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: UPASANA PARESH SHAH

UID Number: 172065 **Date of Submission:** 9th September, 2019

Isolation & Identification of Unknown Protease Producing Bacteria from Compost Soil

Upasana Shah, Josette Misquitta, Nimisha Thomas, Siena D'Souza,
Department of Life Sciences & Biochemistry, St. Xavier's College (Autonomous), Mumbai

Introduction:

Proteases and their Applications: Proteases occur naturally in all organisms. Proteases are enzymes that are capable of carrying out proteolysis by hydrolysing the peptide binds that link the amino acids, in a polypeptide chain, together. Proteases are also referred to as proteolytic enzymes or proteinases and are of two types- exopeptidases and peptidases. On the basis of their behaviour they are classified as acidic (produced by fungi, pH- 2.0 to 5.0), alkaline (pH > 8.0) or neutral (mainly of plant origin, pH=7.0 to 8.0) proteases [1] These enzymes are used throughout an organism for various metabolic processes, are essential for cell growth and differentiation, and are involved in a multitude of physiological processes, ranging from digestion of food proteins to blood clotting and apoptotic pathways. Proteases are generally present in six forms or types- Serine, Cysteine, Threonine, Aspartic acid, Glutamic acid and Metallic proteases. The mechanism of cleavage of peptide bond occurs vis making an amino acid group that has cysteine and threonine or a water molecule, nucleophilic, in order to attack the carbonyl group of the peptide. A histidine residue is generally used to activate serine, cysteine or threonine as a nucleophile. Depending on the amino acid sequence of a protein, proteases can carry out limited (breaking of specific peptide bonds) or unlimited proteolysis (breakdown of a complete peptide to amino acids) [2]. Those organisms that produce a large amount of the enzyme are considered to be of industrial importance. In the present industrial scenario, protease producing organisms form an integral part of the leather tanning, detergent, medical and food industries, wherein a large proportion of the commercial proteases are derived from the neutrophilic *Bacillus* species.

Sources of Bacterial Proteases: All microorganisms have the inherent capacity to produce proteases. A large number of microbial species belonging to Bacteria, Fungi, Yeast and Actinomycete are known to produce proteases, bacteria being the most predominant group of alkaline protease producers. Strains of *Bacillus- B. lichiniformis*, *B. subtilis* and *B. amyloliquifaciens* are some of the alkaline protease producing species. *Pseudomonas, Flavobacterium, Halobacterium, Vibrio, Serratia, Staphylococcus, Brevibacterium, Alcaligenes, Streptomyces, Nocardia and Nocardiopsis, Neurospora, Penicillium, Ophiostoma, Myxococcus, Rhizopus* species have also been isolated as protease producing organisms [3]. Microbial sources of proteases are preferred over the other sources since microbes can be grown in a small space, the growth is fast and easy, generally lack pathogenicity, produce eco-friendly products and genetic modification is possible [4]. The objective of the present study was to isolate protease producing bacterial strains from a compost soil source.

Materials & Methods:

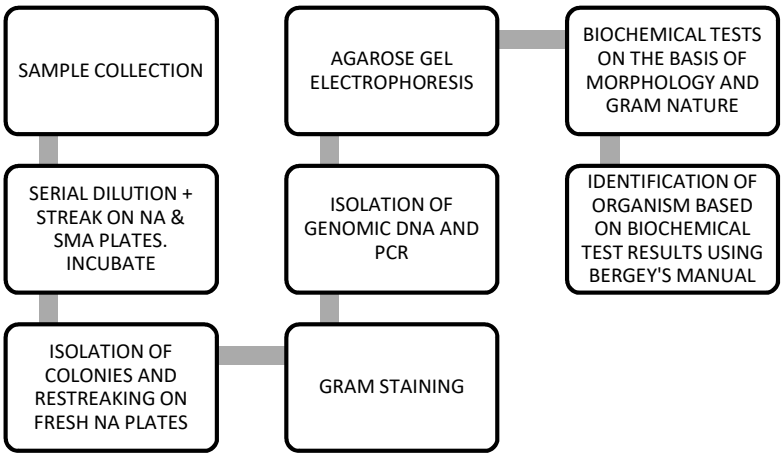


Fig: Flowchart for protocol implemented for isolation and identification

Sample Collection: The compost soil source selected for the present study was taken from plants' soil where composting had been carried out and from compost soil that was found in St. Xavier's College, Mumbai campus. The soil sample was collected in a container which was later transferred to a polythene bag. The date of sample collection was noted.

