryptone, yeast extract, dextrose and agar.[5]	0.1ml of the culture is spread plated on a skimmed milk agar plate under sterile conditions. This plate was then incubated at 37^{O}C for 24 hours.[16]

5	Sugar utilization a) Sucrose b) Maltose c) Galactose d) Lactose e) Xylulose f) Mannitole	Andrade's reagent, Durhams's tube and respective sugar solutions.	a) Test tubes containing it's respective sugar, peptone, Andrade's reagent and a Durham's tube were prepared. b) A loopfull of culture was inoculated under sterile conditions in each of these test tubes containing the respective sugar. c) These test tubes are incubated at 37^{O}C and checked for any change the next day.[6]
6	Citrate utilization	Sodium Chloride (NaCl), Sodium Citrate (dehydrate), Ammonium Dihydrogen Phosphate, Dipotassium Phosphate, Magnesium Sulfate (heptahydrate), Bromothymol Blue, agar. [5]	A loopfull of culture was inoculated on a slant in a zizzag manner starting from the bottom and ending at the top. The test tube was incubated at 37^{O}C for 24 hours.[8]
7	Catalase test	3% H_2O_2 solution	A loopful of the culture was mounted on a clean, grease- free slide. Then a drop of 3% H_2O_2 was added to the culture and the reaction between them was observed.[7]
8	Urea hydrolysis	Peptone, dextrose, sodium chloride, disodium phosphate, monopotassium phosphate & phenol red.[5]	A loopful of culture was inoculated into a tube to check for urea hydrolysis the tube was incubated at 37^{O}C for 24 hours.
9	Indole test	Casein enzyme hydrolysate, sodium chloride.[5]	A loopful of the culture was inoculated into a tryptophan broth and incubated at 37^{O}C. After 24 hours, 0.5 ml of the Kovac's reagent was added to the broth and any changes were observed.[9]

10	Growth in 7% NaCl	Sodium Chloride (7% NaCl), Peptone, dextrose, agar.[5]	A loopfull of culture was inoculated on a slant in a zigzig manner starting from the bottom and ending at the topand was incubated at $37^{O}C$ for 24 hours.
11	Growth in Glucose-6-PO_4		A loopfull of culture was inoculated in a broth of glucose-6-PO_4 and incubated at $37^{O}C$ for 24 hours.
12	Methyl Red (MR) test	Methyl red	In a tube containing the culture add methyl red and observe the colour change.[10]
13	Vogues-Proskauer (VP) test	40% KOH, α-naphthol	In a tube containing the culture add α-naphtol and potassium hydroxide and observe for results.[11]
14	Reduction of Nitrate (Nitratase test)	Peptic digest of animal tissue, meat extract, potassium nitrate and sodium chloride.[5]	A loopfull of the culture was inoculated into a nitrate broth and incubated at $37^{O}C$ for 24 hours. A dropperful of sulfanilic acid and α-naphthylamine was then added and observed. [12]

3) RESULTS:-

A protease producing colony was picked under sterile conditions from skimmed milk agar plate of 10^{-2} diluton factor. This colony was then streak plated on a sterile neutrient agar plate and a skimmed milk agar plate. An isolated pure colony was picked under sterile conditions from the skimmed milk agar plate and was inoculated into a test tube containing neutrient broth. This test tube was kept in an incubator at $37^{O}C$ for 24 hours and stored. The culture in the nutrient broth was used for further biological and biochemical tests.

3.i) Morphological results from Streak plate:-

Colony characteristics	Selected colony
Size (mm)	3 – 4
Elevation	Convex
Margin (edge)	Smooth
Shape	Circular
Optical characteristics	Opaque
Pigmentation	Unpigminted

Table 3: Observations for Streak Plate.

