ISOLATION, QUANTIFICATION, MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF PROTEASE PRODUCING BACTERIA ISOLATED FROM TOMATO

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF

"BACHELOR OF SCIENCE IN BOTANY"

BY

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CERTIFICATE

This is to certify that the dissertation entitled ‘‘**ISOLATION, QUANTIFICATION, MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF PROTEASE PRODUCING BACTERIA ISOLATED FROM TOMATO”** submitted in partial fulfilment for the award of the Degree of Bachelor of Science in Botany is an authentic work carried out by **Ms. Anagha R (Reg.No:AB20BOT022)** under the supervision and guidance of **Smt. Merin Alice George**

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DECLARATION

I hereby declare that the project work entitled **" Isolation, Quantification, Molecular Identification and Phylogenetic Analysis of Protease producing Bacteria Isolated from Tomato"** submitted to The Department of Botany and Centre for Research, St. Teresa's College (Autonomous) Ernakulam, in partial fulfilment of the requirement for the award of the Degree of Bachelor of Science in Botany, is a record of the original project done by me under the supervision of **Smt. Merin Alice George**, Assistant Professor(Contract), Department of Botanyand Centre for Research.

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CONTENT

ISOLATION, QUANTIFICATION, MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF PROTEASE PRODUCING BACTERIA ISOLATED FROM TOMATO

I. INTRODUCTION

Enzymes are specialized proteins produced in an organism which is capable of catalysing a specific chemical reaction. They can work in certain conditions such as presence of cofactors, optimum pH and temperature. Enzymes can be used over and over again due to its unchangeable chemical structure during its action. Almost all biochemical reactions in living organisms demand enzymes. They speed up the biochemical reactions million times faster by providing alternative reaction pathways by lowering the activation energy demand by the process without them. Enzymes are virtually important in our day today life. They help in the production of food we eat, clothes we wear, even in the production of most of the medicines we use. Enzymes are classified into seven categories according to the type of reaction it catalyses. They include hydrolases, lyases, transferases, isomerases, ligases, translocases and oxidoreductases.

To produce environment friendly products and product outputs chemical processes are being replaced by many enzymes such as proteases. Protease is one of the most important enzymes of the biological world. They are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. They likely arose at the earliest stages of protein evolution as simple destructive enzymes necessary for protein catabolism and the generation of amino acids in primitive organisms.

Proteases are the efficient executioners of a common chemical reaction: the hydrolysis of peptide bonds. Most proteolytic enzymes cleave α-peptide bonds between naturally occurring amino acids, but there are some proteases that perform slightly different reactions. (Carlos and Judith, 2008).

Proteolytic enzymes have many physiological functions, ranging from generalized protein digestion to more specific regulated processes such as the activation of zymogens, blood coagulation and the lysis of fibrin clots, the release of hormones and pharmacologically active peptides from precursor proteins, and the transport of secretory proteins across membranes. They are present in all forms of living organisms. Comparisons of amino acid sequences, three-dimensional structures, and enzymatic reaction mechanisms of proteases indicate that there are distinct families of these proteins. Changes in molecular structure and function have accompanied the evolution of proteolytic enzymes and their inhibitors, each having relatively

simple roles in primitive organisms and more diverse and more complex functions in higher organisms. (Hans, 1984).

Alkaline protease is an enzyme that belongs to the hydrolase enzyme group. Its catalytic function is to hydrolyse peptide bonds of proteins. It is capable of hydrolysing, a broad range of peptide bonds in variety of proteins. This enzyme demonstrates for proteolytic activity and high stability that receives their high degree of cross-linking to hydrogen bonds, disulphide bonds and hydrophobic reactions. They are stable in association with chelating agents and perborates. (Ami *et al.*,2009). They are of considerable interest to the biological world due to their stability in highly alkaline medium.

Classification of proteases

Proteases are classified according to their active pH range into acidic, neutral and alkaline proteases. Acid proteases performed best at pH range of 2.0 -5.0 are mostly produced by fungi. Proteases having pH optima in the range of 7.0 or around are called neutral proteases. Neutral proteases are mainly of plant origin while proteases having optimum activity at pH range 8.0 and above are classified as alkaline proteases .

Proteases are broadly classified into 4 major classes (Ellaiah *et al.*, 2002).

1. ***Serine Protease***

   Serine proteases are the most widely synthesized proteases of both microbial and animal origin. They have a reactive serine residue in their active site and are generally inhibited by diisopropyl flurophosphate (DFP) and Phenyl methyl sulphonyl fluride (PMSF). Most of the proteases are inhibited by thiol reagents such as, p-chloromercuric benzoate (pCMB) which might be due to the presence of cysteine residue near their active site, which probably do not participate in the catalytic activity of the enzyme. They are generally active at neutral alkaline pH, optimally between 7-11. They have broad substrate specificities, including considerable estercolytic activity towards many ester substrates.

2. ***Cysteine Proteases***

   Cysteine proteases are sensitive to sulfhydryl reagents like pCMB, Na-tosyl-L-Lysine chloromethyl ketone (TLCK), iodoacetic acid, iodoacetamide, heavy metals, and are activated by reducing agents like potassium cyanide or cysteine, dithiothreitol, and

Ethylenediaminetetraacetic acid (EDTA). The cysteine protease occurrence has been reported only in a few fungi. Intracellular enzymes with properties similar to cysteine proteinases have been reported in *Trichosporon* species, *Oidiodendron kalarai*, and *nannizaia fulva.*Extracellular cysteine proteases have been observed in *Microsporium* species, *Aspergillus oryzea* and *Sporotrichum pulverulentum* . Most of these enzymes are active at pH 5-8. Some are stimulated by reducing agents.

3. *Aspartic Proteases*

Aspartic proteases are characterized by maximum activity at low pH optimally between 3-8 and insensitivity to inhibitors of other three groups of proteases. They are widely distributed in fungi, but are rarely seen in bacterial or protozoan classes. Most aspartic proteases are sensitive to epoxy and diazo-ketone compounds in the presence of copper cations. They are also inhibited by pepstatin or streptomyces pepsin inhibitor.

Most aspartic proteases have molecular weights in the range 30-45 kDa and their isoelectric points are usually in the pH range of 3.4-4.6. Specific action against aromatic or bulky amino acid residues on both sides of cleavage point is a characteristic feature of this group. Catalytic activity involves two aspartic acid residues. They catalytic mechanism requires the initial binding of water molecule at the active site before nucleophilic attack on substrate peptide bonds. Most of the fungal aspartic proteases are non-functional above neutral pH and are not found in cultures growing at neutral or alkaline pH range.

4. *Metallo Proteases*

All the metallo proteases have an optimal pH range between 5-9 and are sensitive to metal – chelating reagents, such as EDTA, but are unaffected by serine protease inhibiting agents or sulfhydryl agents. Many of the EDTA- inhibited enzymes can be reactivated by ions, such as zinc, calcium, and cobalt. These are widespread, but only a few have been reported in the fungal group. A majority of the fungal and bacterial metalloproteases are zinc-containing enzymes, with one atom of zinc per molecule of enzyme. The zinc atom is essential for enzyme activity. Calcium is required to stabilize the protein structure.

Protease production by Bacteria

Proteases constitute 50-65% of the global industrial enzyme market, of which most are alkaline protease. (Pradeep et al., 2012). They are commercially obtained and isolated from living sources like various types of plants, animals, bacteria and fungi. With advancements in the field of biotechnology, the application of proteases has been expanded to fields like clinical, medicinal and analytic chemistry.

To meet the ever-increasing demand of proteases studies on effective production of industrially important proteases have become a need of the hour. Microbial proteases are of better demand and preference over animal and plant proteases due to their possession of all the desired characteristics for their biological application. Microorganisms excrete a wide variety of proteolytic enzymes which are also observed in mammalian systems. They are of smaller size, compact spherical shapes which involves in the hydrolysis of peptide bonds. Among bacteria, *Bacillus* species is specific producers of extracellular proteases. Microorganisms serves as the widely used sources of protease as they can be cultured in large quantities in a relatively short time by establishing the methods like fermentation producing an abundant, regular supply of the desired products.

In general, microbial proteases are extracellular in nature and are directly secreted into fermentation broth by the producer, thus simplifying the downstream processing of the enzymes as compared to proteases obtained from plants and animals (Pradeep *et al.*, 2012).

II. OBJECTIVES OF THE STUDY

1) Isolation of alkaline protease producing bacteria.
2) Estimation of protease activity.
3) Morphological and biochemical characterization of bacteria.
4) Molecular identification of bacteria.
5) Phylogenetic relationship with various other plant derived protease producing organisms.

