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Optimisation, Production, Identification and Screening of Alpha Amylase Producing *Bacillus* Species from Kitchen Soil

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Abstract

Amylases are crucial enzymes which hydrolyze internal glycosidic linkages in starch and produce as primary products dextrins and oligosaccharides. Amylases are classified into α-amylase, β-amylase, and glucoamylase based on their three-dimensional structures, reaction mechanisms, and amino acid sequences. Amylases have innumerable applications in clinical, medical, and analytical chemistries as well as in food, detergent, textile, brewing, and distilling industries. Amylases can be produced from plants, animals, and microbial sources. Due to the advantages in microbial production, it meets commercial needs. The pervasive nature, easy production, and wide range of applications make amylase an industrially pivotal enzyme.

Microorganisms are the most important sources for enzyme production. For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. Due to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes. Starch degrading ***Amylolytic*** enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper. ***Amylases*** are obtained from various origins like plant, animal, bacterial and fungal.

***Key words*:** *Bacillus*, endospore, Amylolytic enzymes, α-Amylase

INTRODUCTION

Microbes are ubiquitous in nature and soil is a source of a variety of microbes. The Gram -positive, aerobic, rod - shaped endospore - forming bacteria of the Genus *Bacillus* are the most widely represented organisms in the soil. Due to their ability to form spores and withstand a range of variable environmental conditions, Bacillus sp. adapt easily to diverse habitats. The diverse physiology of Bacillus sp. requires elaborate biochemical tests for their identification (Ammini Parvathi *et.al*, 2009).

The most appropriate Microorganism for a potential process is found by isolation from a variety of sources most commonly soil. Classical methods of screening to obtain suitable organisms are very time consuming expensive and often without any guarantee (D. H. Bergey, 1994) . Enzymes are chemical substances produced by living

cells that are capable of initiating a chemical reaction without themselves being used in that reaction. They enhance the rate of a chemical reaction (Oyeleke et al., 2009); enzymes are produced by plants, animals but microbial enzyme production is of great importance as these are more economical to produce, calculable, tractable and stable (Burhan et al., 2003). Amylases are very important enzymes now days; Amylases are a group of enzymes that hydrolyze α-1,4 glycosidic linkages of starch to yield dextrin and different monomeric products. There are two major classes of amylases, mostly identified among microorganisms are α- amylase and gluco-amylase (Vijayabaskar et al., 2012). β-amylase, mostly from plant origin, has been recorded from microbial sources (Pandey et al,. 2000). Amylases have been isolated from bacteria, fungi and actinomycetes (Kathiresan *et.al*., 2006). Bacterial amylases are mostly reported from acidophilic, alkalophilic and thermophilic bacteria (Boyer *et.al*., 1972). Most Microbial enzymes are commercially available and have replaced chemical hydrolysis of starch in industry (Pandey *et.al*., 2000). *Bacillus subtilis, Bacillus stearothermophilis, Bacillus licheniformis* and *Bacillus amyloliquifaciens* are most prominent among the bacterial sources of amylase (Pokhrel *et.al*; Sani *et.al*., 2014).

Amylase are very important in recent days with application ranging from food, textile, paper, and fermentation such as baking, brewing, digestive acid's production, chocolate cake's production, fruit juices and starch syrups (Lin *et.al*., 1997; Pandey *et.al*., 2000; Verma *et.al*., 2011; Kaur *et.al*,2012). There are several reports on starch degrading microorganisms from different sources and respective amylase activity (Ryan *et.al*., 2006; Serin *et.al* 2012). The microbial source of amylase is preferred to other sources because of its plasticity and vast availability. Microbial amylase has almost surpassed the synthetic sources in different industries. Amylolytic enzymes are widely distributed in bacteria and fungi. They are categorized into exo-acting, endo-acting and debranching enzymes. Unusual bacterial amylases are found in acidophilic, alkalophilic and thermo acidophilic bacteria (Dipali Parmar and Ajit Pandya, 2012). Soil is one of the rich sources of starch degrading microorganisms as it contains mostly starchy substances. The purpose present investigation is to isolate *Bacillus* species from kitchen soil samples and their screening for amylase production.

The present study deals with isolation and characterization of amylase producing bacteria (particularly *Bacillus*) from kitchen area soil sample. This was followed by isolation of the DNA the characterization of the amylase gene using Polymerase chain reaction (PCR).

AIMS AND OBJECTIVE

- Isolation of Amylase producing organisms from kitchen soil waste.
- Morphological and biochemical characterization of the organisms.
- Screening for amylase producing organisms by starch amylase assay.
- Isolation of DNA and its qualitative and quantitative estimation using AGE (Agarose gel electrophoresis) and Nano Drop Spectrophotometer.
- Molecular characterization of the gene of interest (amylase gene) using specific primer.

REVIEW OF LITERATURE

The enzymes from microbial sources are more stable and obtained cheaply. Amylases are among the most important enzymes and are of great significance in present day industry. Starch degrading bacteria are most important for industries such as food, fermentation, textile and paper. Thus isolating and manipulating pure culture from various soil and waste materials has manifold importance for various biotechnology industries. In the present investigation bacterial strains were isolated from soil sample and growth pattern as well as optimum growth condition was determined. Characteristic feature of the strains indicates them a*s Bacillus subtilis, Pseudomonas florescens, Escherichia coli* and *Serratia marscens.* The optimum temperature for production was 35-40 °C, whereas maximum growth was observed at 1 % dextrose concentration but increases with increase in substrate concentration. The pH range was found to be 7 and incubation time 48 hrs with 1mL as inoculum for optimum growth. Other optimum parameters include Yeast extract as Nitrogen source, Calcium chloride as chloride and Manganese **s**ulphate as sulphate source for amylase production (Alariya *et.al*, 2013).

There was study that aimed at isolation, identification and screening of enzyme producing bacteria from soil samples. For this purpose the soil samples were collected from agriculture fields at sangam dairy, Guntur district, fish culture ponds, timber depot soil at rural areas of Bhimavaram, West Godavari district and oil mills at Vijayawada, Krishna district, Andhra Pradesh. A total of seventeen strains of bacteria were isolated from soil samples. Out of them nine isolates have amylase producing activity, eight have protease producing activity, two isolates have cellulose releasing activity and two have lipase producing activity. Among seventeen strains of bacteria 8 isolates belongs to *Micrococcus sp*., six isolates belongs to *Bacillus sp.*, two belongs to *Staphylococcus sp.* and one belongs to *Streptococcus sp.* Further optimization studies and gene 16S RNA studies needed to confirm the strains (Duza and Mastan, 2013).

Among different types of enzymes obtained from microbial sources, amylases are the most widely used in industries. There was a study in which bacteria were isolated from sewage soil and screened for the production of α-amylase. Among four bacterial isolates, one isolate produced maximum zone of starch hydrolysis. The bacterial isolate was identified as *Bacillus* sp. and was later used for further characterization. Maximum yield of amylase was obtained after 48h of incubation. The optimum pH for enzyme activity was found to be at pH 7 and the optimum temperature for the activity was found to be at 35 °C (Pokhrel et *al*, 2013).

In a study, the gene encoding the alpha-amylase enzyme of native isolated *Bacillus subtilis* was amplified with specific primers containing of N*otI* and Asc*I* restriction sites by PCR and then sequenced. Purified PCR product and shuttle episomal vector p316TDH3 were cut by restriction enzymes and cloned into *Escherichia coli* and yeast hosts. The haploid auxotroph (ura3-) strain of *Saccharomyces cerevisiae* and p316TDH3 were used as the host and vector for cloning and expression of the alpha amylase gene, respectively. In native *Bacillus* sp. the *AmyE* gene without signal sequence was amplified with specific primers that introduced Asc*I* and *NotI* restriction sites. After constructing the recombinant plasmid, it was transformed into *E. coli c*ompetent cells. Then, colonies selection and confirmation were performed and the extracted plasmid was introduced to competent yeast cells using carrier sperm DNA. Recombinant yeast cells could grow on minimal media and produce extracellular enzyme. The presence of alpha-amylase gene in recombinant bacteria was certificated by colony-PCR method. After extraction of recombinant vector from *E. coli*, the competent *S. cerevisiae* cells were transformed using polyethylene glycol and carrier sperm DNA. The recombinant yeast strains were screened by URA3 auxotrophic marker and analyzed for alpha-amylase gene existence. In the other hand, the amylase gene length of native B. subtilis was 1887 base pairs (bp) with an approximately 93.65% similarity with standard bacterial strain. Based on this similarity and our bioinformatics evaluations, this mentioned alpha-amylase gene

can be expressed in *S. cerevisiae* as extracellular enzyme (Fahimeh Afzal-Javan and Mohsen Mobini-Dehkordi 2013).