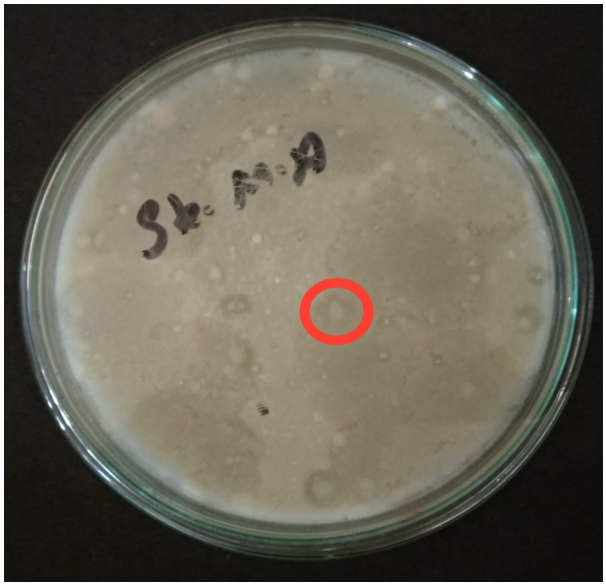

Figure 1: Shows a skimmed milk agar (sma) plate that was spread plated with 0.1ml of 10^{-2} dilution factor. The colony inside the red circle was picked up and streaked on another sma plate.

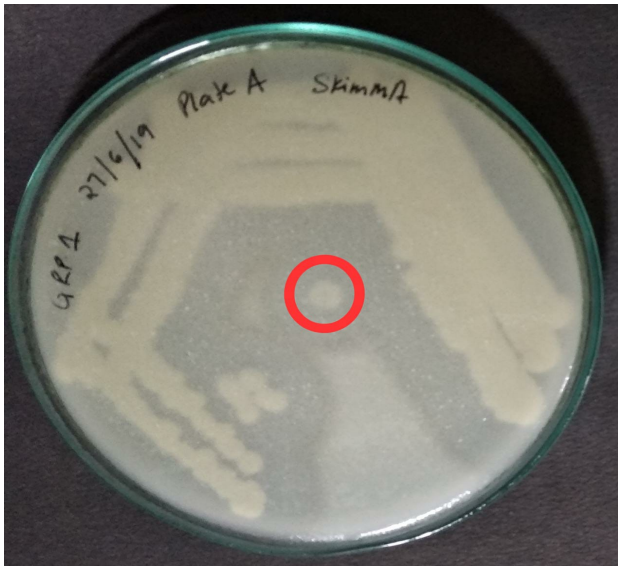

Figure 2: A sma plate that was streak plated. The colony inside the red circle was picked up and inoculated into a neutrient broth, to form a pure isolated culture or the organism.

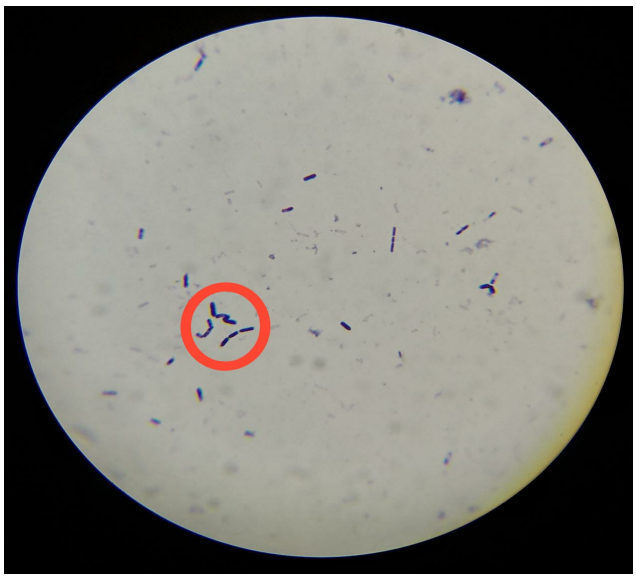

Figure 3:Gram Stain of the isolated orgsmism. Gram positive rods arranged in chains of 2 – 3 cells are seen inside the red circle and in the field of view.