III. REVIEW OF LITERATURE

Oluwaseyi S.O *et al.,* (2021) conducted a study where *Bacillus* species genomes were examined. Their genomes are rich in plant growth-promoting genetic elements. *Bacillus subtilis* and *Bacillus velezensis* are important plant growth promoters; hence, to further improve their abilities, the genetic elements responsible for these traits were characterized and reported by them. Genetic elements that were reported include those of auxin, nitrogen fixation, siderophore production, iron acquisition, volatile organic compounds, and antibiotics. Furthermore, the presence of phages and antibiotic-resistant genes in the genomes are reported.

Diksha.k *et al.,* (2021) studied on the protease producing bacteria present on the mulberry phyllosphere. Serratiopeptidase, the proteolytic enzyme, is one of the most promising enzymes being used in biopharmaceutical industry. The bacteria were further identified as from the genus *Serratia.*

Chandran. M. *et al.,* (2021) Studied and characterized the alkaline protease producing organisms isolated from the leather industry effluent. In their study, they collected samples from Modji leather industrial effluents and stored in the microbiology lab. The bacteria were isolated from effluent using serial dilution and followed by screening of protease-producing bacteria using skim milk agar media. The primary and secondary screening using zonal inhibition methods was conducted to select potential protease-producing bacteria using skim milk agar media. The potential protease producer was finally analysed by using various biochemical methods, genetical analysis and protease assay.

J. Sai Prasad *et al.,* (2020) conducted research to optimize isolation processes and culturing the seed endophytic bacteria from wheat seed endosperm. After isolation on WMM, TSA, NA medium, A total of 18 morphologically different endophytic bacteria were isolated by them from seven wheat genotypes of *Triticum aestivum* and were tested for the production of hydrolytic enzymes. All the 18 isolated were tested positive for proteolytic enzyme production. From the study they inferred that maximum lytic enzyme producers could directly and indirectly promote the seed germination at initial seedling stage of wheat crop. They also noticed that the hydrolases coupled with antagonistic potential could have a major influence on the seed germination.

W.J Sul *et al.,* (2020) conducted a study in order to find the effect of *Bacillus subtilis* species on the growth and life of various plant species. Among several candidates, the application of

Bacillus subtilis strain GOT9, led to the enhancement of drought and salt stress tolerance in *Arabidopsis*. In agreement with the increased stress tolerance phenotypes, its application resulted in increases in the transcripts of various drought stress- and salt stress-inducible genes in the absence or presence of the stresses. Furthermore, the treatment resulted in improved lateral root growth and development in *Arabidopsis*. GOT9 also led to enhanced tolerance against drought and salt stresses and to upregulation of drought-inducible genes in *Brassica*, a closely related crop to *Arabidopsis*. Taken together, these results show that GOT9 could be utilized as a biotic resource that effectively minimizes damage to plants from environmental stresses.

Chakraborthy.A. *et al.,* (2020) isolated the protease producing bacteria from soil and characterized the protease collected from the soil present outside and adjacent areas of a sweetshop in Burdwan. Screening of the bacteria was done by employing skim milk agar plating technique at 37°C and observed after 24 hours. Proteolytic activity of the crude protease extract from the bacteria was detected by using azocasein as substrate. Bacteria isolated were coccus shaped and Gram-positive in nature. The isolated white bacteria were identified by 16s rDNA sequencing as *Staphylococcus* species.

Manavalan T *et al.,* (2020) isolated and identified a unique protease obtained from a proteolytic *Bacillus megaterium*-TK1 strain from a seawater source. The extracellular thermostable serine protease was processed by multiple chromatography steps. The isolated protease displayed a relative molecular weight (MW) of 33 kDa (confirmed by zymography), optimal enzyme performance at pH 8.0, and maximum enzyme performance at 70 °C with 100% substrate specificity towards casein.

Xie. J *et al.,* (2019) Isolated *Bacillus subtilis* an Endophytic bacterium from the stem of mulberry plant. They inferred that the previously studied Bacillus Species *Bacillus tequilensis* and *Bacillus subtilis* exhibited antifungal properties and had a strong capacity to promote plant growth. The strain was studied for its effectiveness as biocontrol agent to reduce mulberry fruit sclerotiniose in the field and as a mulberry growth promoting agent in green house.

B. Asha *et al.,* (2018) conducted an experiment for the optimization of alkaline protease production by *Bacillus cereus* FT 1 isolated from soil. Protease producing bacteria were isolated from organic waste containing soil, screened for protease production on skim milk agar plates and confirmed the protease production through protease assay. The bacterial isolate

showing highest alkaline protease production was selected and identified by microscopic, macroscopic, biochemical and 16 S RNA phylogenetic analyses as *Bacillus cereus FT 1*.

In a study conducted by Marathe S.K *et al.,* (2018) bacteria isolated from sea water samples of Murdeshwar, Karnataka, were screened for the production of alkaline protease by culturing them onto skim milk agar media. Of the isolated bacteria, *Bacillus subtilis, Pseudomonas aeruginosa* and *Alcaligenes faecalis* showed distinct zones of hydrolysis due to enzyme production. Enzyme activity was determined under varying conditions of pH, incubation temperature, different substrates, carbon and nitrogen sources and salt concentrations using sigma's universal protease activity assay. Efficacy of enzyme in stain removal was tested and haemolysis was observed within of 60 s which resulted in removal of the stain. Among the three organisms, enzyme from *Bacillus subtilis* showed highest activity in all cases indicating that it was the most ideal organism for enzyme production.

S.E.D. Hassan (2017) studied on the impact of endophytic bacteria and fungus on the promotion of plant growth in *Teucrium polium* a sub shrub or herb native to Mediterranean region, and a member of the Lamiaceae Family. Seven bacterial endophytes were isolated and identified as *Bacillus cereus* and *Bacillus subtilis.* The study results indicated that microbial endophytes isolated from medicinal plants were possessing a vital role in the improved plant growth and could be used as inoculants to establish a sustainable crop production system.

A comparison between poultry waste contaminated and detergent contaminated soil were carried out by Vishnupriya C S *et al.,* (2016) to detect the most potent alkaline protease producer. Soil samples were serially diluted and plated on skim milk agar to yield 4 potent isolates. Protease activity was compared using protease assay and the best isolate selected was B2 isolate from detergent contaminated soil. It showed a higher protease production. The selected isolate was identified as *Bacillus subtilis* using standard identification parameters such as gram staining and biochemical tests followed by molecular analysis. The 16S rDNA of the selected organism showed 99% similarity in the BLAST search with *Bacillus subtilis.*

Guleria S *et al.,* (2016) conducted and experiment where a thermostable extracellular alkaline protease producing *Bacillus amyloliquefaciens* SP1 was isolated from apple rhizosphere having multifarious plant growth promoting activities. Strain SP1 was purified to 6.48-fold using four-step purification protocol and characterized in detail for its robustness and eco-friendly application in leather and detergent industries. Structural analysis revealed that the protease

was monomeric and had a molecular weight of 43 kDa. It has shown stain removal property and dehairing of goat skin without chemical assistance and hydrolysing fibrous proteins.

Onkar.N.T *et al.,(2015)* studied on the alkaline protease producing bacteria isolated from the undisturbed soil of North Eastern region of India falling under the Indo-Burma biodiversity hotspots. In this study, 5 isolates forming larger zone as a result of casein hydrolysis from many isolates were further studied for quantitative production of extracellular alkaline protease activity. Out of these, isolate no. B-2 was observed as a highest potential protease producer bacterium. Upon 16S rRNA analysis, it displayed maximum similarity with *Bacillus* species.

Mohammad N (2015) carried out a research work to explore the indigenous protease producing soil bacterial flora of Karak, Khyber Pakhtunkhwa, Pakistan. A total of 60 bacteria were isolated from 16 soil samples collected from different sites in district Karak. The isolates were screened for protease production by using skimmed milk agar. Among all the isolates, 18 bacterial isolates were found as protease producers with various degree of protease activity. These isolates were further identified through their morphological, microscopic and biochemical examination. It was concluded that the soil of Karak is rich in protease producing bacteria that exhibit diversified proteolytic activities and biochemical features.

Aruna K. *et al.,* (2014) isolated an alkaline protease producing strain was from spoilt cottage cheese sample which was identified as *Bacillus tequilensis* strain SCSGAB0139 on the basis of morphological, cultural, biochemical characteristics and 16S rRNA sequence analysis. Primary screening for protease production was carried out by observing for zone of hydrolysis on skim milk agar, GYEA milk agar and gelatine agar plates. Physicochemical parameters like pH of the medium, incubation time and temperature, aeration and composition of the medium were optimized for maximum protease production by this isolate.

Jaiswal M. *et al.,* (2014) conducted a work which is an application of 16S rRNA gene sequencing approach to identify a novel, alkaline protease producing bacteria, from poultry farm waste. The sample was collected from a local poultry farm in the Guntur district, Andhra Pradesh, India. Subsequently the sample was serially diluted and the aliquots were incubated for a suitable time period following which the suspected colony was subjected to 16S rDNA sequencing. The results showed the isolate to be a novel, high alkaline protease producing bacteria, which was named *Bacillus firmus* isolate EMBS023.

