



## ISOLATION AND MOLECULAR CHARACTERIZATION OF PROTEOLYTIC AND LIPOLYTIC BACTERIAL ISOLATES FROM THE MARKET VEGETABLE WASTES

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### ABSTRACT

Microbial enzymes are diversified in their properties and substrate specificity, which make them attractive biotechnological tools for various industrial applications. These enzymes are widely derived from plants, animals because they are more stable than their corresponding plant and animal enzymes, their production is more convenient and safer. The aims of this study were to isolate and characterize proteolytic, lipolytic microorganisms from vegetable market waste tanks through morphological, biochemical tests and to confirm the bacterial species through 16S rRNA gene sequencing. The isolation of microorganisms is done by standard plate assay method. The molecular phylogeny of isolated strains shows similarity with *Bacillus* species. The first strain isolate showed sequence identity with *Bacillus pumilus* and the second isolate showed sequence similarity with *Bacillus altitudinis*. The isolates have good proteolytic, lipolytic activity and might have the potential application in several industrial processes. The production of protease and lipase as a metabolite indicates the potential of these isolated cultures for application in large scale production and recovery of proteins and lipids.

**Keywords:** Microbes, Vegetables, Proteolytic bacteria, Lipolytic enzymes, 16s rRNA, DNA Sequencing.

## INTRODUCTION

The biosphere is occupied by a wide variety of microorganisms that perform important functions like global primary energy; element cycling and together form the largest part of living organisms in earth's surface [1]. Microorganisms were found everywhere in the nature. They can be observed in association with plants, animals, deepest ocean sediments, at high atmospheric pressure, cold temperatures and also in highly polluted environments [2]. Microbes break down a variety of carbon, other energy sources and convert them into amino acids, vitamins, carbohydrates and fatty acids by producing specific enzymes [3].

The majority of industrial enzyme market is occupied by hydrolytic enzymes, such as proteases, esterases lipases and amylases [4]. Proteases, amylases have dominated the world market due to their hydrolytic reactions for proteins and carbohydrates [5]. Likewise, lipases are the most important biocatalysts carrying out novel reactions in aqueous and non- aqueous media. This is chiefly due to their ability to utilize a wide spectrum of substrates, high stability towards extreme temperature, pH, organic solvents, and enantioselectivity [6]. Most of the commercially available proteases and lipases are alkaline and biosynthesized mainly by bacteria such as *Bacillus*, *Pseudomonas*, *Clostridium* species and some fungi were also reported to produce these enzymes which are used for several industrial purposes [7]. Bacterial enzymes are preferred over fungal enzymes because of higher activities, neutral or alkaline pH optima, short generation time, simple nutritional needs and easy screening procedures for the desired properties [8]. A variety of proteases and lipases are produced from both Gram-positive and Gram-negative microorganisms. Most of bacterial species were studied for protease, lipase production because they are non-pathogenic, easily cultivable and used for biotechnological applications [9].

Proteases are the enzymes that have been categorized on the basis of their specificity of the peptide bonds they attack [10]. There are four major classes of proteases designated by the principle functional group in their active site as serine, thiol, carboxyl and metallo. Proteases are also classified by their hydrolyzing mechanism such as exoproteinases or endoproteinases [11]. The biochemical diversity of microorganisms significantly contributes in expanding the protease applications and their market value *Bacillus subtilis* and *Bacillus licheniformis* are the major and highly exploited neutralophilic organisms for alkaline protease production [12]. They possess numerous applications in industrial process including food, pharmaceutical, detergents, peptide synthesis, tanning industry and production of useful biomass from waste such as horn, hair and feathers [13].

Lipases are ubiquitous enzymes produced by all biological systems such as animals, plants and microorganisms. In contrast to animal and plant lipases, extracellular microbial lipases can be produced relatively inexpensively by fermentation and in large quantities [14]. With the rapid growth of enzyme technology, many new potential biotechnological applications for lipases have been identified in the areas of food, pharmaceuticals, cosmetics, detergents, paper manufacturing and biosurfactant synthesis [15]. Microbial lipases are more stable than the plant and animal derivatives and their production is easier, safer for research and industrial applications [16].