Amylases are among the most important industrial enzymes and also have great significance in Biotechnological studies. In a certain study cultural, morphological, and metabolic characteristics of the bacterial isolates were studied. Total 18 bacterial cultures were isolated from collected soil samples. Among 18 bacterial isolates, 14 isolates showed the amylolytic activity. These 18 isolate was identified according to Bergey's manual of systemic Bacteriology. These isolates related to *Bacillus* sp. The optimum pH for the growth of all the cultures was observed at pH 7. Submerged fermentation was carried out for the production of amylase was observed in the range of 0.045-1.35 U/min/mL. The maximum activity of amylase was 1.35(U/min/mL) after 48 hours was recorded, have great significance (Dipali Parmar and Ajit Pandya, 2012).

MATERIALS AND METHODS

Sample Collection

Kitchen area soil samples were collected from C.V. Raman Nagar, BEL Circle, Laxmipura Cross located in Bangalore, Karnataka. Sample collection was carried out at sites where garbage from household, vegetable wastes decomposes. Samples were collected in every quarter of the year, i.e., January to March, April to June, July to September, and October to December. Samples were taken from a depth of 5 cm. The samples were brought to the laboratory under aseptic conditions.
These samples have been kept in sealed polythene bags and stored at 4^0C until isolation. The Physio-chemical parameters of soil samples such as pH, temperature and turbidity were determined.

Medium for isolation

Medium used for isolation of Bacillus species was L. B agar (Luria - Bertani Agar). The medium containing per liter (10 gm starch; 1 gm yeast extract; 11 gm tryptone; 8.9 gm Nacl; 8 gm agar; pH - 7).

Isolation of Organism

The soil samples collected were serially diluted through SERIAL DILUTION TECNIQUE (Jamil *et al.*; 2007), (Rasooli I; 2008) to 10^{-5} dilution, 0.1 ml from 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated out on nutrient agar plates. Plates were incubated at 37◦c for 24 hours. Colonies were observed for morphological characterization followed by sub-culturing on Luria - Bertani (LB) agar plates. The colonies were subjected to enzyme assay and biochemical characterization. The pure cultures were maintained in LB slants.

Identification of Isolated Organisms

The isolated colonies were subjected to Gram staining and the colonies showing Gram positive reaction were picked and maintained on L.B agar slants. The Isolates were subjected to different physiological and biochemical parameters and were identified by using Bergey’s Manual of Determinative Bacteriology.

Screening for amylase activity

Amylases break starch into glucose, maltose, maltotriose and dextrin by hydrolysis of glycosidic bonds (Kaur *et.al*, 2012). Mainly microorganisms such as *Bacillus subtilis, Lactobacillus, Escherichia, B. licheniformis, B. stereothermophilus, B. megaterium, B. cereus, Streptomyces* species and *Pseudomonas* species (Pokhrel *et.al*, 2013) produce the amylase enzyme. Microbial production of amylase is beneficial as it is economical, gives high yield and it can be engineered to produce enzymes with desired characteristics. Microbial amylases are used potentially in pharmaceuticals, fine chemical industries, paper industries, food and beverage industry, textiles etc (Kaur *et.al*, 2012).

- The isolated pure strains were screened for the production of extracellular amylase using starch agar i.e. bacterial isolates were screened for amylolytic activity by Starch hydrolysis test on starch agar plates. The microbial isolates were streaked on the starch agar plate and incubated at room temperature for 24-48 hours. After incubation 1% iodine solution was flooded with dropper for 30 seconds on starch agar plates. The isolates that produced clear zones of hydrolysis were considered as amylase producers and were further investigated (Pokhrel *et.al*, 2013).

Morphological and Biochemical Characterization

Gram's Staining

Gram's staining is performed for the identification of a bacterial organism. It differentiates bacteria into two large groups – gram positive and gram negative. Gram's staining differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, which is present in a thick layer in gram – positive bacteria (Bergey *et.al*, 1994). In a Gram's stain test the gram – positive bacteria retain the crystal violet dye, while a counter stain (commonly safranin) added after the crystal violet gives all gram – negative bacteria a red or pink colouring. There are four basic steps of the Gram's stain(Cappuccino and Sherman), Which include

- Primary stain (crystal violet) was applied to a heat –fixed smear of a bacterial culture and allowed to stand for 45 seconds.
- Followed by this is addition of a mordant (Gram's iodine) for 1 minute and rapid decolourization with alcohol or acetone.
- The smear is counterstained with safranin or basic fuchsin for 45 seconds.
- The smear is air dried and viewed under the microscope.

Endospore Staining

Vegetative cells are bacteria that are actively growing, metabolizing and dividing. When vegetative cells are subjected to environmental stresses such as nutrient deprivation they eventually die. However, some bacteria such as the *Bacillus* sp. and the *Clostridium* sp. can circumvent the problems associated with environmental stress by forming endospores. Endospores are dormant or metabolically inactive forms of a bacterium that allow it to survive the harsh environmental conditions. Spores are resistant to heat, UV radiation and chemicals because they are comprised of a tough proteinaceous covering called Keratin.

A differential staining technique (the Schaeffer-Fulton method) is used to distinguish between the vegetative cells and the endospores. A primary stain (malachite green) is used to stain the endospores; endospores have a keratin covering and resist staining; the malachite green will be forced into the endospores by heating. In this technique heating acts as a mordant. Water is used to decolorize the cells; as the endospores are resistant to staining, the endospores are equally resistant to de-staining and will retain the primary dye while the vegetative cells will lose the stain. The addition of a counter stain or secondary stain (safranin) is used to stain the decolorized vegetative cells. When visualized under microscopy the cells should have three characteristics: the vegetative cells should appear pink, the vegetative cells that contain endospores should stain pink while the spores should be seen as green ellipses within the cells. Mature, free endospores should not be associated with the vegetative bacteria and should be seen as green ellipses. The technique involves the following steps:-

- A smear of the bacterial culture was prepared on a clean glass slide and was heat fixed.
- The heat fixed smear is placed in a water bath and primary stain Malachite green is added intermittently without letting the stain dry for 20 minutes.
- The stain is washed under tap water and safranin is added and left over for about 45 seconds to 1 minute.
- The stain is washed and smear is allowed to air dry and it is viewed under the high power objective of a microscope.

Catalase Test:-

Catalase is the enzyme that breaks hydrogen peroxide (H_2O_2) into H_2O and O_2. Hydrogen peroxide is often used as a topical disinfectant in wounds and the bubbling that is seen is due to the evolution of O_2 gas. H_2O_2 is a potent oxidizing agent that can wreak havoc in a cell; because of this, any cell that uses O2 or can live in the presence of O2 must have a way to get rid of the peroxide. One of those ways is to make catalase. The procedure is as follows (Cappuccino and Sherman):

- A small amount of growth from our culture is placed onto a clean microscope slide.
- A few drops of H_2O_2 are added onto the smear.
- A positive result is the rapid evolution of O_2 as evidenced by bubbling and a negative results is no bubbles or only a few scattered bubbles.

Mannitol Motility Test

Mannitol motility test is performed for determining motility and mannitol fermentation of the organism. The highly nutritious peptic digest of animal tissue supports luxuriant growth of fastidious bacteria like Bacillus, Staphylococci etc. Semisolid nature of the medium due to 0.6% agar helps to detect motility. Motile bacteria produce diffused growth throughout the medium while non - motile bacteria grow only along the line of inoculation. Fermentation of mannitol produces acidity in the medium. Phenol red is the pH indicator, which detects acidity by exhibiting a visible colour change from red to yellow. The media used is mannitol agar (Cappuccino and Sherman).

- A loopful of bacteria culture is stabbed into the semisolid mannitol agar and incubated for 24 hours.
- Appearance of turbidity along the line of inoculation to the surface of the media indicates motility. Acid producing bacteria causes change in the colour of the media as they grow often from red to yellow. A negative result is no appearance of turbidity along the line of inoculation.

IMViC Test

These are a group of biochemical tests namely Indole, Methyl red, Voges Proskauer and Citrate utilization test (Cappuccino and Sherman).

Indole Test

Indole test is used to determine the ability of an organism to split amino acid and tryptophan to form the compound indole. Tryptophan is hydrolysed by tryptophanase to produce three possible end products – one of which is indole. Indole production is detected by Kovac's or Ehrlich's reagent which contains 4(p)-dimethyl aminobenzaldehyde, this reacts with indole to produce a red coloured compound. Indole test is a commonly used biochemical test.

- Tryptophan broth was inoculated with bacterial culture.
- Then it was incubated at room temperature for 24-28 hours in ambient air.
- 0.5 ml of Kovac's reagent was added to broth culture. Pink coloured ring formation after addition of appropriate reagent indicates positive results. No colour change even after the addition of appropriate reagent indicates negative result.

Methyl Red Test

Methyl red test determines whether the microbe performs mixed acids fermentation when supplied glucose. These large amounts of acid results significant decrease in the pH of the medium below 4.4. This is visualized by using pH indicator, methyl red (**2-(N, N-dimethyl-4-aminophenyl) azobenzenecarboxylic acid**) or (p-dimethylaminoazobenzene-O-carboxylic acid), which is yellow above pH 5.1 and red at pH 4.4.