Isolation of Protease Producing Bacteria:

Serial Dilution: The process of isolation of the bacteria was carried out on the same day as that of the soil sample collection. The technique used for isolation was that of Serial Dilution and Spread plating. 1gm of the soil sample was weighed and diluted serially with 9ml of Saline to obtain dilutions upto 10^{-5}. 10^{-3} to 10^{-5} dilutions were spread plated on Nutrient Agar (NA) plates, in duplicates. Dilutions 10^{-2} and 10^{-3} were also separately plated on Skimmed Milk Agar (SMA) plates. The plates were then incubated overnight at 37°C, and observed. Out of all the colonies obtained, the colonies showing the highest zone of inhibition or clearance were selected for further studies. Seven such colonies (labelled as 7A, 7B,7C, 7D,7E, 7F and 7G) were picked using a Laminar Flow, and streaked on NA plates, using hexagonal streaking method. The plates were incubated overnight at 37°C. Isolates from the plates (excluding 7G) were picked and streaked on SMA and stored. Post growth, the isolates were inoculated and grown. The cultures were preserved on Nutrient slants and were sub cultured twice, in order to prolong the life of the microorganisms' in the culture and to preserve them.

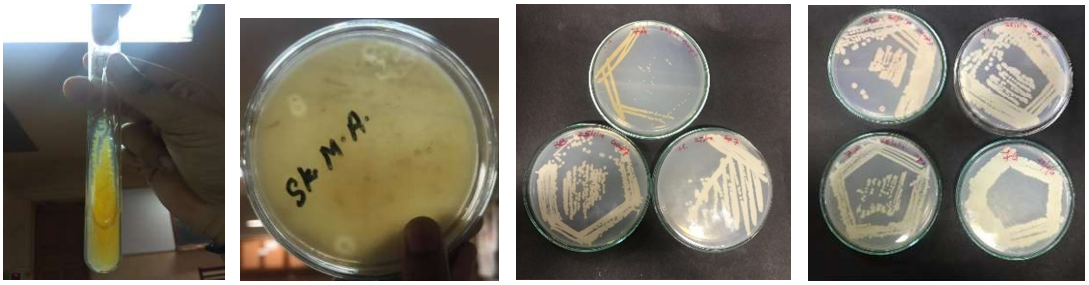


Fig1: 7A streaked on NA slant, Fig2: Colonies showing zone of inhibition on SMA, Fig3&4: 7 isolates restreaked on NA plates

NA and SMA: Nutrient Agar consists of Beef extract (3.0g), Peptone (5.0g), Agar (20.0g), Distilled water (1000ml), at a pH of 7.0-7.2. Skimmed milk agar consists of Skimmed milk powder (28g), Tryptone (5g), Yeast extract (2.5g), Dextrose (1g), Agar (15g), Distilled water (1000ml), at a pH of 7.0 to 7.2. SMA is generally preferred, depending upon the source, in the isolation of protease producers because it is a differential media, commonly used to demonstrate proteolysis by organisms capable of hydrolysing casein present in the medium. This proteolytic action is identified as a clear zone around the colonies.

Identification of Microorganism:

The identification of the bacteria was carried out using preliminary examination using a microscope to test for the Gram nature and motility. Several biochemical assays were also performed, in accordance to the Bergey's Manual. For the initial identification tests, isolates 7A and 7D were selected and all further tests were carried out using the 7A isolate.

Gram Staining (7A &7D): Gram staining is a staining technique used in the classification of bacteria in order to differentiate them as a Gram positive bacteria or Gram negative bacteria, thereby determining the Gram nature of the bacteria. The staining protocol comprises of initial staining of the bacteria using crystal violet, followed by Gram's iodine, decolourising using acetone alcohol, followed by counter-staining using safranin. Gram positive bacteria stain purple due to the presence of a thick peptidoglycan layer in their cell walls, whereas, Gram negative bacteria stain pink, which is due to their thin peptidoglycan layer which does not retain crystal violet during the decolourising process [5].