Isolation, screening and characterization of lipase-producing microbes have been performed from the samples collected from diverse habitats such as industrial wastes, vegetables wastes, vegetable oil processing factories, marine water habitats, soils which are contaminated with oil and oil spills. The application of ribosomal RNA sequence analyses for the identification of microbial community and its composition offers new perspectives on the conventional phenotypic classification system. Differences in nucleotide sequences of 16S rRNA gene facilitate not only a complete understanding of microbial phylogeny but also the identification of bacteria from unknown samples. The present work was carried out to study the microbial genomic diversity of cultivable proteolytic and lipolytic organisms and 16S rRNA gene sequencing to characterize the microbial communities which are isolated from vegetable market waste tanks.

## MATERIALS AND METHODS

### Sample Collection:

The study samples were collected in sterile containers from the market wastes tanks at Maatuthavani Central market, Madurai, Tamil Nadu, India. The collected samples were labeled and covered with aluminum foil to avoid environmental contamination. They were transported to the microbiological laboratory with 4°C and used for further processes.

### Isolation of microorganisms:

The samples were taken and they were cleaned with sterile distilled water to wipe out the extra particles adhering to the vegetable surfaces. Then with the help of sterile mortar pestle the vegetables were grinded, made into a liquid solution and they are serially diluted and from  $10^{-4}$  to  $10^{-7}$  dilutions plated in LB Agar plates incubated at 37°C for 24-48 hours. The colonies were differentiated with the help of colony morphology and they were pure cultured and maintained. The total genomic DNA of bacterial isolates were isolated by HiPer® Bacterial Genomic DNA Extraction Kit using the manufacturer's protocol and confirmed with agarose gel electrophoresis.

### Biochemical characterization:

#### Salmonella Shigella Agar Test

Salmonella Shigella Agar are designated as selective media based upon the degree of inhibition of gram-positive microorganisms that they inhibit due to their content of bile salts, brilliant green and citrates. Differentiation of enteric organisms is achieved by the incorporation of lactose in the medium. Organisms that ferment lactose produce acid which, in the presence of the neutral red indicator, results in the formation of red colonies. Lactose nonfermenters form colorless colonies. The latter group contains the majority of the intestinal pathogens, including *Salmonella* and *Shigella*. The sodium thiosulfate and ferric citrate enable the detection of hydrogen sulfide production as evidenced by colonies with black centers.

### **Citrate Utilization Test**

Simmons Citrate Agar is used for the differentiation of gram negative bacteria on the basis of citrate utilization. Organisms able to utilize ammonium dihydrogen phosphate and sodium citrate as the sole sources of nitrogen and carbon, respectively, will grow on this medium and produce an alkaline reaction as evidenced by a change in the color of the bromthymol blue indicator from green (neutral) to blue (alkaline).

### **Mannitol Salt Agar Test**

Mannitol Salt Agar is a nutritive medium due to its content of peptones and beef extract, which supply essential growth factors, such as nitrogen, carbon, sulfur and trace nutrients. The 7.5% concentration of sodium chloride results in the partial or complete inhibition of bacterial organisms other than *staphylococci*. Mannitol fermentation, as indicated by a change in the phenol red indicator, aids in the differentiation of staphylococcal species.

### **Starch Hydrolysis**

Starch Agar is used for cultivating microbes being tested for starch hydrolysis. Beef extract provides the nitrogen, vitamins, carbon and amino acids in Starch Agar. Starch reacts with Gram Iodine to give a blue color. Organisms hydrolyzing starch through amylase production will produce a clearing around the isolate while the remaining medium is blue. Agar is the solidifying agent.