Santhosh, S., & Dhandapani, R. (2014) reported the production of thermostable protease and its characterization in *Bacillus* species, which is a thermotolerant bacterium; *Bacillus megaterium* is widely used for isolating protease enzyme. Water sample were collected and screened for protease producing bacteria. The selected organism was identified based on Bergey's manual and molecular characterization was performed. Enzyme was partially purified using ammonium sulphate precipitation technique and SDS–PAGE analysis. Efficacies of enzyme was analysed by the effective removal of blood stain from cloth.

O.S.Bindhu. *et al*., (2014) investigated on the haemostatic, milk clotting and blood stain removal potential of *Calotropis gigantea* latex proteases. The protease activity of crude enzyme (CE), obtained by centrifugation followed by ammonium sulphate precipitation and dialysis, was assayed using casein as the substrate. Effect of pH, temperature and specific inhibitors on protease activity was determined.

Protease producing bacteria was isolated by Kumari.P *et al.,* (2014) from soil of Tatapani area of Mandi district of Himachal Pradesh. These bacteria were screened in skim milk agar medium using skim milk as the substrate. The highest clear zone producing bacterial isolate P5 was selected for further optimization studies. The isolate was identified as *Bacillus subtilis* based on morphological, biochemical and molecular characterizations. The isolate was able to grow under alkaline conditions at pH 9.0 and a temperature of 50°C. 1.9fold purification of enzyme following ammonium sulphate precipitation and DEAE-cellulose chromatography was achieved.

Balaji N (2012) worked on the isolation and characterization of the protease producing potent bacteria from gastro intestinal tract of freshwater fish *Cyprinus carpio*. The protease producer was characterized as *Bacillus sp*. morphologically and biochemically. The optimization studies for growth and protease production of the isolate *Bacillus sp*. was carried out. The optimum temperature for the growth of isolated *Bacillus sp*. was 350C but the optimum temperature for the protease production was 450C, the optimum pH was 7.0 in which the isolate produced 18 mMol/ml.

Habib.S.M.A *et al.,* (2012) isolated extracellular protease producing bacteria from tannery effluents. The isolation was performed by serial dilution and plating method. Eight protease producing isolates were screened out on the basis of their clear zone formation on skim milk agar as well as production of protease in protease producing broth. They were identified on the

basis of cultural, morphological and biochemical tests. Among the eight isolates, 3 isolates were found to belong to the genus *Halobacterium*, and the rest 5 isolates were found to belong to genus *Actinobacillus*.

In the study conducted by Khan M A *et al.*, (2011) soil samples from different habitats including tanneries, soap industries, garden soil and soil compost were screened for the presence of alkalophilic *Bacillus* isolates capable of producing alkaline protease in large quantities. One hundred and eighteen (118) isolates were found having proteolytic activity on skim milk agar plates. Isolates forming larger zones, as a result of casein hydrolysis were further studied for quantitative production of extracellular alkaline protease activity in the shake flask studies.

G.Das & Prasad.M.P (2010) conducted an experiment where, screening and isolation of protease producing strains of bacteria were carried out from four different soil samples collected from various places in Bangalore. The isolates were positive on skim milk agar (1%) and thus are selected as protease producing strain. The organisms were tested for various biochemical tests, which lead to their identification as *Bacillus subtilis* producing protease enzyme.

The purification and characterization of alkaline protease from *a Bacillus firmus* strain TAP5, isolated from tannery waste have been reported by Joshi.B.H (2010). This protease was purified to homogeneity by the combination of ammonium sulphate precipitation, DEAE sephacryl ion exchange and phenyl sepharose hydrophobic interaction chromatography. The enzyme was a serine protease with a relative molecular mass of 34 kDa by SDS-PAGE. Proteolytic activity of the enzyme was detected by gelatine zymography, which gave a very clear protease activity zone on gel.

A protease producing bacteria was isolated from meat waste contaminated soil and identified as *Pseudomonas fluorescens* by Kalaiarasi K *et al.*, (2009). Optimization of the fermentation medium for maximum protease production was carried out. The culture conditions like inoculum concentration, incubation time, pH, temperature, carbon sources, nitrogen sources and metal ions were optimized.

IV. MATERIALS AND METHODS

COLLECTION OF SAMPLE

Tomatoes were collected from the local vegetable markets of Ernakulam, Kerala. The samples were stored under sterile conditions under dark place in airtight zip-bags. Date and time were labelled on it.

1. ISOLATION

1.1. SERIAL DILUTION

Serial dilution is a procedure of stepwise dilution of substance in a solution. Here, the substance is grinded Tomato paste and this procedure helps to dilute the tomato to a maximum level in each successive step. It helps to get down to a concentration or number of organisms per area that is much easier to work with.

Materials required

1. Eppendorf tubes
2. Distilled water
3. Micropipette
4. Soil sample 20

Procedure

Using Mortar and pestle the tomato was grinded into fine paste. We then dilute the sample up to 10^{-5} . 5 Eppendorf tubes were taken with 9 ml of distilled water in each. One gram of sample was added to 1 ml water and this stock was added to the first tube. 1ml from the stock was serially diluted to 10^{-5} under aseptic environment of Laminar Airflow Cabinet.

1.2. SPREAD PLATE METHOD

Spread plate technique is employed to plate the liquid sample, for the purpose of isolating the bacteria present in it. A countable number of bacterial cells were evenly distributed on an agar plate.

Materials required

1. Petri plates
2. Nutrient agar
3. L- rod
4. Distilled water

Procedure

According to the standard methods, 100 ml of nutrient agar medium was prepared. After cooling the solution, it was poured into two petri plates and was allowed to solidify inside the laminar air flow chamber. After solidification, 1 µl of diluted sample from 10^{-2} and 10^{-3} dilutions of sample were spread onto the plates using an L- rod. The plates were kept at the incubator for 24 hours.

1.3. STREAK PLATE METHOD

Streak plate method is used to isolate the pure culture of bacteria from a mixed population. The inoculation loop is streaked very thinly over the agar surface to get individual bacteria.

Materials required

1. Petri plates
2. Nutrient agar
3. Agar Powder
4. Inoculation loop

Procedure

As per the standard methods, 40 ml nutrient agar was prepared. The agar was poured into the petri plates and allowed it to get settled by cooling inside the laminar air flow chamber. The plates were inoculated with the primary cultures of different morphologies using single streaking and kept at incubator for 24 hours at inverted position.

2. SCREENING

2.1. SKIM MILK AGAR PLATING

Skim milk agar (SMA)plates were prepared for the confirmation and determination of proteolysis by various bacteria. Here we gave milk powder as the only nutrient source for the bacteria to identify the bacteria with the protease genes.

Materials required

1.Skim milk

2.Casein enzyme hydrolysate

3.Yeast extract

4.Dextrose

5.Agar

6.Water

7.Petriplates

8.Inoculation loop

9.Pure culture

Procedure

First 1.04g of nutrient agar, 0.04g of dextrose, 0.1g yeast extract and 5g casein enzymic hydrolysate was mixed with 40 ml of distilled water by autoclaving. After sterilisation, 28g of skim milk powder was added to the cooled solution and was mixed well. The solution was poured into the petri plates and allowed it to solidify under the laminar air flow chamber. The primary cultures of different morphologies were inoculated onto each plate using single spotting method. The plates were kept inside the incubator at 37 ºC for 24 hours at inverted position.

2.2. QUADRANT STREAKING METHOD

Quadrant streaking method helps us to form discrete colony forming units (CFUs). Here we used this method to isolate and obtain pure cultures of bacteria with proteolytic activity found in skim milk agar plates. 60 ml of nutrient agar medium was prepared as per standard and 20 ml each was poured to 3 different sterilized petri plates. After the agar got settled, quadrant streaking was done on each plate using each culture. The plates were kept in an incubator for one day at 37ºC at inverted position.

3. GRAM STAINING

Gram staining is one of the important staining methods invented by the Danish bacteriologist Hans Christian Gram. This method helps to differentiate the bacteria into two different groups as gram positive and gram negative. This method is based on their differences in their cell wall composition. Gram positive bacteria have a thick peptidoglycan cell wall and retain the crystal violet dye on staining, and appear violet, while gram negative bacteria appear pink after counter stained with safranin.

Materials required

1. Glass slide
2. Pure culture
3. Crystal Violet
4. Distilled water
5. Decolourising agent
6. Safranin
7. Gram's iodine

Procedure

A small drop of culture was smeared into a glass slide using an inoculation loop and made it to heat dry under the heat of a Bunsen burner. A small amount of crystal violet was poured onto the culture, kept for 1 min and washed with tap water. Flooded the smear with gram's iodine for 1min and washed with tap water. The stain was decolourised with ethyl alcohol (95%) by dropping the reagent drop wise until crystal violet fails to wash from smear. Washed it away with tap water and counterstain with safranin for 45 seconds and washed again. After air drying, examine the slide under a microscope in a 100x lens.