Biochemical Assays:

The biochemical tests were performed on the isolate 7A.

Carbohydrate Fermentation Test/ Sugar Utilization Test: Carbohydrate fermentation patterns are essential to determine whether a certain bacterial species can ferment a specific carbohydrate or not. This is useful in differentiating between bacterial species. The basal media (5-7ml) used contained 2% peptone, Andrade red indicator and a Durham's tube was added. Andrade Peptone water is a basal media to which various carbohydrates can be added, to study fermentation reactions. This is then inoculated with the test organism (7A). If the test isolate metabolises the added carbohydrate, acids are produced, thereby lowering the pH of the the medium. This subsequently causes a colour change of the indicator, from colourless to pinkish-red. If the added carbohydrate is not metabolized, the medium remains pale tan to straw coloured. Andrade indicator is a solution of acid fuschin. When the pH decreases, the indicator changes colour from yellow to pink. Colour change occurs only when sufficient amount of acid is produced, as bacteria may utilise the peptone producing alkaline by products. The Durham's tube is added to collect the gas produced during fermentation. If the organism produces gas, the gas displaces the media present inside the tube, producing a visible air bubble. The sugars used in the current study were Glucose, Sucrose, Maltose, Galactose, Lactose, Xylose and Mannitol. A positive test result indicates fermentation of the sugar in the media, whereas a negative test result indicates that the bacteria cannot ferment the sugar present in the media. No gas production is an indicative of an anaerogenic organism [6].

Indole Production Test: The Indole test is used to determine the ability of the organism to split the amino acid Tryptophan to form the compound Indole. Indole production can be detected using Kovac's reagent, which contains 4- dimethylamino benzaldehyde that reacts with Indole to produce a red coloured compound. The Indole test is commonly used in the IMVIC test. The isolated colony of the test organism is emulsified in tryptophan broth and incubated overnight post which, Kovac's reagent is added. A positive result shows the presence of a pink coloured ring upon addition of the reagents. No change in colour is an indicative of a negative test result.

Methyl Red (MR) Test: The methyl red (MR) test detects the production of sufficient acid during the fermentation of glucose. Some bacteria have the ability to utilize glucose and convert it to a stable acid like lactic acid, acetic acid or formic acid as the end product. These bacteria initially metabolise glucose to pyruvic acid, which is further metabolized to produce the stable acid. The acid so produced decreases the pH to 4.5 or below, which is indicated by a change in the colour of methyl red from yellow to red. The pH at which methyl red detects acid is considerably lower than the pH for other indicators used in bacteriologic culture media. Thus, to produce a colour change, the test organism must produce large quantities of acid from carbohydrate substrate being used. In the MR test, the test bacteria is grown in a broth medium containing glucose. If the bacteria have the ability to utilise glucose with production of a stable acid, the colour of the methyl red changes from yellow to red, when added into the broth culture.

Voges Proskauer (VP) Test: Certain organisms produce acetoin as the chief end product of glucose metabolism and form smaller quantities of mixed acids. 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, which is an indicative of a positive test result.

Citrate Utilization Test: Citrate utilization test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ammonium dihydrogen phosphate ($NH_4H_2PO_4$) is the sole fixed nitrogen source. This test generally forms a part of the IMViC tests. In this test, the citrate medium most commonly used is the formula of Simmons. The medium is poured into a tube on a slant. 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. The carbon dioxide that is thus released, via metabolic breakdown, will subsequently react with water and the sodium ion in the medium to produce sodium carbonate, 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 (Citrate positive).

Motility Test: In the present study, the Hanging Drop method was implemented in order to test for motility. It consists of preparing a wet mount of a loop full of culture using a cavity slide and cover slip (adding petroleum jelly) which are then inverted and observed under a light microscope. The difference between Brownian motion and motility should be observed clearly.

Catalase Test: Catalase is an enzyme that is largely produced by aerobic species in order to neutralize hydrogen peroxide. The enzyme neutralises the bactericidal effects of hydrogen peroxide, thereby protecting the organism. It leads to the breakdown of hydrogen peroxide to water and oxygen (gas bubbles). A loop full of the bacterial isolate is mixed with hydrogen peroxide solution and observed for the rapid release of oxygen bubbles. A catalase negative test result can be seen by weak bubble production. Catalase positive test results are seen in species that have the ability to respire using oxygen as the terminal electron acceptor.