### **Lipid Hydrolysis**

Lipid hydrolysis test is used to differentiate bacteria based on their ability to produce exoenzyme lipase. Lipase will cleave the ester bonds in triglycerides to form a glycerol and fatty acid. If lipid is hydrolyzed in spirit blue agar plates, there will be a clear zone of lipolysis around the growth and a rancid odour smell.

### **Hydrogen Sulfide Test**

SIM Medium is used to differentiate enteric bacilli on the basis of sulfide production, indole formation and motility. Hydrogen sulfide production, indole formation and motility are distinguishing characteristics which aid in the identification of the *Enterobacteriaceae*, especially *Salmonella* and *Shigella*. SIM medium, therefore, is useful in the process of identification of enteric pathogens.

### **Triple Sugar - Iron Agar Test**

Triple Sugar Iron Agar (TSI Agar) is used for the differentiation of gram-negative enteric bacilli based on carbohydrate fermentation and the production of hydrogen sulfide. Carbohydrate fermentation is detected by the presence of gas and a visible color change (from red to yellow) of the pH indicator, phenol red. The production of hydrogen sulfide is indicated by the presence of a precipitate that blackens the medium in the butt of the tube.

### **Ceasin Hydrolysis Test**

Skim Milk is used for preparing microbiological culture media. Skim Milk Medium may be used for the cultivation and differentiation of microorganisms based on the coagulation and proteolysis of casein. Skim Milk is a source of lactose and casein and other nutrients required for the growth of lactobacilli. *Clostridial* species can be differentiated based on their ability to enzymatically degrade proteins to peptones (peptonization) or coagulate milk. It may be used to detect the stormy fermentation produced by *Clostridium perfringens*.

### **Gelatin Hydrolysis**

Nutrient Gelatin is used for the detection of gelatin liquefaction by microbial species. Gelatin liquefaction is one of the characteristics used in the classification of members of the Enterobacteriaceae and non-fermenting gram-negative bacteria. Nutrient Gelatin is used chiefly for identification of pure cultures of bacteria that are not particularly fastidious in regard to nutritional requirements. The peptone and beef extract supply sufficient nutrients for the growth of nonfastidious bacterial species. The gelatin is the substrate for the determination of the ability of an organism to produce gelatinases, which are proteolytic-like enzymes active in the liquefaction of gelatin.

### **Methyl Red and Voges Proskauer Test**

Some organisms produce acid from the metabolism of glucose in a sufficient quantity to produce a pH of 4.4 in the media. These acids are not further metabolized and are said to be stable acids. At a pH of 4.4 or less the pH indicator methyl red is a bright cherry red. The production of extracellular enzymes namely gelatinase, protease, alkaline, lipase phosphatase and chitinase was studied following the protocol described by Smibert and Krieg [17]. Those cultures that were positive for proteolytic and lipolytic were further characterized by molecular techniques.

### **16s rRNA gene amplification:**

Using the primers 27F5'- AGAGTTTGATCCTGGCTCAG - 3' and 1492 R5' -GGTTACCTTGTTACGACTT-3', [18] synthesized from Sigma Genosys, India, the 16S rRNA gene was amplified with 30 µL reaction mixture containing 1X reaction buffer (10 mM Tris [pH 8.3], 50 mM KCl, 1.5 mM MgCl<sub>2</sub>), 200 mM dNTPs, 0.5 U Taq DNA polymerase enzyme (Sigma, USA), 2.5 µL each primer and 20 ng template DNA. The PCR composition for 16s rRNA gene amplification has been shown in Table-1. The thermal cycling conditions were: 5 mins at 94°C for initial denaturation; 31 cycles of 30s at 95°C, 1 min 30s at 54°C, 2 mins at 72°C, and a final extension for 5 min at 72°C. The amplification reaction was performed with a thermal cycler (MyCycler, Bio-Rad, USA) and the PCR amplicons were resolved by electrophoresis in 1% (w/v) agarose gel to confirm the expected size of the product.