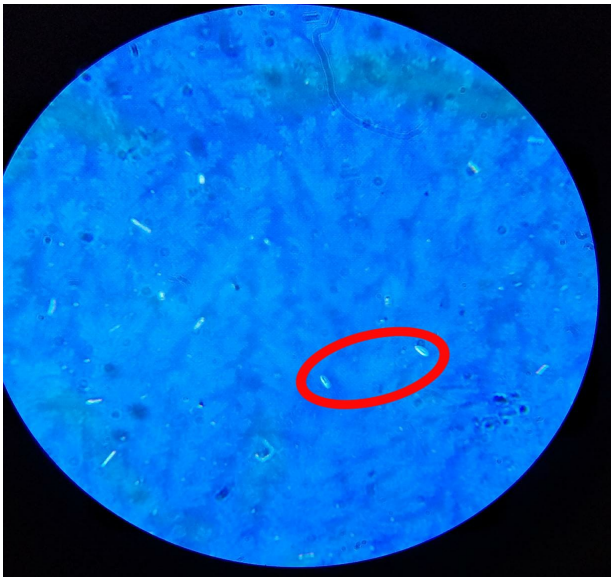

Figure 4: Capsule Staining of the organism. A pink cell inside a colourless halo can be seen on a blue background. This indicates that the organism produces a capsule.

Figure 5: Isolated organism growing in a neutrient broth.

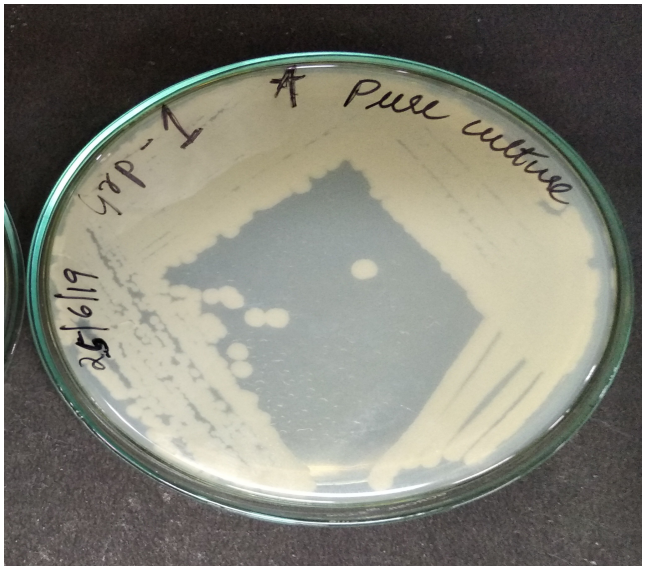

Figure 6: Results of a streak plate performed to verify if the isolated organism is still in a pure cultue form without any contaminations. In the plate we can see only one type of colony growing indicating that the culture is not contaminated.

3.ii) Biological and Biochemical test results:-

The test results of the various biological and biochemical tests performed are given in the format of a table below –

Key :- Positive test (+ ve test) and Negative test (- ve test)					
Sr. No.	Name of Test	Principal of Test	Expected Results	Observed Results	Inference
1	Gram Stain	Gram positive cells due to their thick peptidoglycan layer are able to resist decolorization and retain the primary stain while the gram negative cells are decolorized and take up the counterstain, safranin.[13]	Purple coloured gram positive cells or pink coloured gram negative cells.[13]	Purple coloured gram positive rods.	The organism is gram positive in nature and rod shaped in morphology.
2	Capsule staining	The background of the cell is stained with an acidic stain like Congo red and the cell itself is stained with a basic dye like methylene blue to be able to clearly visualize the capsule. The capsule is non-ionic in nature.[14]	Capsule present: Blue background with stained cells in colourless halo zones. Capsule absent: Blue background with no stained cells in colourless halo zones.[14]	Blue background with stained cells in coloueless halo zones.	The organism has a capsule.
3	Motility test	Since the cavity is sealed with wax, the hanging drop won't evaporate easily and the organism whether or not its moving can be observed at the edge of the drop.[15]	Motile or non motile cells.	Motile cells	The organism is slightly motile. (can be caused by brownian motion)