Urea Hydrolysis (Urease) Test (Christensen's Method): Many organisms contain the enzyme urease which can split urea, in the presence of water, to release ammonia and carbon dioxide. They combine together to form ammonium carbonate which turns the medium alkaline. The indicator, phenol red, now changes its colour from yellowish orange to bright red-pink.

Nitrate Reduction (Nitratase) Test: This test is used to differentiate between organisms based on their ability to produce Nitrate reductase enzyme which can hydrolyse Nitrate to Nitrite which can then further be degraded to give gas. A loop full of the isolate is inoculated in nitrate broth, which is then tested for the reduction of nitrate by the addition of reagents like sulfanilic acid and α- naphthylamine. If reduction has taken place, the nitrous acid formed will react with sulfanilic acid to form diazotized sulfanilic acid which in turn reacts with α- naphthylamine to form a red coloured compound. Therefore, a red medium is an indicative of a positive test. In case there is no red coloured medium, a small amount of powdered Zinc is to be added. If the medium now turns red, it indicates that there was some amount of unreduced nitrate and therefore a red colour in this step is an indication of a negative result.

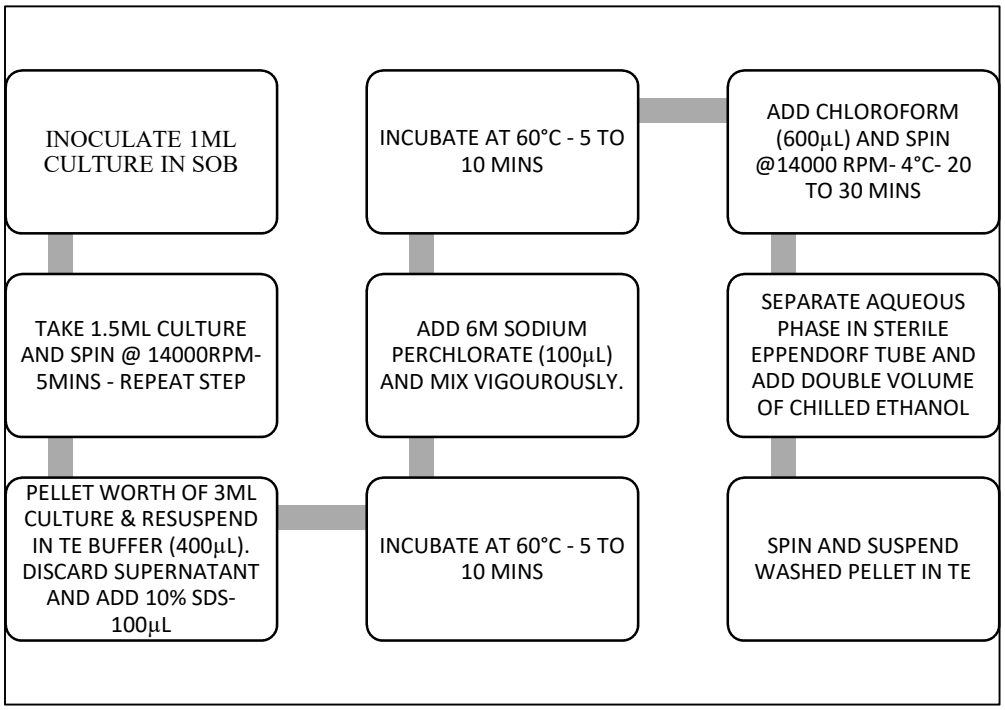
7% NaCl Test: This test is used in order to differentiate between bacteria on the basis of their salt tolerance. NA slants with 7% NaCl are used and a loop full of culture is streaked on the slants and incubated overnight. A positive test result shows growth of the organism on the slant and indicates the presence of a salt tolerant species (since salt acts as a selective agent and interferes with membrane permeability and osmotic equilibrium).

Isolation of Genomic DNA from Bacterial Isolates (7A & 7D):

DNA isolation was carried out for two isolates- 7A and 7D. A SOB Media i.e. super optimal broth media was used for the isolation. An SOB Medium is a nutrient rich bacterial growth medium that is used for microorganisms. It consists of Tryptone, Yeats extract, NaCl, KCl, $MgCl_2$, $MgSO_4$ and distilled water.