PCR Components	Composition
Buffer	5.0 µl
dNTPs	2.0 µl
Template DNA	2.0 µl
Reverse primer	2.5 µl
Forward primer	2.5 µl
Taq DNA polymerase	1.0 µl
Water	15.0µl

**Table 1:** PCR composition for 16s rRNA gene amplification

### PCR product purification:

The PCR product was purified by Himedia PCR product purification kit according to their kit instruction. The purified PCR products were visualized on 1.2% agarose gel under Gel Documentation System (GeNei TMGel documentation). Finally, the purified amplicons were confirmed using automated DNA Sequencer (Applied Biosystems 377 XL, Foster City, USA).

### Phylogenetic analysis of microorganisms:

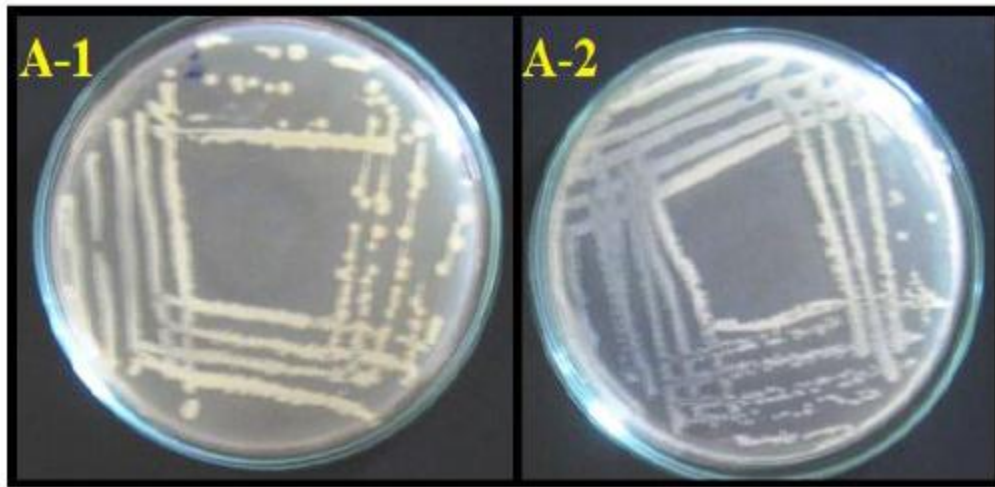
The 16S rRNA gene was sequenced by Applied Biosystems 3130xl Genetic Analyzer. The sequences were analyzed and phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA, V.7) software and the sequences were matched using NCBI-BLAST [19] tools. Then for predicting the structure of protein structure prediction suite (PS)<sup>2</sup> was used for both the protein sequences derived from the submitted nucleotides.

## RESULTS

### Analysis of pure-culture microorganisms:

Microorganisms were isolated by spread plating technique in the Luria-Bertani (LB) Agar medium. After 12 hours incubation the colonies appeared as translucent whitish yellow color. The colonies were observed under Olympus Research Binocular Microscope (Model: CX-21i) to determine their morphological variations. They showed different morphological characteristics and were stored in glycerol stock at -20°C for further use. Two different bacterial cultures were pure cultured and used in this present study. Taxonomical studies on the pure-cultured isolates showed that the isolates were gram (positive and negative), rod-shaped and were aerobic bacterium. The bacterial cultures were named as A1-A2 and their growth characteristics

were shown in figure 1.



Bacterial colonies on a petri dish (agar plate) isolated on Luria-Bertani (LB) Agar medium.

**Figure 1: Bacterial strains isolated from vegetable samples after pure culture**

### **Biochemical characterization:**

The biochemical and metabolic characteristics of pure-cultured isolates were summarized in Table 2. The A1 isolate was capable of producing catalase, oxidase, lipase and protease. From these results, this isolate was classified into the genus of *Bacillus* according to Bergey's manual lipase-producing microorganisms. The A2 isolate was capable of producing catalase, oxidase, amylase, protease and gelatinase. From these results, this isolate was classified into the genus of *Bacillus* according to Bergey's manual protease-producing microorganisms.