- Tubes containing MRVP broth was inoculated with bacterial culture and incubated at room temperature for 24 hours.

- Few drops of methyl red indicator solution were added to the tubes. A positive test is indicated by formation of red coloured ring within few minutes.

Voges Proskauer test

This test determines whether the microbe produces 2, 3-butanediol as a fermentation product from glucose. The production of 2, 3-butanediol is indirectly detected when the pathway intermediate acetoin reacts with reagents to turn red.

- The pure cultures are inoculated into MRVP broth and incubated at room temperature for 24-48 hours.
- After incubation, five drops of Barritt's A (5% α-naphthol in ethanol) was added and the tube was shaken gently to mix the ingredients.
- Then, five drops of Barritt's B (40% KOH) was added. Development of a red color indicates a positive test.

Citrate Utilisation Test

The citrate test is commonly employed as part of a group of tests, the IMViC tests, that distinguish between members of the Enterbacteriaceae family based on their metabolic by products(1, 2, 4). In the most common formulation, citrate is the sole source of carbon in the Simmons citrate medium while inorganic ammonium salt ($NH_4H_2PO_4$) is the sole fixed nitrogen source(3, 4, 7, 8). When an organic acid such as citrate is used as a carbon and energy source, alkaline carbonates and bicarbonates ultimately are produced (5, 7). The visible presence of growth on the medium and the change in pH indicator color due to the increased pH are the signs that an organism can import citrate and used it as a sole carbon and energy source; such organisms are considered to be citrate positive (Cappuccino and Sherman).

- Simmon citrate agar slants were prepared and the pure cultures were inoculated into the slants and incubated at room temperature for 48 hours.
- Citrate positive: growth will be visible on the slant surface and the medium will be an intense Prussian blue. The alkaline carbonates and bicarbonates produced as by products of citrate catabolism raise the pH of the medium to above 7.6, causing the bromothymol blue to change from the original green color to blue.
- Citrate negative: trace or no growth will be visible. No colour change will occur; the medium will remain the deep forest green colour of the uninoculated agar. Only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons citrate medium, thus a citrate-negative test culture will be virtually indistinguishable form an uninoculated slant.
- Selection of the right organism plays a key role in high yield of desirable enzymes. Micro-organisms have become increasingly important as producer of industrial enzymes. Due to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been

isolated from complex eukaryotes. Starch degrading ***Amylolytic*** enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper. ***Amylases*** are obtained from various origins like plant, animal, bacterial and fungal.

Isolation of DNA

To isolate DNA from bacterial cell there is a need to lyse the cell wall and denature the protein. DNA is very easily damaged by shear forces; even rapid stirring of solution can break high molecular weight DNA into shorter segments. Therefore DNA is recovered from cells by using "lysis buffer". This lysis buffer contains EDTA, Tris HCl and SDS.

EDTA: Ethylene diamine tetra Acetic acid is a chelating agent, which forms a complex (chelates) with several kinds of metal ions. Divalent metal cations such as Mg^{2+} are required co factors by the majority of DNases. Since the complexed Mg^{2+} cannot be utilized by this enzyme therefore the DNA extracted is protected from DNA degradation.

SDS: Sodium dodecyl sulphate is an anionic detergent, which acts as a surfactant. It dissolves the cell wall and denatures the proteins.

TRIS: Protein present in the sample is denatured by using phenol: chloroform mixture (phenol is saturated with 1M Tris). To wash out the phenol, chloroform: isopropanol mixture is used. Lastly DNA is precipitated in the form of fibrous structure by using cold absolute alcohol. Tris HCl is a common buffer for buffering at pH 8 if pH is desired. Buffers stabilise pH while the cells are being lysed.

NaCl: NaCl provides an osmotic shock to the cell. NaCl salt may also be used to raise the ionic strength, the total concentration of solutes outside the cell.

Requirements:

- Lysis Buffer:
  - Solution A: 10mMTris HCI
  - Solution B: 5mM EDTA
  - Solution C: 0.5% SDS
  - Solution D: 1M NaCl
- Prepare 10mI of each of the stock solutions and mix in the following in the ratio 2.5 ml: 2.5 L: 5 mL: 1.5 ml. Make up the solution upto 15mL to give the final lysis buffer.
- Nutrient broth
- Phenol: Chloroform mixture in the ratio 1:1.
- Chloroform: Isoamyl alcohol mixture in the ratio 24:1.
- 3M-sodium acetate: To 8.39 mL of 3M Sodium acetate add 1.61 mL of glacial acetic acid.
- Tris EDTA buffer:
  - 10mM Tris
  - 1mM EDTA.

Procedure:

1. *Bacillus* cultures were inoculated in Luria-Bertani broth and incubated at room temperature for 48 hours.
2. The broth was centrifuged at 8000 rpm for 10 minutes.
3. The cells were collected in the form of pellets at the bottom. The supernatant was discarded.
4. The above step was repeated to get an increased concentration of cells.
5. The pellet was suspended in 1ml of lysis buffer.
6. This was incubated at 45^0C in boiling water bath for 10 minutes.
7. 1ml of phenol: chloroform mixture was added and centrifuged at 10,000 rpm for 10 minutes.
8. To the supernatant (upper aqueous layer) equal volume of chloroform: isoamyl alcohol mixture was added followed by $1/20^{th}$ volume of 3M sodium acetate and centrifuged at 10,000 rpm for 10 minutes.
9. To the upper aqueous layer double volume of chilled ethanol was added and incubated at -20^0C for 20minutes.
10. Then it was centrifuged at 12,000 rpm for 10 minutes and the pellet was air dried.
11. The DNA pellet was dissolved in 20-50 µL TE buffer.

A. QUANTIFICATION OF DNA BY NANO DROP

Fig. 1: Nano drop ND-1000

Principle

Nucleic acids absorb light at a wavelength of 260 nm. If a 260 nm light source shines on a sample, the amount of light that passes through the sample can be measured, and the amount of light absorbed by the sample can be inferred. For double-stranded DNA, an Optical Density (OD) of 1 at 260 nm correlates to a DNA concentration of 50 ng/µL, so DNA concentration can be easily calculated from 0D measurements. These measurements were traditionally taken with standard spectrophotometers, but now a tabletop spec is used, called a NanoDrop that requires only 2 µL of a sample for quantification. The principle of action is the same, but the practical usage is much easier.

Materials required:

- Sample to be measured
- NanoDrop
- P2 or P10 micropipette with tips
- Lint-free lab wipes or tissue paper.
- Purified water
- Blanking solution (H_2O, TE, EB, Tris, or other depending on the sample)

Procedure:

1. The computer attached to the NanoDrop was opened and turned on
2. The NanoDrop pedestal was washed with purified water was added to the lower pedestal, and then the arm was lowered again. Waited for 30-60 seconds. The upper arm lifted and the wipe was used to vigorously scrub both the upper and lower pedestals.
3. The NanoDrop software was opened on the computer by double –clicking the "ND-1000" icon that looks a bit like an hourglass.
4. The NanoDrop was initiated-
    a. The "Nucleic Acid" option was clicked in the NanoDrop software and then 'OK' was clicked after water was added.
    b. 2µL of purified water was added to the lower pedestal, then the upper arm was placed above the lower one and it was closed gently.
    c. Then 'OK' option was clicked and in 20 seconds the NanoDrop was initialized.
    d. After it was done, the upper arm was lifted and the pedestal was wiped with a tissue paper or wipe.
    e. 2µL of the buffer was added to the sample. The upper arm of the NanoDrop was closed and the "Blank" option was clicked.
    f. In about 20seconds the blank measurement was made by clicking on the 'Measure' option.
    g. When it was done, the upper arm was lifted and the pedestal was wiped with a wipe or tissue paper.

h. The step was repeated for all the samples.

i. In the 'Sample ID" box, the name of the sample was typed and the window was saved for each of the samples.

5. Data was collected:

a. The measurements were written down. The cursor could be moved to check the absorbance number at various wavelengths.

b. The "Print Screen" button was clicked to print the complete spectrum.

c. After finished making all measurements, "Print Report" was clicked to get a table of all data.

6. The computer was shut down.

B. AGAROSE GEL ELECTROPHORESIS

Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Most every molecular biology research laboratory routinely uses agarose gel electrophoresis for the preparation and analysis of DNA. We will be using agarose gel electrophoresis to determine the presence and size of PCR products.

Background

Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatin – like slab. During electrophoresis, the gel is submerged in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA itself is not visible within an agarose gel. The DNA will be visualized by the use of a dye that binds to DNA.

Purpose: To determine the presence or absence of PCR products and quantify the size (length of the DNA molecule) of the product.

Materials needed:

- Agarose
- 1X TBE Buffer
- 6X Sample Loading Buffer
- DNA ladder standard
- Electrophoresis chamber
- Power supply

- Gel casting tray and combs
- DNA stain
- Staining tray
- Gloves
- Pipette and tips

TBE Buffer

- Tris Base
- Boric acid
- 0.5 M EDTA

(pH 8.0); Volume of buffer may vary according to the requirement.