Tris EDTA (TE) buffer is used to resuspend the culture. The TE buffer serves the purpose of solubilizing the DNA, while preventing its degradation, via chelation of Mg^{2+} cations. DNA denaturation occurs even with a slight increase in pH and it is damaged at an acidic pH. A buffer like the TE buffer needs to be added in order to stabilize the pH. Sodium Dodecyl Sulphate (SDS) is a strong anionic detergent that can solubilize proteins and lipids that constitute the membranes and destroys the protein conformations [7]. Due to the protein damage, the cell membrane gets damaged and the cell is broken by virtue of which the cell membranes and nuclear envelopes breakdown and expose the DNA containing chromosomes.

It thereby helps in the release of DNA from histones and other DNA binding proteins, via denaturation. Addition of sodium perchlorate in the DNA isolation process aids in the complete deproteinization via removal of the SDS and the protein complexed with it, by precipitation [8]. Chloroform is added in order to further separate out the proteins. Generally, a phenol-chloroform extraction is preferred as chloroform forms a distinct phase separation between the phenolic and aqueous phase thereby allowing the separation of soluble DNA in the aqueous phase. Chilled ethanol is used for the final extraction since low temperatures protect the DNA by slowing down enzymatic activity (DNAases, etc). Cold ethanol aids in the faster precipitation of DNA while also increasing its yield.



DNA Isolation Protocol

Agarose Gel Electrophoresis of Isolated Genomic DNA (7A): Agarose Gel Electrophoresis (AGE) is a technique used to observe and confirm the presence of the isolated genomic DNA. The basic principle of AGE is the separation of DNA fragments based their molecular weight or mass and their charge. The negatively charged DNA molecules migrate towards the positive charge under the influence of a current. As the concentration of the agarose increases, the size of the pore decreases (leading to more impedence) and vice versa and therefore the larger molecules cannot run faster than in comparison to the smaller DNA fragments, which migrate faster towards the positive end. A known molecular weight marker is used to calculate the exact size of the DNA. In the present study, a DNA ladder with a range of 250- 10,000bp was used. Approximately 5µL of the sample and dye was loaded in each well. A tagged fluorescent dye such as EtBr (Ethidium bromide) – 0.5µg/ml is used in order to visualise the DNA bands. EtBr acts as a mutagenic and intercalating agent and intercalates itself in the DNA molecule, within the nucleotides, in a concentration dependent manner. It has a positive charge itself and thereby reduces the DNA migration rate by 15%. The loading dye used in the present study was

Bromophenol Blue. Loading dyes add density to the DNA sample, allowing it to sink to the bottom of the gel, they provide colour thereby aiding in the loading process and they move at standard rates (Bromophenol blue runs ahead of the DNA), allowing for the estimation of the distance travelled by the DNA fragments. The present study made use of a 0.8% agarose gel making use of TAE- 1X (Tris acetic acid EDTA), as the running buffer [9].