4. SELECTION OF SUPERIOR COLONY

For the further analysis, one, high protease producing colony was to be selected. For this all the identified colonies were spotted on 2 different Nutrient agar plates. The length of clear zones of all the colonies were measured. A graph was plotted and the colony was selected on analysing the graph.

5. QUANTIFICATION

5.1. PRODUCTION OF BROTH

The enzyme production media has to be prepared and autoclaved at 121°C, 15 psi for 15 minutes. The selected isolate had been inoculated with fermentation media and incubated in a shaker for 3-4 days. After incubation, this is centrifuged at 12,000rpm for collecting the supernatant. This was used for further processes.

5.2. PROTEASE ASSAY

Protease assay was done for both culture broth and protein sample to estimate the amount of protease enzyme present in it. The assay was carried out by using casein as a substrate.

Materials required

1. Test tubes
2. Peptone broth
3. Casein
4. Water bath
5. Trichloroacetic acid
6. Sodium carbonate
7. Folins -Ciocalteu reagents
8. Spectrometer

Procedure

Standard:

Table No 1: Protease assay for protein sample

Reagent	Std 1	Std 2	Std 3	Blank
L – tyrosine	0.05	0.10	0.20	0
Distilled water	1.95	1.90	1.80	2
Na_2CO_3	5.00	5.00	5.00	5.00
F-C reagent	1.00	1.00	1.00	1.00
Incubate at 37°C for 30 minutes and record the absorbance at 600 nm				

Sample :

Table No 2: Protease assay for culture broth

Reagent	Test	Blank
Casein	5.00	5.00
Enzyme solution	1.00	-
Incubate at 37℃ for 30 minutes		
TCA	5.00	5.00
incubate at 37℃ for 30 minutes		
Filter the contents		
Filtrate	2.00	2.00
Na2CO3	5.00	5.00
F-C reagent	1.00	1.00
Incubate at 37℃ for 30 minutes and record the absorbance at 600 nm		

6. MOLECULAR IDENTIFICATION

6.1. DNA ISOLATION

DNA extraction is required for a variety of applications in molecular biology. Here we isolate the DNA to carry out the polymerase chain reaction using 16S RNA for amplifying the protease producing gene.

Materials required

1. 0.5 M EDTA
2. 0.85% NaCl solution
3. Chloroform: Isoamyl alcohol (24:1)
4. 3M sodium acetate
5. TE buffer
6. 70% alcohol

Procedure

The broth needed for the process was inoculated a day before, using the pure sample plate and kept inside the incubator. The broth was collected in 2 ml Eppendorf tube and centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded and the pellet was treated with 0.85% NaCl. The tubes were again centrifuged for 12 minutes at 12,000 rpm. The supernatant was discarded and 600 µl of lysis buffer was added in the tube and vortex mixed. For the next 30 minutes, the tube was kept in a water bath followed by centrifugation at 1200 rpm for 10 minutes. The supernatant was collected and an equal amount of

chloroform isoamyl alcohol was added to it. The vortex mix was done until it turned to milky colour and centrifuged at 12,000 rpm for 10 minutes. The upper aqueous layer was collected and mixed with 1/10th volume of 3M sodium acetate. The pH was maintained as 5.2 and an equal amount of ice-cold isopropanol was added. The tube was centrifuged at 12,000 rpm for 5 minutes. The pellet was rinsed with 70 % alcohol and centrifuged at 8000 rpm for 5 minutes. The ethanol was discarded and DNA was kept in the refrigerator at 4℃ for further use.

6.2. GEL ELECTROPHOROSIS

Materials required

- Ethidium bromide
- 1.2% agarose
- Micropipette
- Casting tray
- Cellophane
- Running tray

Principle

Under the influence of constant current flow, the negatively charged DNA can migrate towards the positive charge. Thus, the separation depends on the charge and mass of DNA. DNA has to move through the pores of the agarose and this can cause the separation based on the size. That is, the DNA with lower mass can move faster and those with higher mass are slower. The basis of this separation technique is the separation of macromolecules based on its size, charge and binding affinity.

Factors affecting the migration of DNA are:

- The temperature of the buffer .
- The hydrophobicity of the DNA.
- The size and shape of the DNA.
- The ionic strength of the DNA.
- The strength of the field.

DNA marker: commercially available DNA markers were used as the standard molecular weight DNA marker.

Procedure

The running tray was installed by placing it firmly on the casting tray and the comb was placed on the rim of the casting tray and the bottom of the comb was adjusted so that it is about 1.0mm from the running tray. 1.2 % agarose (0.24g in 30ml 1X TBE) solution was prepared by heating and cooling. Ethidium Bromide (0.5ml EtBr for 10 ml TBE) was added to the gel. The agarose solution was poured into the tray. The comb was checked to be vertical to prevent well shape distortions. A minimum of 30 minutes was allowed for the gel to set. Once the gel was set, the comb was removed carefully. Then the running tray was removed with the gel and it was placed on the running platform of the electrophoresis unit. 10µl of DNA with 4µl loading dye was loaded in agarose gel. The gel was run at constant voltage of 100V till the dye travelled 3 cm from the wells and the gels were viewed on UV transilluminator with the safety shield.

6.3. POLYMERASE CHAIN REACTION

Polymerase Chain Reaction (PCR) is a method used to amplify a particular fragment of DNA using the primers. It helps to copy the DNA rapidly about millions to billions within a short time period. The isolated DNA on the above step was used for PCR. 16SrDNA was used as the primer. PCR was developed by Kary Mullis in 1983. The method is based on thermal cycling; consisting of repeated heat variations given to the sample. This helps to denature the double stranded DNA into two single stranded DNA. Thus, the primers can bind and ligate to the single strands to form new copies of DNA. PCR consists of 3 main steps:

Denaturation: The double stranded DNA is heated up to separate it into two single strands. The temperature will be more than 90 ℃ in this step.

Annealing: The primers that we supply get attached to the DNA strands.

Extension: The dNTPs that are complementary to the strands get attached to it with the help of the enzyme Taq polymerase.

Table No 3: Reaction mix for PCR

SI NO	**Components**	**Vol. Per reaction**
1	Reaction mixture	10 l
2	Forward primer	1 µl
3	Reverse primer	1 µl
4	DNA sample	1 µl
5	Deionised water	7 µl

Table no: 4 Steps of PCR

Cycle	**Step**	**Process**	**Temperature**	**Time**
1	**I**	Initial Denaturation	94°C	3 Minutes
1	**II**	Denaturation	94°C	30 Seconds
1	**III**	Annealing	59°C	30 Seconds
1	**IV**	Primer Extension	72°C	30 Seconds
Go to step 2 for 29 times				
30	**V**	Final elongation	72°C	7 Minutes
1	End or 4°C forever			

GENE AMPLIFICATION

Forward primer: 5'-GACACACGTGCTACAATG-3'

Reverse primer: 5'-TGACCTCACCCTTATCAG-3'

AGAROSE GEL ELECTROPHOROSIS

1.2% agarose gel was prepared and electrophoresis of PCR amplified sample was carried out.

6.4. SEQUENCING

PCR product was sent for sequencing.

6.5. BLAST

The edited sequences (16srDNA) were then used for similarity check using BLAST (Basic Local Alignment Search Tool) programme in the NCBI GenBank DNA database for identifying the sample.

FINDING SIMILAR ISOLATIONS USING BLAST

Using BLAST 10 other most similar sequences of microorganisms producing proteases isolated from various plant species were identified. The sequences were downloaded for further analysis.

6.6. MEGA

Using MEGA (Molecular evolutionary Genetics Analysis) multiple sequence alignment was conducted. Using this, a phylogenetic tree was established and the most related microorganism isolate from various other research works was identified.

V. OBSERVATIONS AND RESULTS

SAMPLE COLLECTION

The tomato samples were collected from local market which is shown in (Figure 1). The samples were grinded for isolation of bacteria. (Figure 2).

Figure no: 1 : Tomato sample

Figure no: 2: Tomato paste being weighed

1. ISOLATION

1.2 SPREAD PLATE METHOD

Spread plate technique was performed in skimmed milk agar after the serial dilution of sample(Figure no: 3). After incubation the plate spread with 10^{-3} and 10^{-2} serial dilution gave several colonies of bacteria. (Figure no: 4 and 5).

Figure no:3: Preparation of spread plate

Figure no: 4 : 10^{-2} Spread plate

Figure no: 5: 10^{-3} spread plate

1.3. STREAK PLATE METHOD

The colonies formed after spread plating technique was streaked on Nutrient Agar in a quadrant streak manner. After Incubation, Different colonies were found distinctly. (Figure no:6).