4	Skimmed milk agar	To check if the organism produces an enzyme casease that hydrolyses casein. Casein is present in the agar giving it a white or opaque colour.[16]	+ ve test: A transparent zone of clearance around the colony. - ve test: No transparent zone of clearance.[16]	A transparent zone of clearance around the organism is observed.	The organism is capable of hydrolysing casein by producing enzyme casease.
5	Sugar utilization a) Sucrose b) Maltose c)Galactose d) Lactose e) Xylulose f)Mannitole	If a carbohydrate is used as a energy source by an organism, an acid or an acid and gas is produced. If acid is produced, pH of the medium is lowered that is detected by an indicator present in the medium. If a gas bubble is seen in the Durham's tube it indicates that the organism produces gas on metabolism of carbohydrates. If the organism uses peptone as an alternate source of energy, acidic products wont be formed and the medium wont change colour. [6]	+ test: Pink colour of the solution indicates acid produced from carbohydrate metabolism. - ve test: Solution turns yellow or lighter pink.[6]	No gas was produced in any of the tubes. Acid produced by Sucrose and Maltose. Galactose, Lactose, Xylulose and Mannitole didn't produce any acid.	The organism utilizes sucrose and maltose but not galactose, lactose, xylulose and mannitol.

6	Citrate utilization	Citrate agar contains sodium citrate and inorganic phosphates as the sole of carbon and phosphates respectively. Only organisms with enzyme citrate permease can grow on this agar. Citrate is metabolised to form alkaline products, increasing the pH. The bromothymol blue indicator is green in neutral pH and turns blue as the pH imcreases.[8]	+ ve test: Change in clour from green to blue. - ve test: No change in coloue.[8]	No colour change of the citrate agar.	No colonies grew. The organism cannot detecatably use citrate as a carbon source.
7	Catalase test	Catalase mediates the rapid break down of hydrogen peroxide into oxygen and water. Hence, the rapid effervescence of oxygen bubbles appears on the test region.[7]	+ ve test: Effervescence with gas bubbles. - ve test: No effervescence and gas bubbles.[7]	Slight effervescence with few gas bubbles.	The organism produces catalases. Hence it is an anarobe or facultative anaerobe.
8	Urea hydrolysis	Organisms that hydrolyse urea produce ammonia as a by product that turns the pH of the medium alkaline. This causes the phenol red indicator to change colour from yellow to red.	+ ve test: Colour of the media changes to pink. - ve test: The media does not change colour.	Colour of the media was yellow, it did not change to pink.	The organism cannot hydrolyse urea.
9	Indole test	To determine weather the organism produces tryptophanase to break down tryptophan into indole(one of the end products). Indole reacts with Kovac's reagent to form a red/pink ring.[9]	+ ve test: Red ring is formed. - ve test: Red ring is not formed.[9]	A red ring was not formed after adding Kovac's reagent.	The organism does not break down tryptophan.

10	Growth in 7% NaCl	Salt acts as a selective agent and interferes with membrane permeability and osmotic equilibrium. Only salt tolerant organisms can grow here.	+ ve test: Growth of organism. - ve test: No growth on organism.	Growth of organism is seen on the slant.	The organism is salt tolerant and capable of growing on a 7% NaCl slant.
11	Growth in Glucose-6-PO_4	To check is an organism can utilize glucose-6-PO_4 as a carbon source. A turbid solution indicates growth of the organism.	+ ve test: Turbid solution. - ve test: No turbidity, clear solution.	The solution was Turbid indicating a positive test.	The organism can utilize glucose-6-PO_4 as a carbon source.
12	Methyl Red (MR) test	The fermentation of glucose leads to acidic end products which are detected by addition of the methyl red indicator which turns red in the acidic pH.[10]	+ ve test: Red colouration on addition of methyl red - ve test: Yellow colouration on addition of mrthyl red.[10]	Formation of red colour on addition of methyl red.	The organism produces acids on fermentaion of glucose.
13	Vogues-Proskauer (VP) test	Acetoin produced by metabolism of glucose, is converted to diacetyl when α-naphthol is added to it in presence of atmospheric oxygen and 40% KOH. Diacetyl is converted into a red coloured complex by the catalytic action of α-naphthol and creatine.[11]	+ ve test: Pink-red colour on the surface of the medium. - ve test: Yellow colour on the surface of the medium.[11]	Appreance of yellow colour on the surface of the medium.	The organism does not produce acetoin during metabolism of glucose.