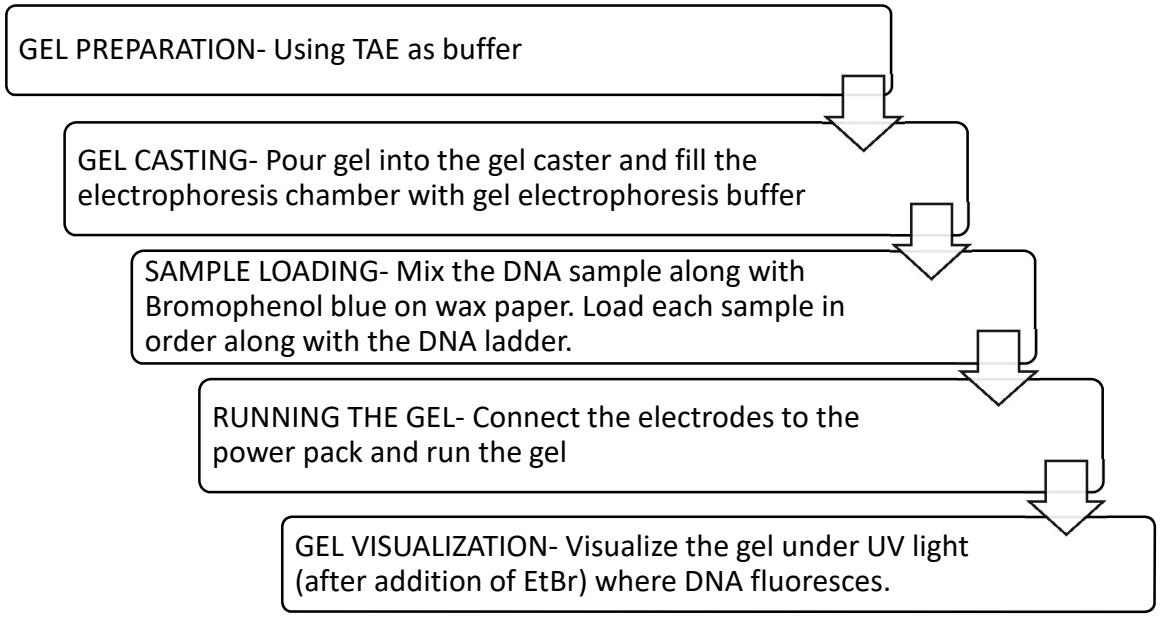
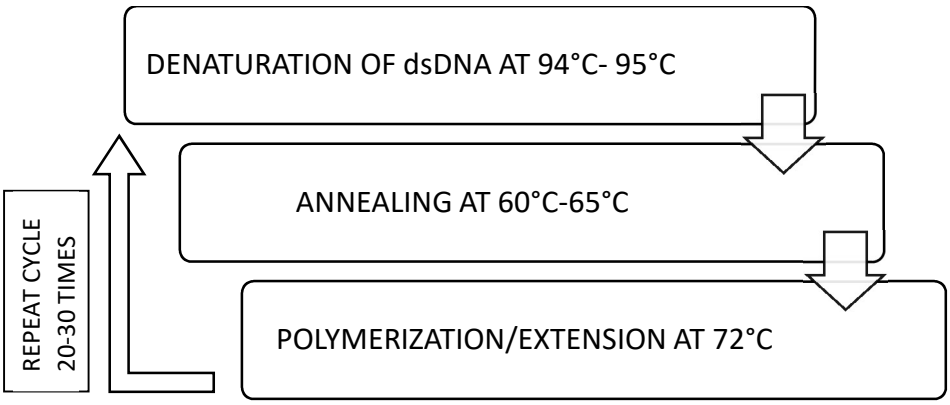


Fig: Agarose Gel Electrophoresis protocol

16S rRNA PCR (7A): PCR or Polymerase Chain Reaction is a method widely used to make several copies of a particular DNA segment, by virtue of which, copies of DNA sequences are exponentially amplified to generate thousands of copies of that particular segment. In order to set up a PCR, there are some general requirements, such as, the DNA template to be copied, in this case – the isolated gDNA- 1µL, forward and reverse primers- 1µL each- that are short stretches of DNA that are used to initiate the PCR reaction by binding to either side of the section of DNA that is to be copied, and a 2X Taq Master Mix- 5µL- which is a 2X concentrated solution of Taq DNA Polymerase (enzyme required for the addition of new bases), dNTPs and all the components required for PCR except the primers and the DNA template. In addition to this 2µL of water was added for injection (WFI). There are three main stages involved:



The process of denaturation consists of separation of the dsDNA template into two single strands, which will act as templates for new strand production. via heating at higher temperatures, causing the breakage of the Hydrogen bonds between the base pairs. During annealing, the temperature is lowered in order to enable the DNA primers to attach to the template DNA. Since the two separated DNA strands are complimentary and run in opposite directions, a forward and reverse primer is used. Polymerization/extension consists of raising the temperature again in order to form the new strand by the action of Taq polymerase enzyme, which is an enzyme taken from a heat loving bacterial species, *Thermus aquaticus*. It adds DNA bases only in the 5'-3' direction. Broad range 16S rRNA gene PCR is generally used for the detection and identification of bacterial species. The primers used are universal primers that target the highly conserved regions of the 16S Ribosomal subunit that is present in all bacterial ribosomal genes, and the PCR of the DNA sequence that codes for 16S rRNA is carried out. After sequencing, the sequences can be compared with known nucleotide sequences using pre-existing databases. In the present study, after the PCR was completed, AGE was used to determine the quantity and size of the DNA fragments produced [10].