Figure no: 6: Quadrant Streak on Nutrient Agar

2. SCREENING

2.1. STREAK PLATE METHOD FOR SCREENING

The colonies formed after Streak plating technique was streaked on one skimmed milk agar plate and spotted on another skim milk agar plate. After incubation showing zone of clearance on skim milk agar. Out of the 8 Chosen colonies that were streaked and spotted, only 3 colonies belonging to quadrants I,IV and VII exhibited zone clearance indicating the presence of protease production. (Figure no: 7 and 8).

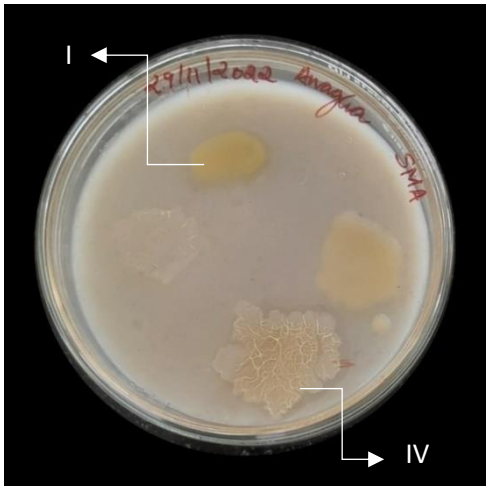


Figure no: 7: Quadrant 1-4

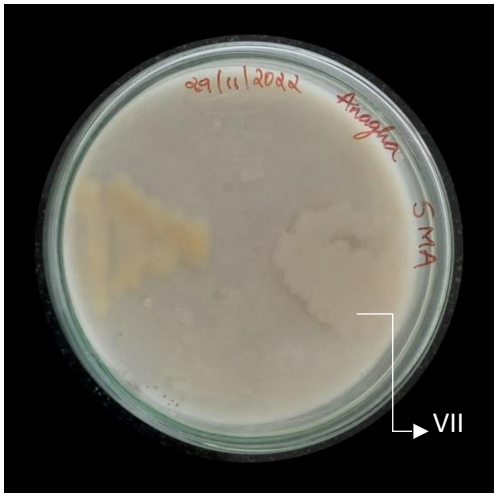


Figure no:8: Quadrant 5-7

2.2. QUADRANT STREAKING OF THE PROTEASE PRODUCERS

The colonies showing zone clearance were quadrant streaked on Nutrient Agar for further study and analysis.

Figure no:9: Colonies I, IV, VII respectively

3. GRAM STAINING

Gram's staining was performed and the slides were observed under oil immersion 100X and the organisms were observed as follows.

Colony I: Gram Positive (Purple coloured, rod Shaped) (figure no: 10).

Colony IV: Gram Negative (Pink coloured, Spherical) (Figure no: 11).

Colony VII: Gram Positive (Purple coloured, rod shaped) (Figure no: 12).

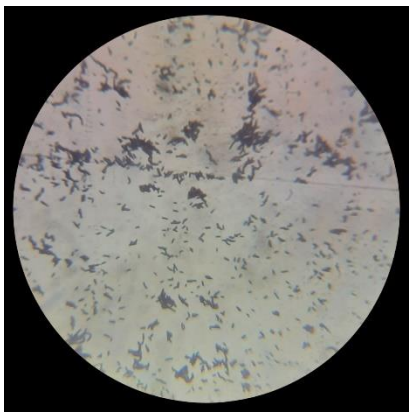


Fig no:10: Colony I

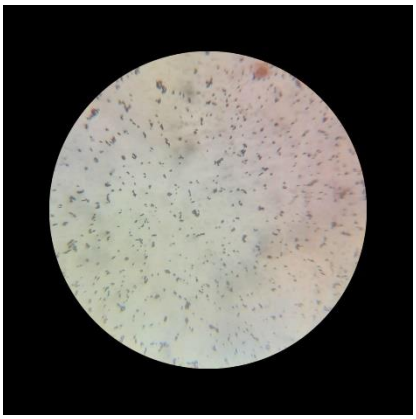


Fig no: 11: Colony IV

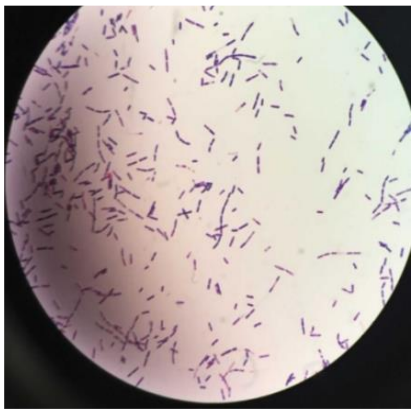


Fig no: 12: Colony VII

4. SELECTION OF SUPERIOR COLONY

The 3 protease producing colonies were spotted on 2 different Skim Milk Agar Plates (Fig no.13). The lengths of the colonies and the length of their clear zone were noted. A graph was plotted for identifying the superior colony.

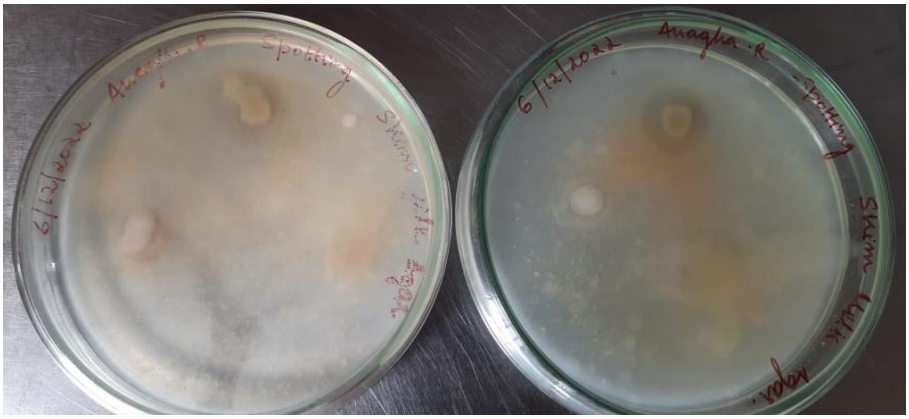


Figure no.13: Spotted plates for selection of superior colony

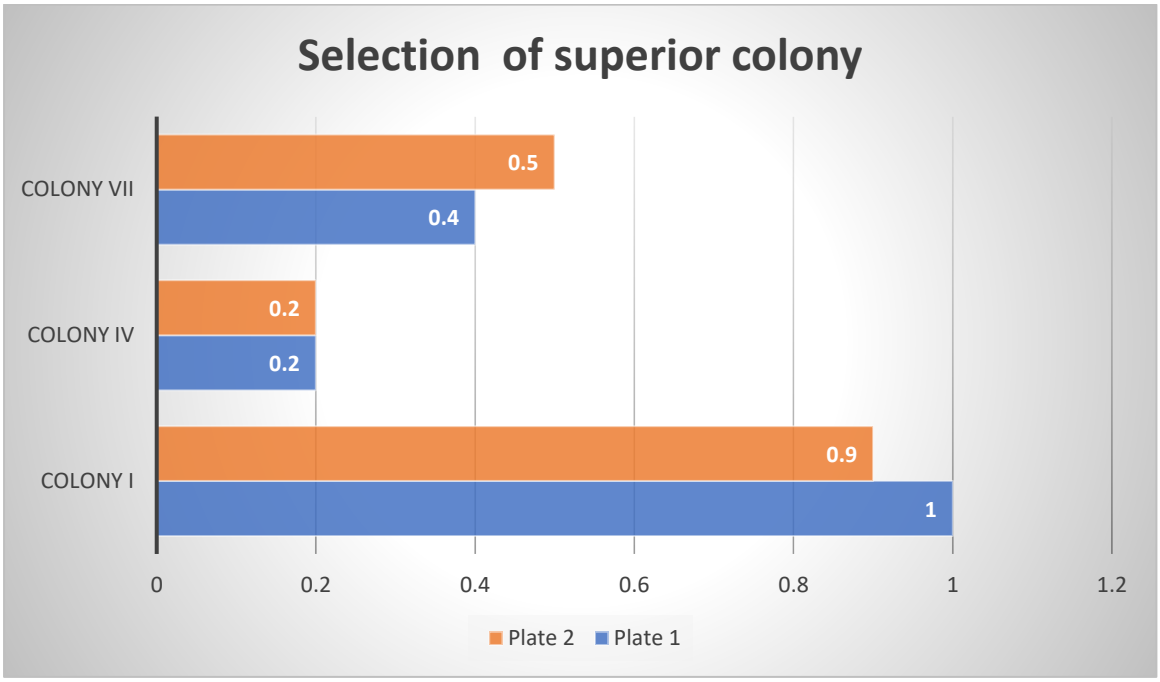
Table no. 5: Length of zones of Plate 1

Colony	Size of colony with zone (cm)	Size of colony without zone (cm)	Size of zone (cm)
Colony I	2 cm	1 cm	1 cm
Colony IV	3.6 cm	3.4 cm	0.2cm
Colony VII	1.2 cm	0.8 cm	0.4 cm

Table no.6: Length of zones of plate 2

Colony	Size of colony with zone (cm)	Size of colony without zone (cm)	Size of zone (cm)
Colony I	2.3 cm	1.4 cm	0.9 cm
Colony IV	3.4 cm	3.2 cm	0.2 cm
Colony VII	1.2 cm	0.7 cm	0.5cm

Graph no.1: Selection of superior colony



From analysing the graph colony I was identified as the superior colony and was chosen for further studies.