6X Sample Loading Buffer

- 1ml sterile distilled water
- 1 ml Glycerol
- bromophenol blue (~0.05 mg)

For long term storage, sample loading buffer is kept frozen.

Agarose Gel Electrophoresis Protocol

Preparing the agarose gel

- 1% agarose was prepared i.e 1 g agarose was measured and mixed with 100ml of TBE buffer (the total gel volume may vary depending on the size of the casting tray).
- The agarose was heated in a microwave or hot water bath until it dissolves completely in buffer and a clear solution is obtained.
- The solution was cooled to about 50-55℃, swirling the flask occasionally to cool evenly and about 7-10 µl ethidium bromide (EtBr) was added.
- The ends of the casting tray were sealed with two layers of tape.
- The melted agarose solution was poured into the casting tray and allowed to cool until it solidified. The combs were placed in the gel casting tray.
- The combs were carefully removed followed by the tape. The gel was placed in the electrophoresis tank containing buffer.

Loading the gel

- 1 µL of 6X Sample Loading Buffer was added to each 25 µL PCR reaction. (The order of each sample to be loaded on the gel was noted).
- 26 µL of each sample/Sample Loading Buffer mixture was carefully pipetted into separate wells in the gel.

Running the gel

- The lid was placed on the gel box, connecting the electrodes.
- The electrode wires were connected to the power supply, making sure the positive (red) and negative (black) are correctly connected.
- The power supply was connected to about 80 volts. Maximum allowed voltage will vary depending on the size of the electrophoresis chamber. It should not exceed 5 volts/ cm between electrodes.
- To ensure proper working of the power pack we need to look for bubbles forming on each electrode. To make sure that the current is passing in the right direction we need to observe the movement of the blue loading dye (it runs in the same direction as DNA).
- The power is run until the blue dye approaches near the end of the gel.
- Power was then turned off.
- The wires were disconnected from the power supply and the lid of the electrophoresis chamber was removed.
- Using gloves, the tray with gel was moved out of the tank and placed it in the gel doc for visualisation of DNA bands.

C. GEL DOC

Purpose:

To provide instruction the proper use of the gel documentation system (Gel Doc).

Application:

Users of the Gel Doc within the College of Biological Science should be familiar with the procedures described below.
Safety Precautions:

a. All operators must receive training prior to using the equipment. Training may be delegated to a qualified individual, but it remains the responsibility of the supervisor to ensure their personnel are adequately trained.
b. Ethidium bromide is a known mutagen. Always wear a lab coat and gloves when handling ethidium bromide solutions and stained agarose gels.
c. Do not look into the UV light source without face or eye protection.

Procedure:

Starting the program

a. Ensuring we are using an ungloved hand, the mouse is clicked to activate the monitor.
b. The Gel Doc software is opened.
c. On the menu bar, 'File' is selected, followed by ‘Acquire’ option and 'Gel Doc'.

Setting up the gel

a. The chamber door was opened with the ungloved hand and the gel is loaded into the chamber with the gloved hand. The gel is placed at centre of the gel doc for proper visualization.
b. The door is closed and UV light is switched on.
c. With an ungloved hand, the focus, zoom, and aperture on the camera was adjusted to obtain the optimal image.
d. In the Gel Doc Window, ‘Capture’ was clicked and the hatched-box icon was selected in this window and dragged to select the area of interest.
e. On the menu bar, ‘Edit’ was selected followed by ‘Extract’. A new window will appear with the final picture. The image properties (brightness/contrast) can be adjusted at this point.

Printing

a. On the menu bar, ‘File’ was selected and then ‘Video Print’ option was selected to print.
b. If the roll has a pink stripe, after the image has been printed, a new roll is obtained from the stock room and installed as per the directions on the printer.
c. The windows containing the extracted and original images were clicked. The ‘Don’t Save’ in the pop-up dialog box was clicked.

Closing the program

a. UV light was switched off.
b. With a gloved hand, gel was removed from the chamber and the surface inside was wiped with a tissue or paper towel.
c. The door of the doc was closed with a clean hand.
d. The name and the number of photos taken were recorded in the log book.

D. POLYMERASE CHAIN REACTION (PCR)

Polymerase Chain Reaction is one of the most important ingenious scientific research tools of the 20th century in molecular biology that allows exponential amplification of short DNA sequences within a longer double stranded DNA molecule. It was invented by Kary Mullis in association with Fred Faloona, Henry A. Erlich, and Randall K. Saiki in the year 1983, while he was working in Emeryville, California for Cetus Corporation. Mullis summarized the procedure: “Beginning with a single molecule of the genetic material DNA, the PCR can generate 100 billion similar molecules in an afternoon. The reaction is easy to execute.

It requires no more than a tube, a few simple reagents, and a source of heat. The reaction is easy to execute. It requires no more than a test tube, a few simple reagents, and a source of heat". In 1993 K. Mullis won the chemistry Nobel Prize for developing PCR.

Components:

The basic components and reagents required to set up a 25 µL PCR reaction are:

1. **Thermal cycler**

It is an apparatus used to amplify segments of DNA. It has a thermal block with holes where tubes holding the PCR reaction mixtures can be inserted. The cycler works on the principle of Peltier effect, which raises and lowers the temperature of the block in a pre-programmed manner by reversing the electric current. Thin-walled reaction tubes permit favourable thermal conductivity to allow for rapid thermal equilibration.

2. **DNA template**

It is the nucleotide sequence of interest that gets amplified.

3. **Primer**

These are oligonucleotides that bind complementarily to the sequence to be amplified. Two primers that are complementary to the 3' ends of each of the sense and anti-sense strand of the DNA target (Tm 52-58℃) are preferred. Primers with melting temperatures above 65 ℃ have a tendency for secondary annealing. The GC of primer should be 40-60 %.

Formula for primer Tm calculation:

Melting Temperature Tm (K) = {ΔH/ ΔS + R ln(C)}, Or Melting Temperature Tm (^{o}C) = {ΔH/ ΔS + R ln(C)}-273.15 where,

ΔH (kcal/mol): H is the Enthalpy. Enthalpy is the amount of heat energy possessed by substances. ΔH is the change in Enthalpy. In the above formula the ΔH is obtained by adding up all the di-nucleotide pair enthalpy values of each nearest neighbour base pair.

ΔS (kca/mole): S is the amount of disorder a system exhibits is called entropy. ΔS is change in Entropy. Here it is obtained by adding up all the dinucleotide pair's entropy values of each nearest neghbour base pair. An additional salt correction is added as the Nearest Neighbour parameters were obtained from DNA melting studies conducted in 1M Na^{+} buffer and this is the default condition used for all calculations.

ΔS (Salt correction) = ΔS (1M NaCl) + 0.368 x N x ln ([Na^{+}])

Where, N is the number of nucleotide pairs in the primer (primer length -1).

$[Na^+]$ is salt equivalent in mM.

The primer annealing temperature is defined by the formula:

Ta = 0.3 x Tm (primer) + 0.7 Tm (product) -14.9

where, Tm(primer) = Melting Temperature of the primers

Tm (product) = Melting temperature of the product

4. Tris-HCI

The recommended buffer solution is 10 to 50 mM Tris-HCI (pH 8.3 - 8.8) at 20 ^oC.

5. $MgCl_2$

It is the cofactor of the enzyme. It is beneficial to optimize the magnesium ion concentration. The magnesium ion affects the primer annealing, strand dissociation temperatures of template and PCR product, product specificity, formation of primer-dimer artifacts and enzymatic activity and fidelity. Taq DNA polymerase requires free magnesium that binds to template DNA, primers, and dNTPs.

6. Distilled water.

Autoclaved distilled water was used. The volume depends on the reaction.

7. Deoxyneuclotide triphosphates.

These are the DNA building blocks. Dntp (dTTP- deoxythymidine triphosphate, dCTP- deoxycytidine triphosphate, dATP- deoxyadenosine triphosphate and dGTP-deoxyguanosine triphosphate) solutions neutralized to pH 7.0. Primary stock solutions are diluted to 10 mM, aliquoted, and stored at -20^oC. A working stock containing 1 mM each dNTP is recommended. The stability of dNTPs during repeated cycles of PCR is such that approximately 50% remains as dNTP after 50 cycles (Corey Levevenson, personal communication). dNTP concentrations between 20 and 200 µM is best for the reaction. The 4 dNTPs should be at equivalent concentrations to minimize misincorporation error.

8. DNA polymerase.

It is an enzyme that catalyzes the reaction. *Taq* DNA polymerase isolated from *Thermus aquaticus* growing in hot springs acts best at 72^oC and the denaturation temperature of 90^oC does not destroy its enzymatic activity. Other thermostable enzyme like *Pflu* DNA polymerase isolated from *Pyrococcus furiosus* and *Vent* polymerase isolated from *Thermococcus litoralis*, were discovered and were found to be more efficient. A recommended concentration of *Taq* polymerase (Perkin-Elmer Cetus) is between 1

and 2.5 units (SA=20 units/pmol) per 100 µL reaction. However enzymatic activity will vary with respect to individual target templates or primers.