Results:

Gram Staining & Biochemical Assays:

Fig: Carbohydrate Fermentation – Negative for all the tubes except Lactose (last picture)

Fig: Test results for Urease, VP, Indole, Nitratase, Citrate Utilisation and 7% NaCl

Table: Biochemical Characterization

BIOCHEMICAL TEST/ STAINING	RESULT
Gram Staining	Positive (Short rods)
Carbohydrate Fermentation: • Glucose • Maltose • Galactose • Lactose • Xylose • Mannitol • Sucrose	 • Negative • Negative • Negative • Positive • Negative • Negative • Negative
Indole Test	Negative
Methyl Red Test	Negative
Voges Proskauer Test	Negative
Citrate Utilization Test	Negative
Motility Test	Sluggishly Motile
Catalase Test	Negative
Urease Test	Positive
Nitratase Test	Positive
7% NaCl Test	Negative

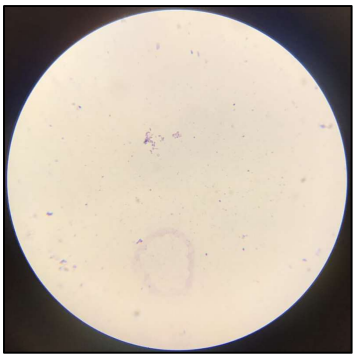


Fig: Gram staining of 7A

Isolation of gDNA and AGE:

On running the gel, the DNA fragments were separated, and the gel was visualized under UV light. Two wells were loaded for isolate 7A wherein, one of the wells shows more DNA migration. The isolated gDNA from the top well containing test organism 7A, shows two bands of DNA, in non-equimolar amounts. The band closer to the well indicates a longer DNA molecule and the well below shows the farthest migration of the genomic DNA indicating a shorter DNA fragment.

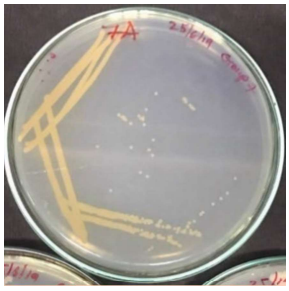


Fig: NA plate of isolated 7A colonies

Fig: gDNA isolation

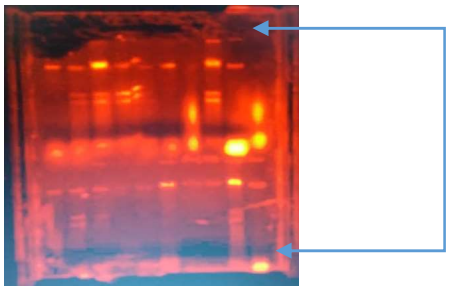


Fig: Visualization of gel under UV
(Arrows showing wells with 7A sample)

16S rRNA PCR and AGE:

After performing the PCR, the DNA was run on an Agarose gel via AGE in order to check for accurate amplification and for the presence of DNA. On visualizing the gel under UV light, DNA from the isolate 7A shows no migration.

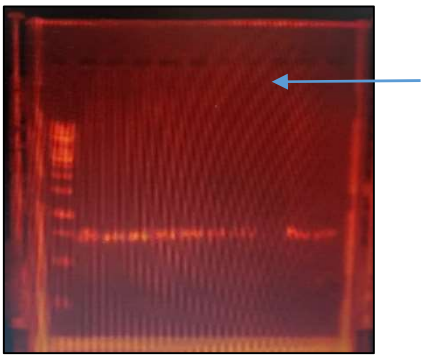


Fig: AGE of PCR products
(Arrow indicates well with 7A)

Discussion:

From the results obtained, the test isolate 7A is found to be a yellowish - Gram positive organism with short rods. The organism tests positive only for lactose fermentation without the production of gas, and does not ferment any of the other carbohydrates. The organism shows slight motility and is therefore sluggishly motile. The organism shows a distinct colour change for urea hydrolysis and thus tests positive for it. In the AGE conducted for the isolated gDNA from isolate 7A, there is a difference in the DNA migration observed between the top and bottom wells. A reason for the same could be due to unequal loading of the sample in both the wells. The bottom well possibly contained a large amount of gDNA and hence maximum

migration was seen. The well at the top possibly contained a moderate amount of gDNA and thus the migration is not a lot. The DNA obtained from the PCR showed no migration when run on the gel. This could be due to loading a less amount of DNA in the wells. While performing the PCR, an assumption was made that all of the strains that were used were conserved, and the universal primer selected was on this basis. There is a possibility that the primer selected for the PCR is not suitable for the test isolate 7A. The gDNA that was used for the PCR was the same as the one that was initially isolated and run on the gel. Since the initial AGE showed DNA migration, improper isolation of gDNA may not be one of the reasons for no DNA migration on the gel. Post PCR, DNA sequencing was not carried out and identification of the test isolate was done on the basis of biochemical tests.

Conclusion: Identification of bacteria was carried out based on morphological characteristics and biochemical tests. It's identification at genus level was done with the help of the Bergey's Manual of determinative Bacteriology. Possible genera were identified as *Brevibacterium, Aneurinibacillus* and *Nocardia.* The genus was identified to be *Nocardia,* based on the various biochemical tests performed. Organisms belonging to the *Nocardia* genus are those that are weakly staining Gram Positive, rod shaped bacteria. Some species are non-pathogenic while some can cause Nocardiosis. *Nocardia* species are found worldwide, mainly in soil that is rich on organic matter. This falls in line with the source of the soil sample (compost soil) chosen for the present study.

In order to further strengthen the identification a few possible tests that could have been run on the isolate, is the acid fast test, testing for growth on blood agar, antibiotic susceptibility tests. Based on the tests conducted and the information known about the organism, the organism *Nocardia flavescens,* can be identified as the test isolate 7A. The isolate 7A shows resemblance to several species under the genus *Nocardia,* such as *N. fordii, N. gibsonii, N. maculata.*

Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Nocardiaceae
Genus	*Nocardia*
Species	*flavescens*

References:

1. Dalal, R. (2015). Screening and Isolation of Protease Producing Bacteria from Soil Collected from Different Areas of Burhanpur Region (MP) India. *International Journal Of Current Microbiology And Applied Sciences*, *4*(8), 597-606.
2. Sinha, S., Choubey, S., Ajay Kumar, A., & Bhosale, P. (2017). Identification, Characterization of Pigment Producing Bacteria from Soil and Water and Testing of Antimicrobial Activity of Bacterial Pigments. *International Journal Of Pharmaceutical Sciences Review And Research*, *42*(2), 119-124.
3. Satpathy, D. Screening and Isolation of Protease Producing Bacteria from Kitchen Exhaust | PharmaTutor. Retrieved 5 September 2019, from https://www.pharmatutor.org/articles/screening-and-isolation-of-protease-producing-bacteria-from-kitchen-exhaust
4. Vishnu Priya V G, Preethi S, et. al. (2016). Isolation and Identification of Protease Producing Bacteria from Soil. *International Research Journal Of Engineering And Technology (IRJET)*, *03*(08), 1362-1365.
5. Bruckner, M. (2016). Gram Staining. Retrieved 5 September 2019, from https://serc.carleton.edu/microbelife/research_methods/microscopy/gramstain.html
6. Tests - Microbeonline. (2018). Retrieved 6 September 2019, from https://microbeonline.com/bacterial-identification-methods/
7. He, F. (2011). E. coli Genomic DNA Extraction. *BIO-PROTOCOL*, *1*(14). doi: 10.21769/bioprotoc.97
8. Wilcockson, J. (1973). The use of sodium perchlorate in deproteinization during the preparation of nucleic acids. *Biochemical Journal*, *135*(3), 559-561. doi: 10.1042/bj1350559
9. Lee, P., Costumbrado, J., Hsu, C., & Kim, Y. (2012). Agarose Gel Electrophoresis for the Separation of DNA Fragments. *Journal Of Visualized Experiments*, (62). doi: 10.3791/3923
10. Patel A, Harris KA, Fitzgerald F. (2017). What is broad-range 16S rDNA PCR? *Archives of Disease in Childhood - Education and Practice*.;102:261-264.