14	Reduction of Nitrate. (Nitratase test)	To determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2). It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen. If the medium turns red after the addition of the nitrate reagents, it is considered a positive result for nitrate reduction. If the tube turns red after the addition of the zinc, it means that unreduced nitrate was present.[12]	On addition of nitrate reagents + ve test: Red ring formed in broth. - ve test: No colour change. In the -ve test add Zn powder. + ve test: If the colour does not change to red. - ve test: If colour changes to red.[12]	A red ring was formed after adding the nitrate reagents.	The organism is capable of reducing nitrate and nitrite.

Figure 7: Left t.t shows a +ve MR test, red colouration of medium. Right t.t. shows a -ve VP test, yellow colouration of the medium.

Figure 8: The organism doesn't synthesize citrate permease hence it is not capable to grow on a citrate slant.

Figure 9: Growth of the organism on a 7% NaCl slant is seen.

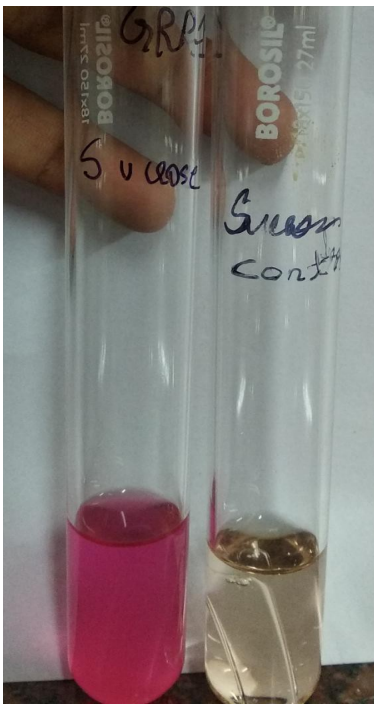

Figure 10: Left t.t. shows a +ve test for sucrose utilization forming acids that gives a pink colour to the media. Right t.t. is a control t.t.

Figure 11: Left test tube (t.t.) shows a +ve test for maltose utilization forming acids that gives a pink colour to the media. Right t.t. is a control t.t.

Figure 12: Formation of a red ring on addition of nitrate reagents indicates a +ve Nitratase Test.

3.iii) Agarose Gel Electrophoresis (AGE) Results:-

a) AGE of genomic DNA:

The gDNA from nine different organisms was sucessfully isolated and this gDNA was run on a 0.8% agarose gel with 20 wells. The gDNA sample was run in duplicates on the gel. Several bands of different base pairs were observed using a UV transilluminator. Few conserved bands were also observered from the nine different organisms.

b) AGE of 16S rRNA PCR product:

The PCR product of 16S rRNA from nine different organism was run on an agarose gel with 10 wells. A DNA ladder was spotted in the first well, so as to check the kilo base pairs of the PCR product after it is let to run on the gel. 16Sr RNA is suposed to be a conserved sequence among a vast variety of bacterial species. Although certain exceptions are possible.