Principle

Polymerase Chain Reaction or PCR is an in vitro technique based on the principle of DNA polymerization reaction. It relies on thermal cycling consisting of repeated cycles of heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA using thermostable DNA polymerase, primer sequence (complementary to target region) and dNTPs. It thus can amplify a specific sequence of DNA by as many as one billion times. Most PCR methods can amplify DNA fragments of up to ~10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size.

Procedure

l. Initialization

It is the first step of the cycle which consists of raising the temperature of the reaction to 94 - 96ºC or 98ºC if extremely thermostable polymerases are used, which is held for 1-9 minutes. This process activates the DNA polymerase used in the reaction.

2. Denaturation

It consists of heating the reaction to 94-98ºC for 20-30 seconds. This helps in breaking of the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

3. Annealing

The mixture is now cooled to a temperature of 50 - 65ºC for 20-40 seconds which helps in annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are only formed when the prime sequence very closely matches the template sequence that permits annealing of the primer to the complementary sequences in the DNA. As a rule, these sequences are located at the 3'- end of the two strands of the segment to be amplified. The duration of annealing step is usually 1 min during the first as well as the subsequent cycles of PCR. Since the primer concentration is kept very high relative to that of the template DNA, primer-template hybrid formation is greatly favoured over re-annealing of the template strands.

4. Extension /Elongation

It is a DNA polymerase dependent process. *Taq* polymerase has its optimum activity temperature at 75-78ºC. The temperature at this step depends on the DNA polymerase used; *Taq* polymerase has its optimum activity temperature at 75-80ºC. The temperature is now so adjusted that the DNA polymerase synthesizes the complementary strands by utilizing the 3'-OH of the primer. The primers are extended towards each other so that the DNA segment lying between the two primers is copied; this is ensured by employing primers complementary to the 3'-ends of the segment to be amplified. The duration of primer extension is usually 2 min at 72ºC.

Taq polymerase usually amplifies DNA fragments of up to 2 Kb; special reaction conditions are necessary for the amplification of longer segments. At its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute, leading to exponential (geometric) amplification of the specific DNA fragment.

5. **Final elongation.**

This step is performed at a temperature of 70-74^{o}C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

6. **Final hold.**

In this step the mixture is allowed to cool to a temperature of 4-15^{o}C for short term storage of the reaction.

PCR master mix

To set up a 25 µL reaction the concentration of the reagent are as follows for Master Mix.

Reagents	**Quantity(in µl)**
Taq buffer	17.5
Forward primer(AmyF)	7
Reverse primer(AmyR)	7
dNTps	10.5
Taq polymerase	14
DNA sample	7
Water	112
Total volume	**175**

From the master mix 24 µL was added to separate tubes and 1mLDNA was added separately to each of the tubes.

The PCR was run by using following conditions.

Steps	Temperature	Duration	Cycles
Initial denaturation	**94°C**	**5 min.**	**1**
Denaturation	**94°C**	**1min**	**40**
Annealing	**54°C**	**1min**	
Extension	**72°C**	**3min**	
Final extension	**72°C**	**15min**	**1**
Final hold	**10°C**	**10min**	**1**

RESULTS

Isolation of the amylase producing organisms

The organisms isolated on nutrient agar plates were analysed for study of colony characteristics. Some colonies showed irregular margin while some showed circular. The elevation of the colonies was found to be flat and margin had ridges. All the colonies were cream white in color.

Fig. 2 : Mother cultures and subcultured colonies on Nutrient agar and LB agar plates

Morphological characterization of the isolates

The isolated colonies were further confirmed by morphological characterization. The results obtained are given as follows.

Table 1: Gram's staining and Endospore staining characteristics of organisms under study.

S.No.	Culture no.	Gram's stain characteristic	Shape	Endospore forming characteristic
1.	X_3	+	Rods	+
2.	X_4	+	Rods	+
3.	X_7	+	Rods	+
4.	X_{10}	+	Rods	+
5.	X_{11}	+	Rods	+
6.	X_{12}	+	Rods	+
7.	X_{13}	+	Rods	+
8.	PA_1	+	Rods	+
9.	PA_2	+	Rods	+
10.	PB_2	+	Rods	+

+ positive; -- negative

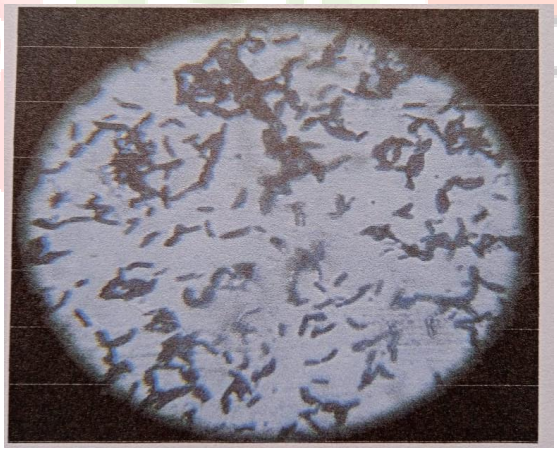

Fig. 3: The isolates were found to be Gram positive rods.

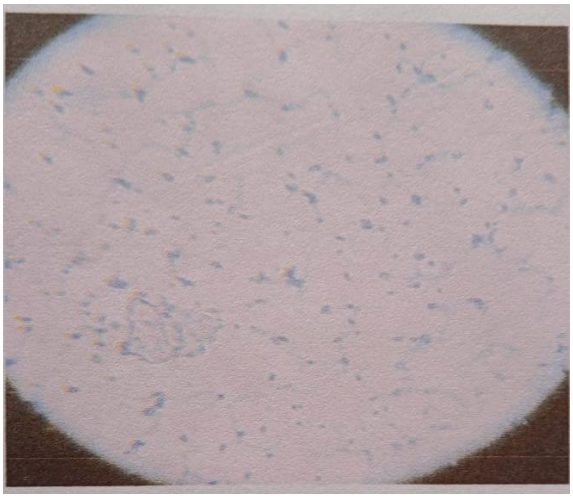

Fig.4: The isolates were found to be Endospore positive.

The morphological characterization i.e. the prevalence of gram positive short rods and spore production ascertained that the selected isolates were of *Bacillus* sp.

Screening for Amylase producing *Bacillus* sp.

The isolates were screened for their amylase production by patch culturing them on selective media are Starch agar and the indicator used was iodine. The results obtained are given as follows:

Table 2 : Amylase producing characteristics of the organisms under study.

S. N0.	Colony no.	Amylase activity on starch agar plate.
1.	X_3	--
2.	X_4	--
3.	X_7	++
4.	X_{10}	++
5.	X_{11}	++
6.	X_{12}	+
7.	X_{13}	--
8.	PA_1	+
9.	PA_2	++
10.	PB_2	++

+ positive — negative ++ highly positive

The cultures which were amylase positive were further confirmed with the help of biochemical characterization.