5. QUANTIFICATION

5.2. TYROSINE STANDARD CURVE -PROTEASE ASSAY

The enzyme production was quantified using Tyrosine standard curve protease assay. The rate of absorbance was noted for Tyrosine solution, taken as Blank and for the test solution which was the culture broth. (Fig no. 14)

Figure no.14: Enzyme Assay

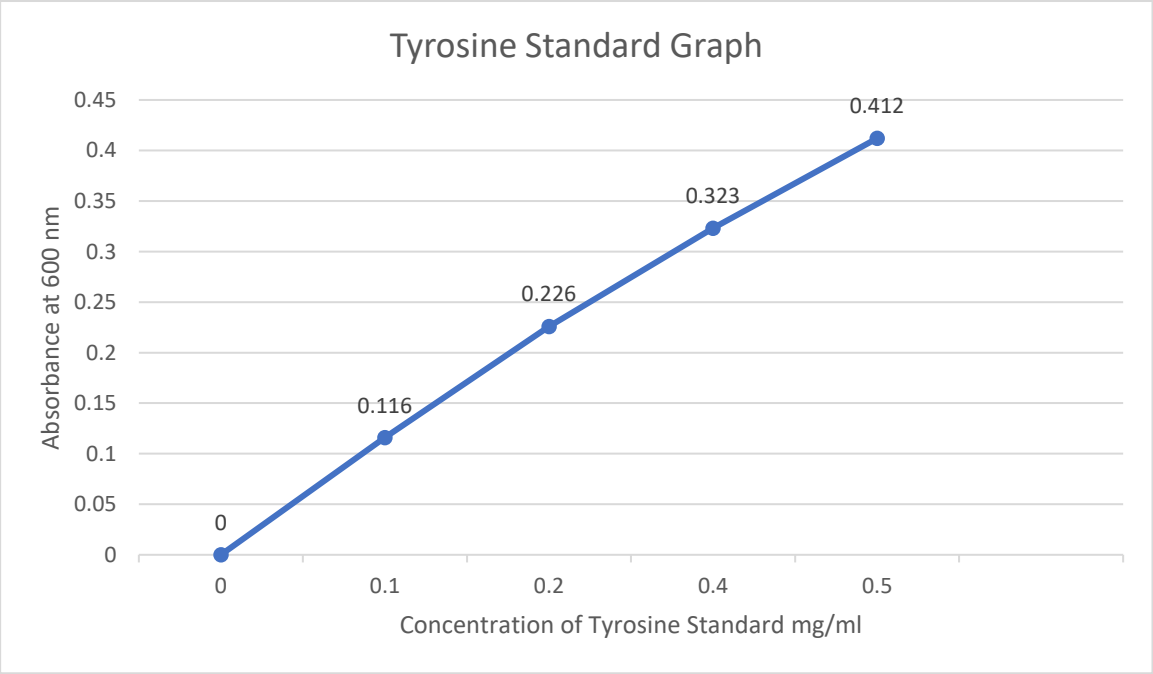
Table No 7: Tyrosine standard curve

Volume of Tyrosine	Absorbance (at 660 nm)
0.05	0.0531
0.10	0.116
0.20	0.226
0.40	0.323
0.50	0.412

Absorbance of supernatant: 0.379

Absorbance of purified enzyme: 0.331

Graph no.2: Tyrosine Standard Graph



Equation:- Y= 1.039x+0.0065

Interpretation: The activity of enzyme in purified sample and supernatant was measured by tyrosine standard curve and it was found as **0.17204 U/ml/min** and **0.197395U/mL/min** respectively.

Calculation

From graph, Y=mx+c = 1.039x+0.006

In the case of supernatant;

x=y-c/m=> 0.379-0.006/1.039= 0.3589 (U mole of tyrosine equivalents released)

In the case of purified enzyme;

x=y-c/m =>0.331-0.006/1.039 =0.3128 (U mole of tyrosine equivalents released)

Unit/ml enzyme =U mole of tyrosine equivalents released *(11)/ {(1) * (10) *(2)}

Where, the vales represent the following:

11- Total volume of Assay in millilitre.

1 - Volume of enzyme used in millilitres.

10 - Time of assay (here in minutes) as per the units taken.

2- Volume of enzyme used in colorimetric observation.

By substituting the values in the equation:

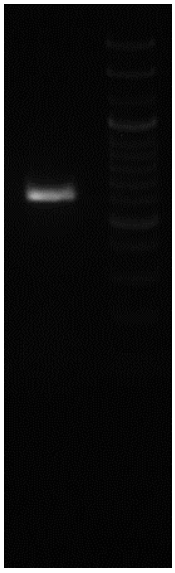
In the case of supernatant;0.3589*(11)/{(1) *(10) *(2)}= **0.197395U/mL/min**

In the case of purified sample 0.3128*(11)/ {(1) * (10) * (2)}= **0.17204 U/ mL /min**

6. MOLECULAR IDENTIFICATION

6.2. AGAROSE GEL ELECTROPHOROSIS

0.8% Agarose gel electrophoresis was conducted on the DNA molecules after which the molecules got separated based on their molecular weight.(Figure no. 15)



Lane 1 : DNA

Lane 2 : Ladder

Figure no: 15 : Agarose gel Electrophoresis of Isolated DNA

6.3. POLYMERASE CHAIN REACTION

The DNA fragment obtained after agarose gel electrophoresis was amplified using specific primers and PCR Copies were loaded on 1.2% Agarose gel electrophoresis and bands were observed under gel documentation. (figure no. 16)

Figure no. 16: Agarose gel Electrophoresis of PCR Amplified DNA

6.4. DNA SEQUENCING AND BLAST

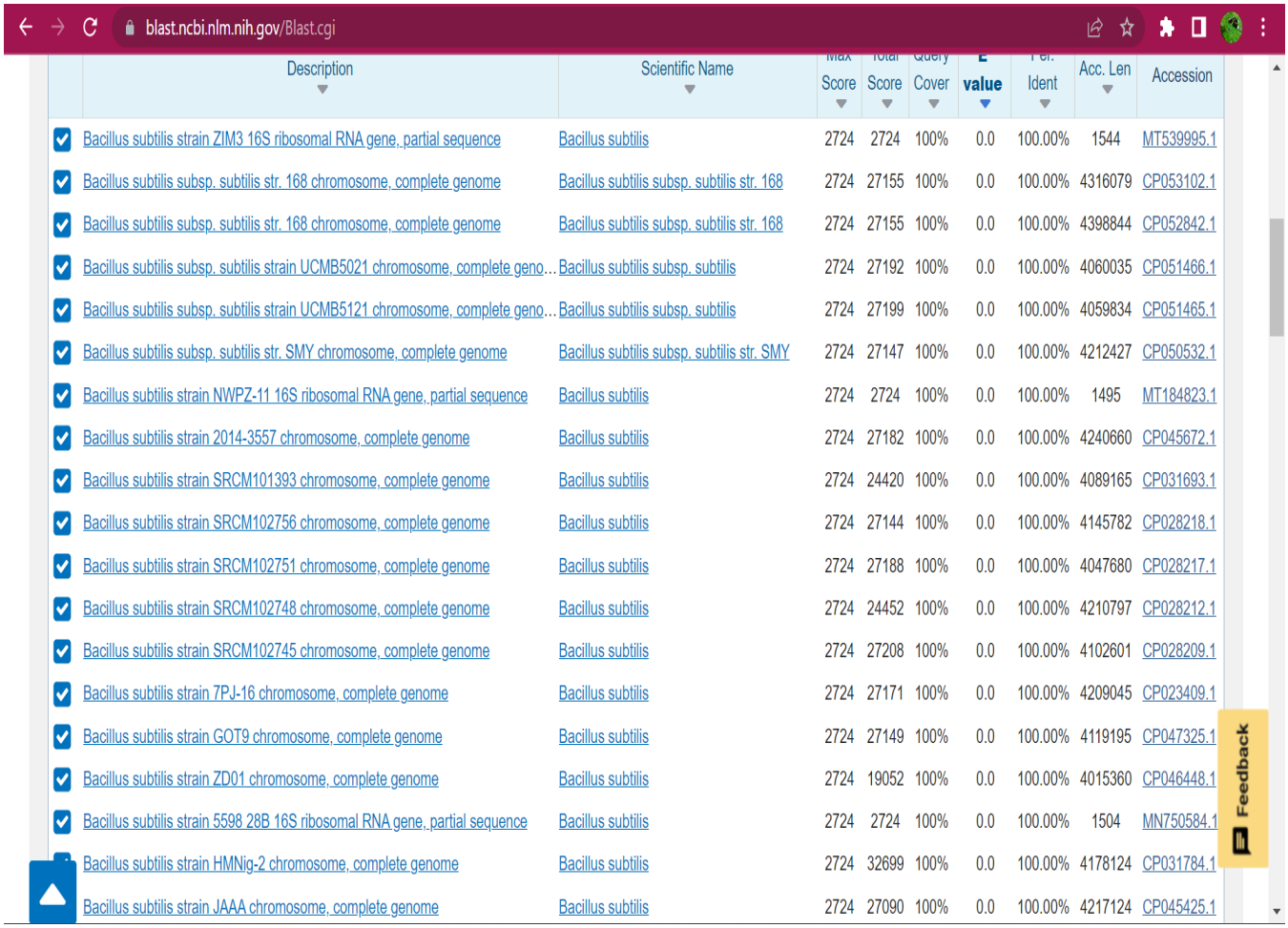
Nucleotide sequence determination PCR products of the 16 s rDNA of strains were analysed for nucleotide sequence. The homology of partial sequence obtained were compared with the sequences of the DNA databases and similarity showing above 95 % were retrieved by nucleotide Basic Local Alignment Search Tool (BLAST) program at the national centre for Biotechnology information (NCBI) BLAST server (www.ncbi.nlm.nih.gov/BLAST). Sequences obtained were compared against the sequences available in NCBI, database using BLAST (Figure no. 18). The result obtained was found to be *Bacillus subtilis.* The results of *Bacillus subtilis* sequence described in figure (17) below.

TGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGAC
AGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGG

ATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACT
TACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAA
GGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA
CGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTA
AAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTG
ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGG
CGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATT
GGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAG
CGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCT
GGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTT
CCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG
GTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACAT
CCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC
CCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCG
AAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAG
TCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCAT
GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGA
GAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCG
AAGGTGGGACAGATGATTG

Figure no. 17 : 16s r DNA sequence

6.5. BLAST



Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus subtilis strain ZIM3 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	2724	2724	100%	0.0	100.00%	1544	MT539995.1
Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome	Bacillus subtilis subsp. subtilis str. 168	2724	27155	100%	0.0	100.00%	4316079	CP053102.1
Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome	Bacillus subtilis subsp. subtilis str. 168	2724	27155	100%	0.0	100.00%	4398844	CP052842.1
Bacillus subtilis subsp. subtilis strain UCMB5021 chromosome, complete geno...	Bacillus subtilis subsp. subtilis	2724	27192	100%	0.0	100.00%	4060035	CP051466.1
Bacillus subtilis subsp. subtilis strain UCMB5121 chromosome, complete geno...	Bacillus subtilis subsp. subtilis	2724	27199	100%	0.0	100.00%	4059834	CP051465.1
Bacillus subtilis subsp. subtilis str. SMY chromosome, complete genome	Bacillus subtilis subsp. subtilis str. SMY	2724	27147	100%	0.0	100.00%	4212427	CP050532.1
Bacillus subtilis strain NWPZ-11 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	2724	2724	100%	0.0	100.00%	1495	MT184823.1
Bacillus subtilis strain 2014-3557 chromosome, complete genome	Bacillus subtilis	2724	27182	100%	0.0	100.00%	4240660	CP045672.1
Bacillus subtilis strain SRCM101393 chromosome, complete genome	Bacillus subtilis	2724	24420	100%	0.0	100.00%	4089165	CP031693.1
Bacillus subtilis strain SRCM102756 chromosome, complete genome	Bacillus subtilis	2724	27144	100%	0.0	100.00%	4145782	CP028218.1
Bacillus subtilis strain SRCM102751 chromosome, complete genome	Bacillus subtilis	2724	27188	100%	0.0	100.00%	4047680	CP028217.1
Bacillus subtilis strain SRCM102748 chromosome, complete genome	Bacillus subtilis	2724	24452	100%	0.0	100.00%	4210797	CP028212.1
Bacillus subtilis strain SRCM102745 chromosome, complete genome	Bacillus subtilis	2724	27208	100%	0.0	100.00%	4102601	CP028209.1
Bacillus subtilis strain 7PJ-16 chromosome, complete genome	Bacillus subtilis	2724	27171	100%	0.0	100.00%	4209045	CP023409.1
Bacillus subtilis strain GOT9 chromosome, complete genome	Bacillus subtilis	2724	27149	100%	0.0	100.00%	4119195	CP047325.1
Bacillus subtilis strain ZD01 chromosome, complete genome	Bacillus subtilis	2724	19052	100%	0.0	100.00%	4015360	CP046448.1
Bacillus subtilis strain 5598 28B 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	2724	2724	100%	0.0	100.00%	1504	MN750584.1
Bacillus subtilis strain HMNig-2 chromosome, complete genome	Bacillus subtilis	2724	32699	100%	0.0	100.00%	4178124	CP031784.1
Bacillus subtilis strain JAAA chromosome, complete genome	Bacillus subtilis	2724	27090	100%	0.0	100.00%	4217124	CP045425.1

Figure no. 18 : BLAST Results

6.6. Molecular Evolutionary Genetics Analysis (MEGA)

Other works on isolation of *Bacillus subtilis* from plants were identified and their DNA sequences were taken from BLAST. Using Molecular Evolutionary Genetic Analysis (MEGA) 11 software, the sequences were aligned and a phylogenetic tree was created in order to analyse the evolutionary relationship between the different organisms isolated (Figure 19). The plants used for MEGA analysis were:

1. Mulberry
2. Wheat
3. Arabidopsis and Brassica
4. *Teucrium polium*

Bacillus subtilis isolated from tomato showed maximum similarity with that isolated from wheat endophyte bacteria.

Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model [1]. The tree with the highest log likelihood (-350.06) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 5 nucleotide sequences. There were a total of 107 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2].

1. Tamura K. and Nei M. (**1993**). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**:512-526.
2. Tamura K., Stecher G., and Kumar S. (**2021**). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution* https://doi.org/10.1093/molbev/msab120.