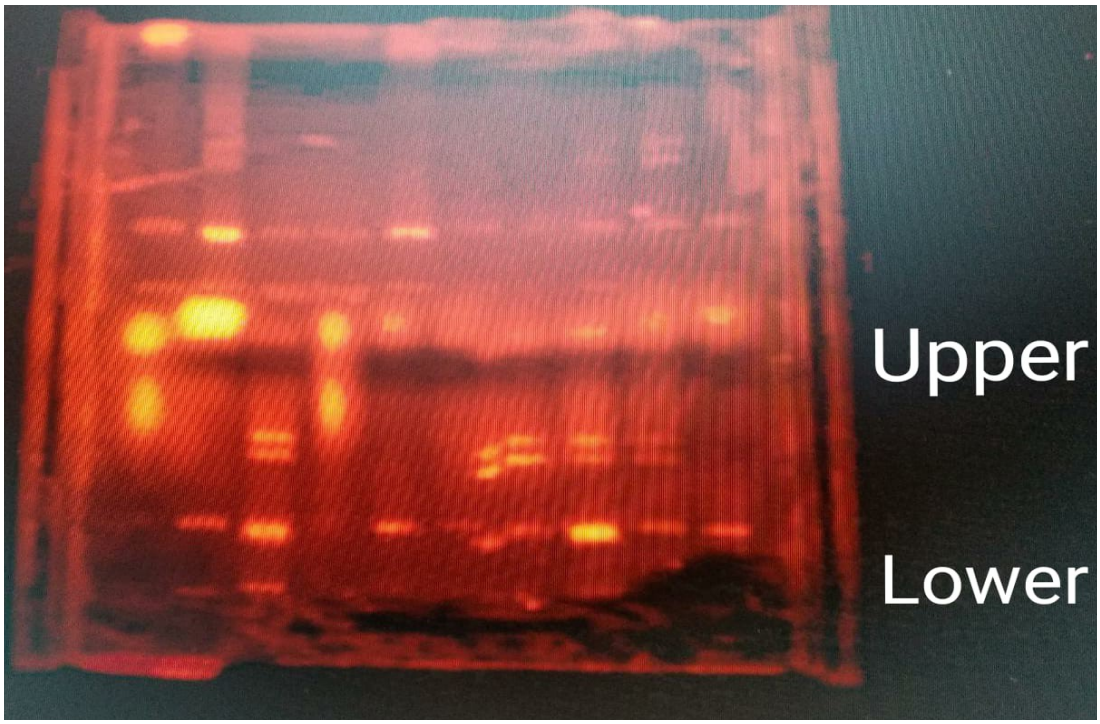

Figure 13: AGE results observed in a UV transilluminator of gDNA from nine different organisms. Lower and Upper (text) in the picture indicates the position of the wells.