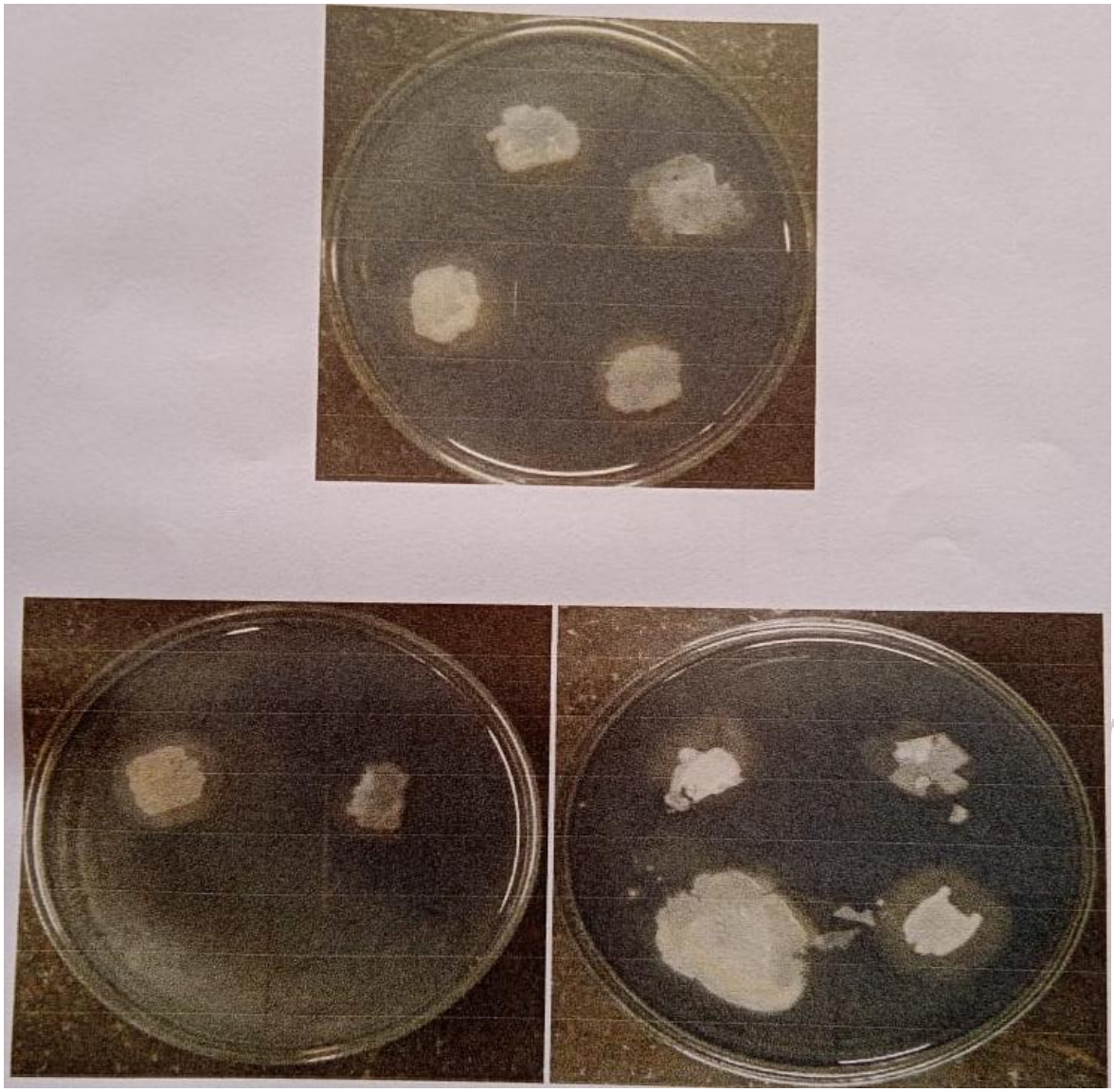

Fig. 5: Amylase hydrolysing activity on star agar plates.

Biochemical characterization

The screened isolates showing maximum amylase production were biochemically characterized. The results obtained are given as follows:

Table 3 : Substrate utilization characteristics of the organisms under study.

S. No.	Culture no.	Catalase test	Indole test	MR	VP	Citrate utilization test
1.	PA_1	+	--	+	--	--
2.	PA_2	+	--	+	--	--
3.	PB_2	+	--	+	--	--
4.	X_7	+	--	+	--	--
5.	X_{10}	+	--	+	--	--
6.	X_{11}	+	--	+	--	--
7.	X_{12}	+	--	+	--	--

+ positive -- negative

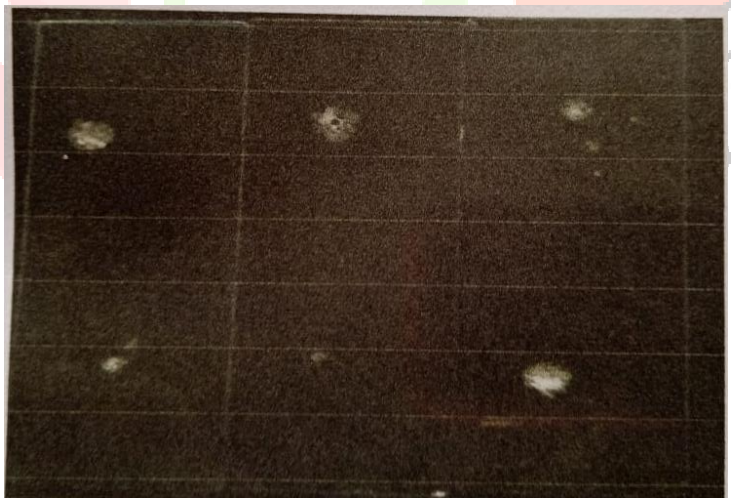

Fig.6: Catalase test (positive).

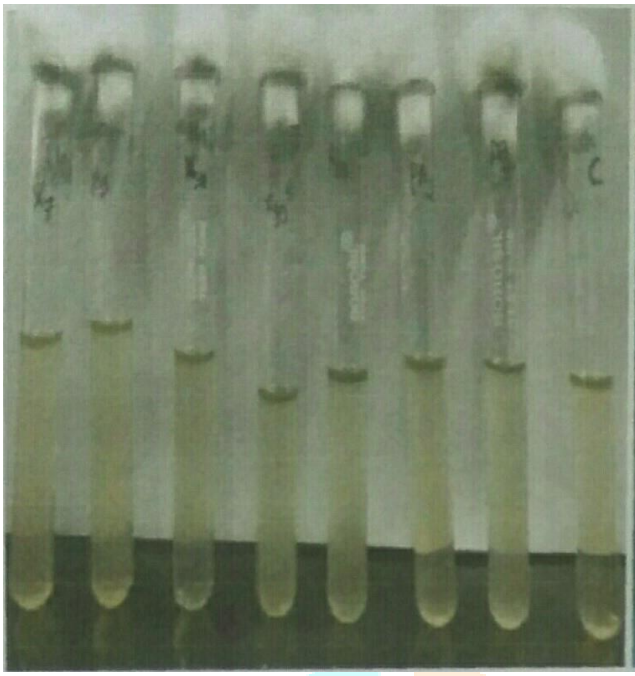

Fig.7: Indole test.

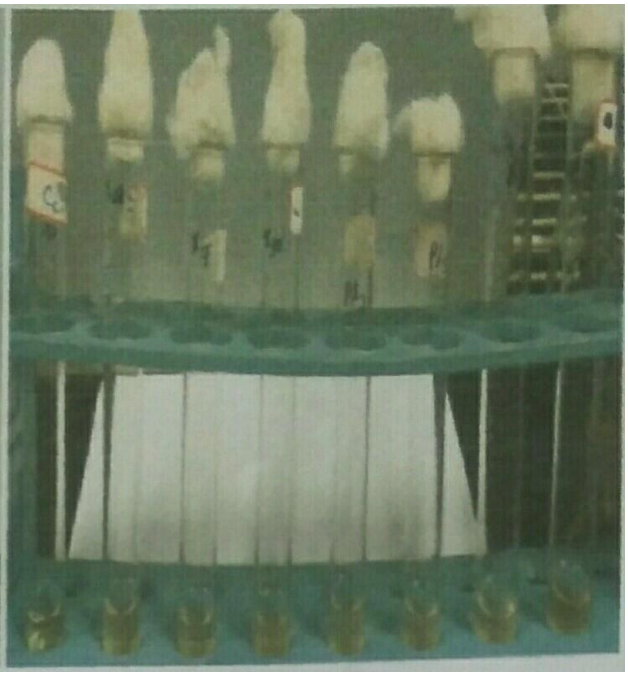

Fig.8: Voges Proskauer test.

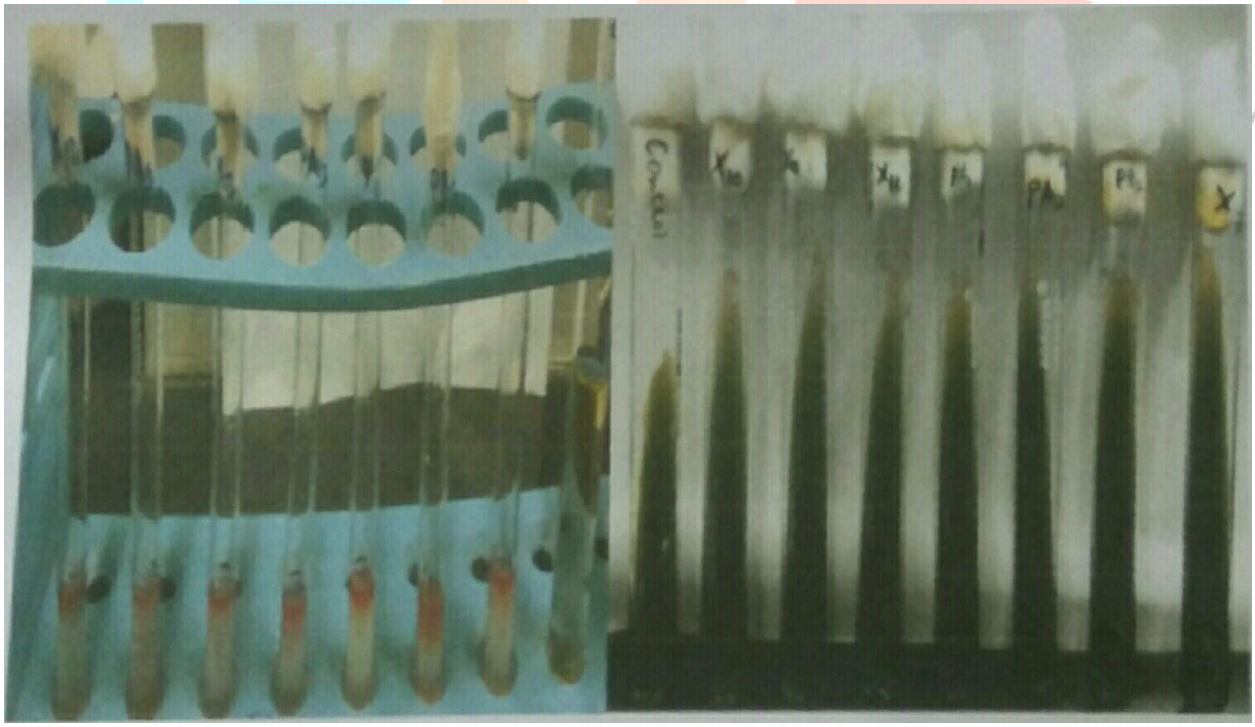

Fig. 9: Methyl Red test

Fig 10: Citrate utilization test.