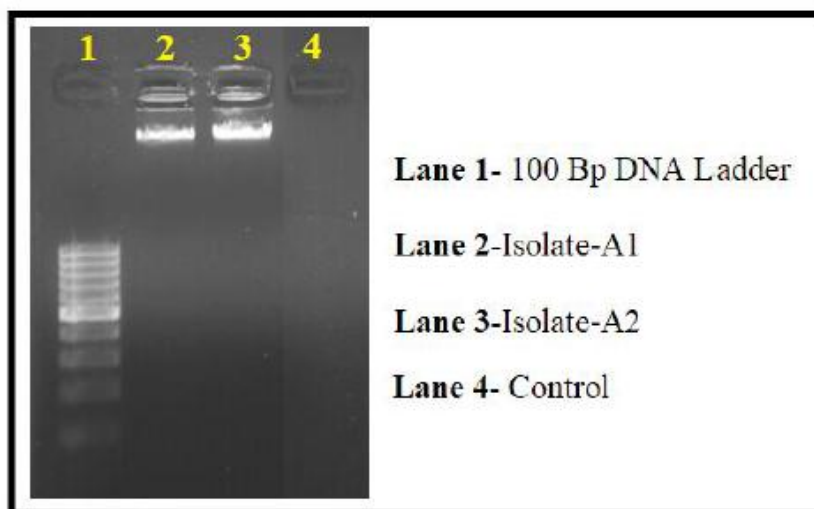
Bio-chemical Characteristics	Isolate -A1	Isolate -A2
Indole production	-	-
Voges-Proskauer	+	-
Citrate utilization	-	-
Catalase	+	+
Oxidase	+	+
Amylase	-	+
Protease	+	+
Lipase	+	-
Gelatinase	-	+
Chitinase	-	-
Phosphatase	-	-