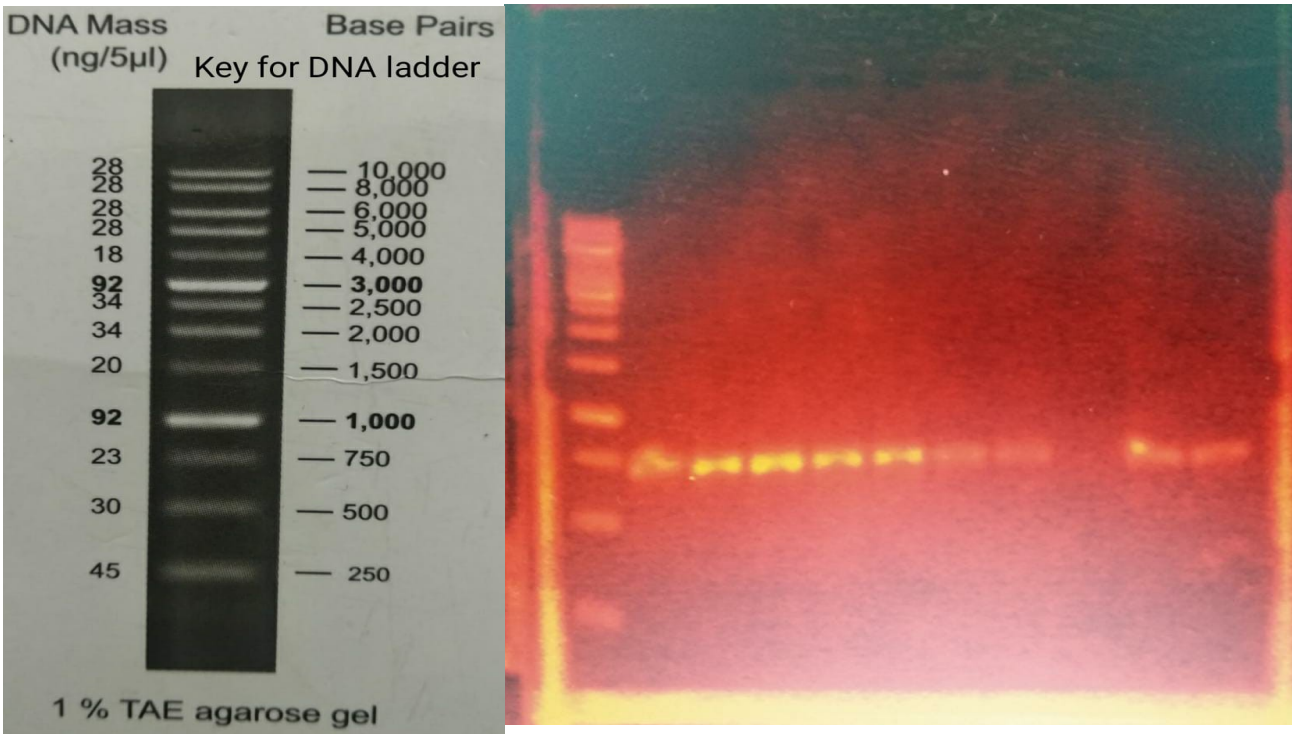

Figure 14: AGE results of 16SrRNA orserved in a UV transilluminator on the right and a key of the DNA ladder used on the left. Wells are located from the top left to right region. It is noticed that the 16S rRNA from the different organism almost have the same amount of base pairs. On comparision with the DNA ladder the bands formed corelates with the 750 base pair band.

4) IDENTIFICATION OF THE ORGANISM:-

4.i) GENUS:-

Characteristics of the organisms used for the identification of the genus.
a) Rod shaped cells, sometimes occuring in chains.
b) Mostly motile.
c) Gram positive in nature.
d) Catalase positive.
e) Aerobic or facultatively anerobic.
f) Occurance of capsule.
g) Carbohydrates are generally fermentated with the production of more or less acids.
From all the possible genera, ***Bacillus* is the only genus that possesses all the characteristics mentioned above**.
Hence the organism belongs to the genus *Bacillus*.[17]

4.ii) SPECIES:-

After the genus of the isolated bacteria was detemined, Bergey's Manual of Systematic and Determinative Bacteriology was refered to identify what species it could be. There were 95 existing species of *Bacillus*, each of them had their distinguishing test results. The species that did not fit in the test results obtained from the experiments performed were eliminated and the list was narrowed down to a few possible species.
Folllowing are the test results used to identify the species:-
1) Coloured colony (dark brown/black or red, yellow, pink): The organism formed an opaque, white colony with no pigmentation.
2) Motility: The organism was motile.
3) Catalase test: The organism is catalase positive.
4) Hydrolysis of protein: The organism is capable of hydrolysing caesin.
5) Vogues–Proskauer test: The organism is VP negative.
6) Citrate utilization: The organism does not utilize citrate to grow.
7) Nitrate reduction: The organism produces nitratase enzyme to reduce nitrates.
After considering these seven results, the possible species of *Bacillus* that the organism could be was reduced to 8 species i.e., *B. decolorationis, B. firmus, B. galactosidilyticus, B. novalis, B. psychrosaccharolyticus, B. siralis, B. soli, B. vireti.* [17][18]

Further tests were done to narrow down the posible species isolated:-
1) Sugar utilization: The organism produced acids from sucrose and maltose.
2) Urease test: The organism cannot hydrolyse urea.
3) 7% NaCl slant: The organism grows on a 7% NaCl slant.
Through these tests the possible species were further narrowed down to four i.e., *B. firmus, B. decolorationis, B. galactosidilyticus or B. soli.*[18]

5) DISCUSSION:-

Even though the study of micro flora in soil began a cenury ago, there are many microorganisms that are yet to be classified into an existing bacterial genus or a completely new genus, or even to be discovered. It is an understatement that “One gram of fertile soil can contain upto one billion bacteria”. As many of these organisms are not only bacteria as soil contains other microbes such as archae, fungi, algae, protozoa, actinomycetes. All though isolating a bacteria from a soil sample, identifying it's characteristics, studying it's effect on environment and human beings, it's function as a soil organism seems to be quite chalenging still at times the results accuried are astonishing and unexpected that drives one's curiosity to discover and identify as many organisms as one could.