Table 4: Results for Mannitol motility test.

S. N0.	Culture no.	Acid production	Motility
1.	PA_1	+	+
2.	PA_2	+	+
3.	PB_2	+	+
4.	X_7	+	+
5.	X_{10}	+	+
6.	X_{11}	+	+
7.	X_{12}	+	+

+ positive — negative

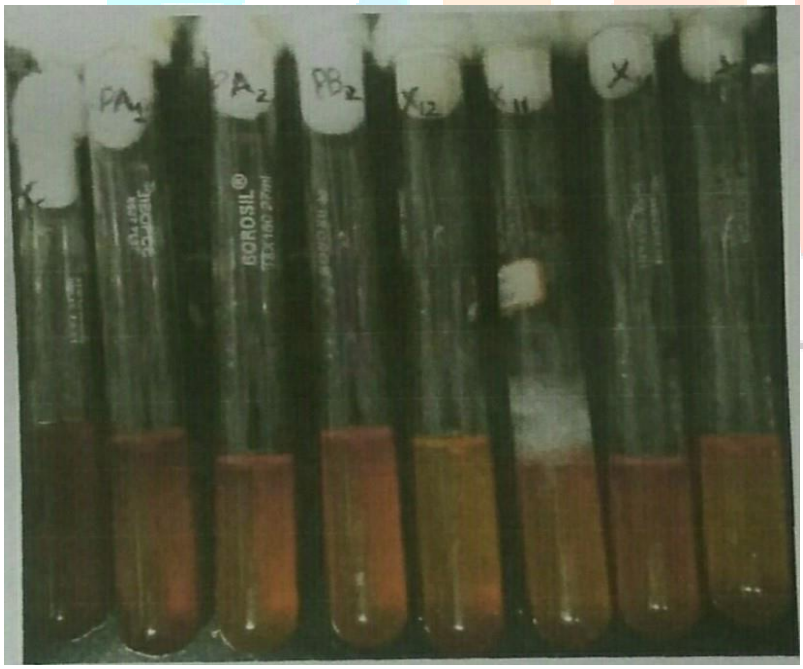

Fig.ll: Mannitol motility test.

Based upon the morphological and biochemical characterization the organisms were identified as *Bacillus* species.

Quantification of DNA using NanoDrop spectrophotometer

DNA was isolated from the isolates which showed maximum amylase production. These cultures were: X_7, X_{10}, X_{11}, PA_2 and PB_2. Quantification of DNA was carried out to check the purity of the DNA isolated from the different samples. The concentration of DNA (in µg/mL) for each sample was also determined from the NanoDrop spectrophotometer.

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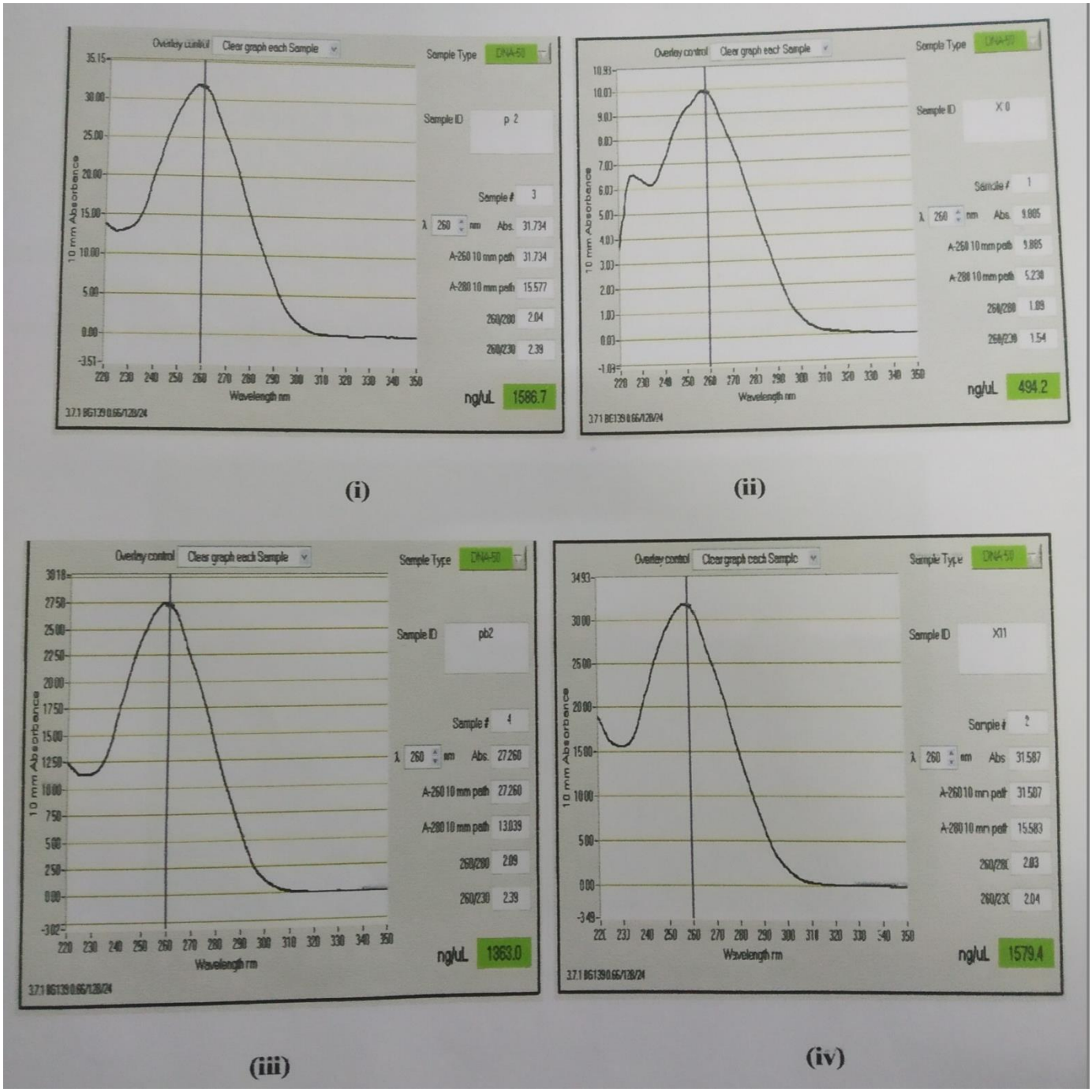

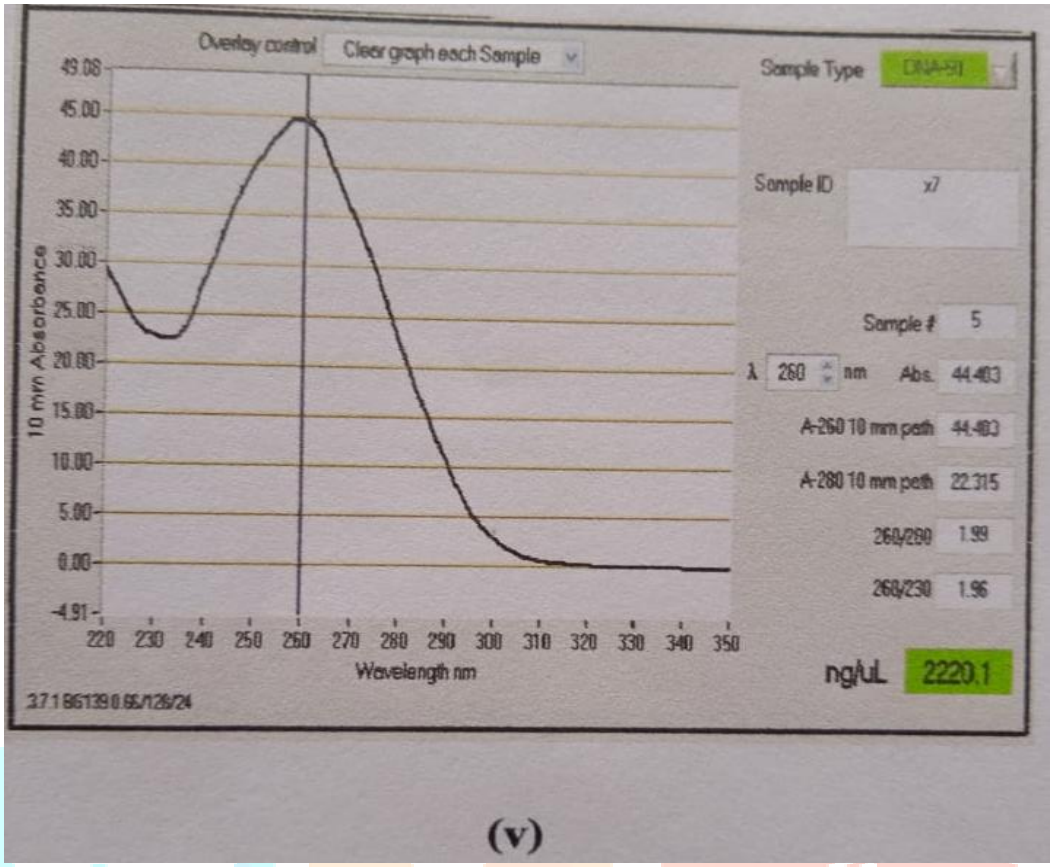

Fig 12: (i) to (v) DNA Quantification using NanoDrop Spectrometer.