MEGA

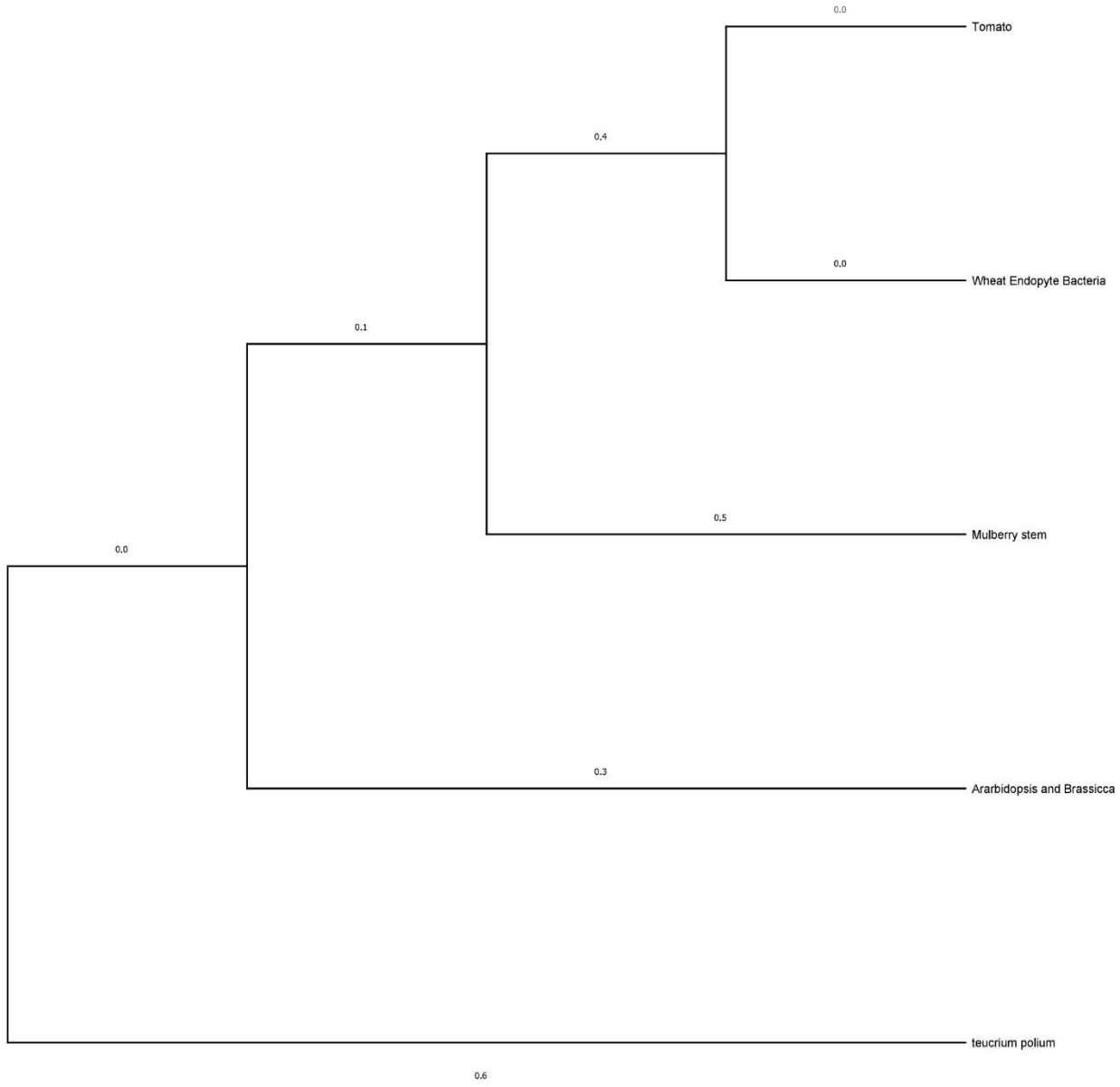


Figure no. 19: Phylogenetic Tree

VI. DISCUSSIONS

Protease producing organisms are generally isolated from soil and most of the work is focused on alkaline protease. Therefore, the present study deals with the isolation of protease producing bacteria from tomato collected from local market. Members of the genus *Bacillus* produce a large variety of extracellular enzymes of which protease are of particularly significant industrial importance. A major commercial use is the addition of microbial protease to domestic detergents for the digestion of proteinaceous stains of fabrics (Sharma *etal*.,1980).

Diksha.k *et al.*,(2021) studied on serratiopeptidase, a proteolytic enzyme produced by a non pathogenic endobacterium named *Serratia marcescens.* Serratiopeptidase is widely used in pharmaceutical industry as an anti-inflammatory drug. The bacteria was isolated from the mulberry phyllosphere which is the main source of serratiopeptidases in silkworms. These peptidases secreted by the bacteria help the silkworm to dissolve the cocoon aiding in the emergence of the moth. In the present study we have isolated the protease producing bacteria from raw tomato. The presence of the bacteria in the phyllosphere of tomato can also be further studied in the future which can help on the formulation of an efficient method for the production of proteases from the parts that are otherwise not consumed. The study on the nature of the protease can also be studied for analysing their pharmaceutical relevance.

J. Sai Prasad *et al.*,(2020) conducted a research to optimize isolation processes and culturing of the seed endophytic bacteria from wheat seed endosperm. Seeds of popular wheat genotype of *Tritiicum aestivum* were taken for sourcing the isolation of seed bacterial endophytes. Seeds of same genotype were pooled to make a composite sample, from which 15 healthy seeds were used on each media for after processing. The isolation technique for analysis of the endophytic bacteria included many steps like serial dilution, NA plating etc which were also conducted in the present study. They isolated 18 proteolytic bacteria from the wheat endosperm while in the current study 3 proteolytic bacteria were isolated from the fruit of tomato. The current study can be expanded in order to inspect the presence of proteolytic bacteria in the seeds of tomato. This will be helpful for the utilisation of non-viable seeds in a more productive manner.

Oluwaseyi S.O *et al.*,(2021) examined the genome of *Bacillus subtilis* isolated from Maize Rhizosphere, which are bacterial species rich in plant growth promoting genetic elements. Genetic elements reported include those of auxin, nitrogen fixation, siderophore production, iron acquisition, volatile organic compounds, and antibiotics. Furthermore, the presence of

phages and antibiotic-resistant genes in the genomes are reported. This study brings an insight into the genetic components of the plant growth-promoting abilities of the isolated strains of *Bacillus subtilis* strains and shows their potential biotechnological applications in agriculture and other relevant sectors. Similarly studies can be conducted on the *Bacillus* Species isolated from tomato which can be further used to produce genetically altered crops modified with the help of the enzymes it produce.

W.J.Sul *et al.*,(2020) studied on the impact of various strains of *Bacillus subtilis* on the promotion of plant growth on selected plants. Here from about 149 bacteria analysed, the strain GOT9 was found to have enhanced the drought and salt stress in *Arabidopsis*. Its application resulted in increases in the transcripts of various drought stress- and salt stress-inducible genes in the absence or presence of the stresses. It also increased the lateral root growth and development in *Arabidopsis*. It also improved the salt stress tolerance of *Brassica*, a closely associated specie of *Arabidopsis.* The strain of *Bacillus* isolated from tomato in the present study can also be tested for their Plant growth-promoting rhizobacteria (PGPR) activity, which can help in the production of more efficient bio-fertilizer for plant growth.

Xie. J *et al.*,(2019) Isolated *Bacillus subtilis* an Endophytic bacterium from the stem of mulberry plant. The isolated strain was showing strong anti-fungal properties which was the core reason for the inhibition of Mulberry Sclerotinioise by *Bacillus subtilis*. Similar studies on antifungal properties of Bacillus proteases isolated from tomato can be conducted, and this could be formulated into new antifungal agents and disinfectants of organic origin. The genetic sequence of the Bacillus subtilis isolated from Mulberry and Tomato were aligned in the MEGA software and their phylogenetic analysis revealed that the bacterias were closely associated. Thus there is a greater probability for the plant isolates to show similar properties.

S.E.D.Hassan (2017) isolated endophytic bacteria and fungi from *Teucrium polium* and analyzed the plant growth promoting endophytic characters of the bacteria. Several strains of *Bacillus subtilis* were present among the identified bacteria that showed strong antimicrobial and enzymatic activities along with the production of Indole Acetic Acid (IAA) and ammonia. They also exhibited variable capacity for phosphate solubilization. The action of various proteases can be further studied for their utilisation in fertilizer and agricultural industries.

Furthermore, currently the researches on proteases from *Bacillus subtilis* is entirely focused on alkaline soil source. This has a major impact on the biodiversity sustainability as other

microorganisms in the soil may also get affected by the collection of soil for detergent industries.

The presence and activity of microbial proteases in rotten tomato are to be further analysed. Their presence can help in the utilization of the rotten tomatoes for a better purpose which can be further flourished for the reduction of risk for the farmers and shop owners.

VII. CONCLUSION AND SUMMARY

Bacterial alkaline proteases are important participants of biofertilizer industry. Investigations are ongoing for the invention of new sources of alkaline protease. Any experiment which is related to alkaline protease production gains importance in the scientific world. Existing knowledge on bacterial alkaline protease is increasing its depth day by day due to the innovative findings on the same. In the present study tomato collected from local market showed presence of protease producers. The sample was serially diluted to reduce the microbial load and it was spread plated in Nutrient Agar. After incubation the plate spread with 10^{-3} and 10^{-2} dilution gave several colonies of bacteria from which about 8 bacterial colonies were isolated and plated on Skim Milk Agar for screening. Gram staining was performed on the 3 colonies of which 2 colonies were gram positive and 1 gram negative. A selection based on amount of clear zone production was conducted in which one of the 3 colonies was selected for further study by analysing a graph. Quantification of protease production was conducted using Tyrosine standard curve – protease assay. The amount of protease produced by the isolated bacteria was **0.197395U/mL/min**. Genomic DNA was isolated and gel electrophoresis was carried out to separate DNA fragments. To produce multiple copies of DNA PCR was carried out. The sequence of DNA obtained from DNA sequencing was analysed for similarity using BLAST. The protease producing organism was identified as ***Bacillus subtilis***. Similar works, in which protease producing organisms isolated from plants were identified and phylogenetic analysis were conducted using MEGA. *Bacillus subtilis* isolated from tomato showed maximum similarity with that isolated from wheat. Researchers have found that *Bacillus subtilis* showed plant growth promoting properties which has also been identified as a function of proteases. Because of this property, various strains of *Bacillus subtilis* are highly used in biofertilizer industries. Protease produced by *Bacillus subtilis* are also used in the detergent industry due to its cleansing action. So, these findings on alkaline protease producing *Bacillus subtilis* are definitely helpful for the industrial purposes and it definitely show its own importance in the scientific world. Thus, the isolated strain could be used as a promising agent for protease production and making novel enzyme-based detergents and fertilizers. The use of microorganisms to produce enzymes has a number of technical and economic advantages and in recent years has become the predominant mode of enzyme production. However, the cost of producing this enzyme is high and the cost of procurement by developing countries can be even higher, hence present potential of *Bacillus sp.* could be an alternative for industrial and commercial use.

VIII. BIBLIOGRAPHY

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