One important fact that needs to be kept into consideration is that many organisms are variable in their test results (E.g. Gram variable organisms). Also because they had been removed from their natural environment and made to grow in a lab setting under artificial conditions, with all nutrients available and no competition to their survival, could have caused the organisms to adapt to the change in their environments and not act the way they would in their natural environment and hence give an error in certain test results leading to an error being made in its identification.

It can safely be said that the isolated organism is an aerobic or facultative anarobe. Because the fact that anaerobes would not grow in aerobic conditiond and thermophilic, acidophiles, psychrophiles or halophiles would not grow in normal lab conditions.

Certain errors could have occured while carrying out the asepitic techniques that could have lead to contaminating the pure culture. These errors could be if a nichrome loop was not cooled enough before using it, if a sterile surface was exposed to an area outside the sterile zone or due to talking while performing an aseptic transfer. If a contamination in the pure culture has occured, then sub culturing techniques can be performed to obtain the pure culture again.

While performing the catalase test the observed results was not as promising as what was expected, this could have mislead the inference as being a catalase negative organism. From the sugar utilization tests the organism showed a good reaction and high amounts of acid production with sucrose and maltose, weak reaction and low amounts of acid production with xylulose and galactose and low to no reaction or acid production with lactose and mannitole.

Genomic DNA was sucessfully isolated from the organism and the 16S rRNA gene sequence was also sucessfully amplified using PCR technique, but this sequence was not outsourced for sequencing. Hemce, identification of the isolated bacteria was done only on the basis of biochemical tests performed.

Based on the data from the Bergey's Manual of Systematic Bacteriology, the following species of *Bacillus* were shortlisted :

1) *B. decolorationis*: Isolated from mural paintings, discoloured by microbial growths. It is an aerobic, gram-variable, motile, rods and coccoid rods, that occur singly, in pairs and in short chains. Gives a negative urease test.[18] However the organism was not isolated from a mural painting discoloured by microbial growth. Hence it cannot be said for sure that the isolated organism is a *B. decolorationis.*
2) *B. galactosidilyticus*: Isolated from raw milk. It is a facultative anaerobe, gram-positive or gram-variable, motile, round-ended rods, that occur singly and in pairs. Gives a negative urease test.[18] Hence, the isolated organism could be a *B. galactosidilyticus*.
3) *B. soli*: Isolated from soil in Drentse, a agricultural reearch area in Netherlands. It is a facultative anerobe, gram-positive or gram-variable, motile, round-ended rods, that occur singly, in pairs and in chains.[18] Since the result for urease test is unknown, it cannot be definitively said that the isolated organism is a *B. soli.*
4) *B. firmus*: Isolated from soil and other environments. It is a facultative anaerobe, gram-variable, motile, round-ended rods, occur singly and in pairs or ocassionally in pairs. Gives a negative urease.[18] Hence, the isolated organism could be a *B. firmus*.

   Therefore, the organism might be *B. firmus, B. decolorationis, B. galactosidilyticus, B. soli*. Out of wich **the organsim is most likely to be *Bacillus firmus, Bacillus soli* or another undiscovered species of *Bacillus***.

   Further tests such as hydrolysis of starch and gelatin, endospore stainig, utilization of propionate can be carried out the get a better understanding of what species the organism is.

Although the process of identification of the isolated organism during the course of this project was quite challenging since there wasn't enough time to perform few extra biochemical tests, it was gave a good exposure to the world of microbiology and its chalenges.

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