Qualitative estimation using Agarose gel electrophoresis

The DNA isolated was run on agarose gel to determine the presence of DNA.

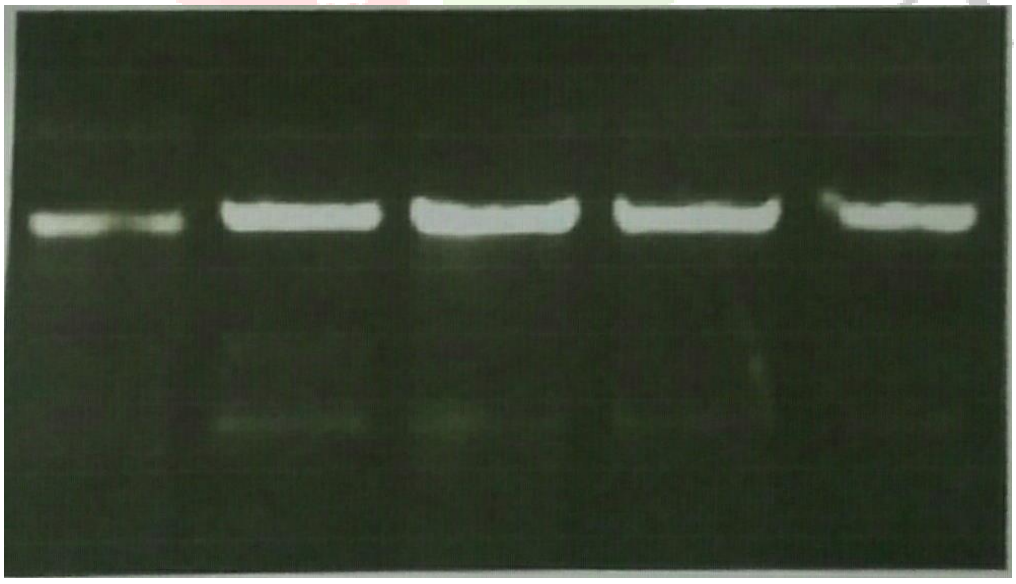

Fig. 13: DNA bands visualized on Agarose gel.

PCR amplification

The isolated DNA subjected to PCR amplification to amplify the gene of interest. The PCR products were then run on agarose gel to check whether the gene of interest was amplified. The gene of interest was the amylase producing gene and specific primers namely AmyR and AmyF were used to amplify the gene. All five DNA samples were amplified by the above mentioned primer.

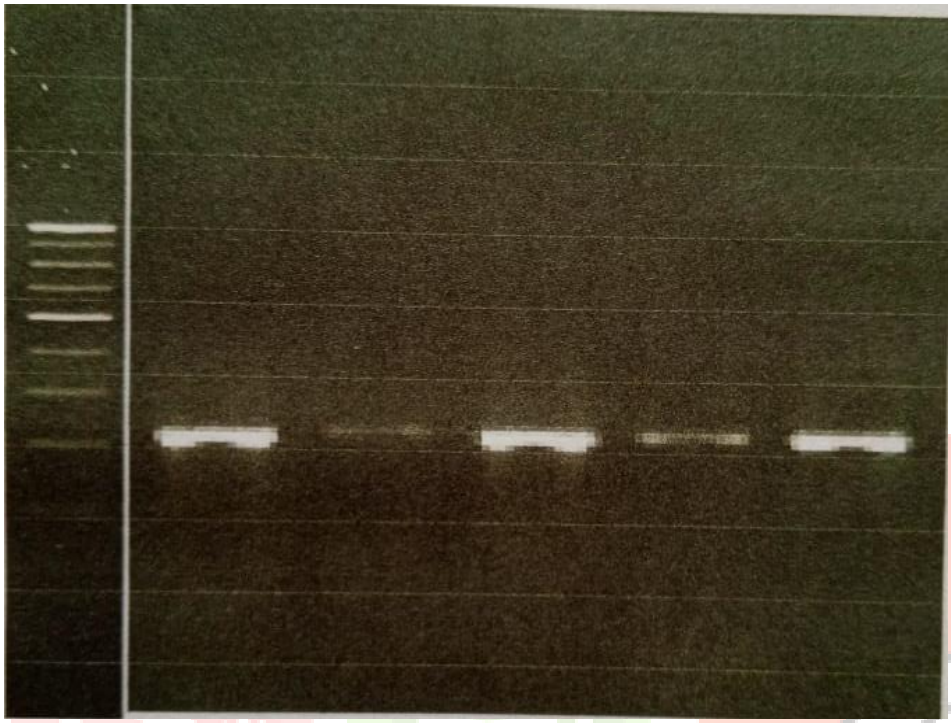

Fig. 14: PCR amplification of the amylase gene using primers AmyF and AmyR.

DISCUSSION

Soil is known to have a rich microflora of which the *Bacillus* species are one of the dominant inhabitants. Microbes in soil are known to produce a variety of enzymes such as proteases, amylases, lipases, ribonucleases, restriction endonucleases etc. Members of the genus *Bacillus* have been the organisms of interest in this study, precisely the amylase producers to characterize the gene responsible for production of the enzyme. *Bacillus* can exist as free living non-pathogenic or parasitic pathogenic organism.

Amylases are enzymes which hydrolyse starch and since kitchen area soil are particularly found to be rich on starch content hence it was selected for isolation of *Bacillus*. The focus has been on *Bacillus* as variety of its species produce amylases and is of great importance in industries such as food, textiles, paper, detergents and fermentation. The organisms isolated were characterized morphologically as well as biochemically for confirmation of the genus. The organisms showed exact characteristics to that of Bacillus i.e., the colonies were cream white in colour, flat, had irregular margins with ridges and some were circular and the organisms were Gram positive, rod shaped, spore forming and catalase producers.

The amylase production was checked by the intensity of the zone of clearance formed by the colonies around them on starch agar plates. Different colonies produced different zones of clearance. The colonies producing amylase were subjected to DNA isolation by phenol chloroform method and it gave a good amount of DNA. The quality and quantity of the DNA is an important criterion that needs to be confirmed before amplification of the amylase producing gene using PCR. The NanoDrop spectrophotometer was used to check the quantity and quality of the DNA. The quantity of DNA ranged from 332.6 ng/µL to 2220.1 ng/µL. The samples of DNA were devoid of any protein contaminations. The DNA isolated was run on agarose gel to further confirm its presence.

PCR amplification was done for the samples of DNA using specific primers AmyF and AmyR which is also a universal primer that can be used for both alpha and beta amylases. This study does not specifically focus on a particular type of amylase enzyme but rather on the amylase gene in general hence a universal primer was used. However different studies reveal that a variety of other primers can be used like AmyE (Javan and Dehkordi, 2013), ldh-R, ycgB-F, Amy3-F etc (Munoz *et.al*. 2011). The DNA amplification could be seen after running the PCR products on agarose gel followed by visualisation under Gel Doc. Thus it can be concluded that the presence of the amylase gene in *Bacillus* species is evident from the amplified bands of the gene which could be seen when viewed under UV light.

CONCLUSION

Bacillus was isolated from the soil sample near kitchen area which is considered to show high amylolytic activity. The nature of culture conditions, temperature and pH for the optimal growth of microbes and production of amylase by the isolated bacterial strains has been observed in this study. The study was carried out on a small scale to find out whether the isolated bacterial strains were amylase producing organisms and it showed positive results. The study holds a variety of future applications in the industrial and research fields. The microbial production of amylase is beneficial as it is economical, gives high yield and it can be engineered to produce enzymes with desired characteristics. Microbial amylases are used potentially in pharmaceuticals, fine chemical industries, paper industries, good and beverage industry, textiles etc (Kaur *et.al*, 2012). The gene for amylase production further be sequenced and cloned into other organisms and the process of amylase production can be optimized. Thus, the present study holds a diverse range of future applications.

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