+ -Positive, -Negative

**Table 2:** Biochemical characteristics of the pure-cultured isolates

**Genomic DNA isolation and 16S rRNA bacterial typing of the isolates by PCR:**

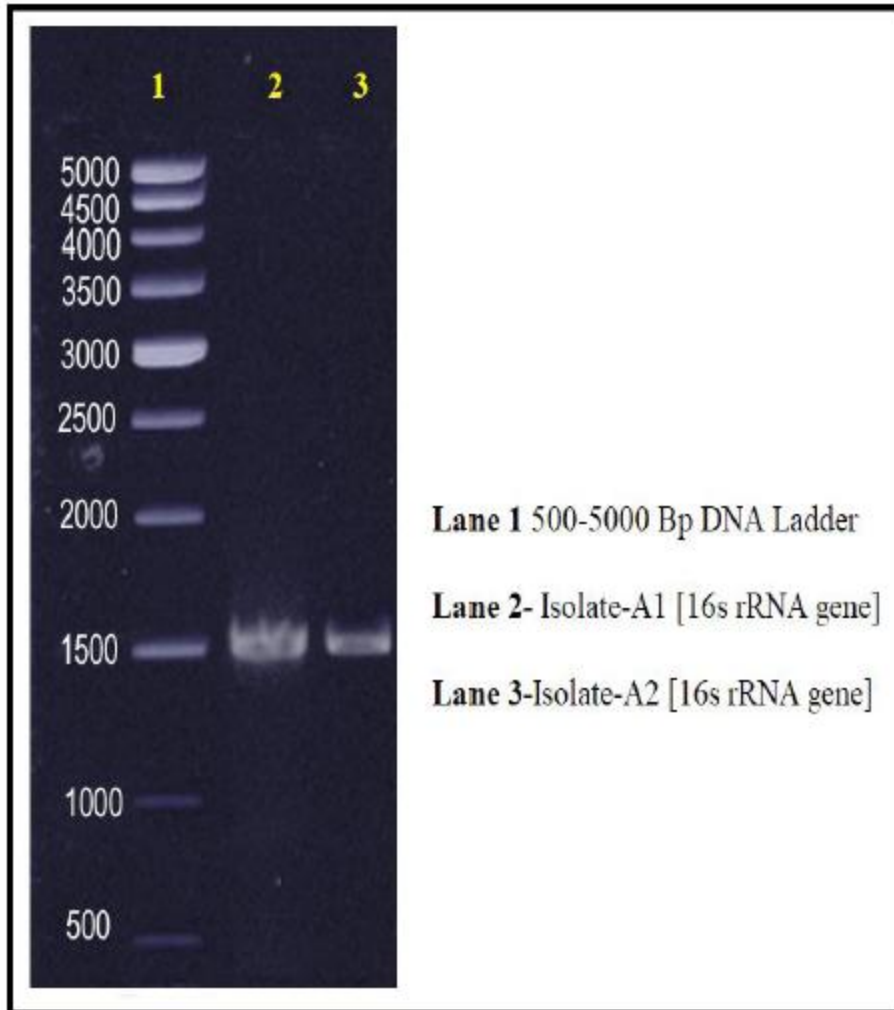
The genomic DNA of bacterial isolates were isolated and analyzed by agarose gel electrophoresis (fig 2). The total genomic DNA of the bacterial isolates (A1 and A2) was taken as the template and prokaryotic 16S rRNA specific primers showed good amplification. The PCR amplicon is electrophoresed in 1.2% agarose gel with a 500 bp DNA marker (Himedia). The molecular weight of PCR amplified product was approximately 1500 bp. The gel photograph of the PCR amplified product has been shown in figure 3.



Lane 1, 2, 3 and 4 denotes 100 Bp DNA ladder, genomic DNA of isolates A1, A2 and no template control (NTC).

**Figure 2:** Genomic DNA of bacteria isolated from vegetable samples





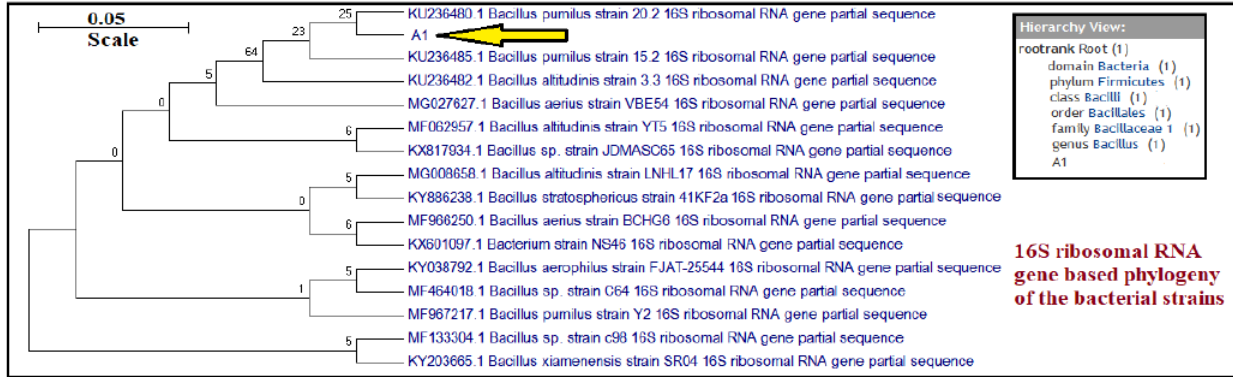
Lane 1 denotes the DNA molecular weight marker. Lane 2-3 denotes the 16S rRNA gene of isolate A1 to A2.

**Figure 3:** Agarose gel electrophoresis of PCR 16S rRNA amplified products from cultured microbes

### **BLAST analysis of 16S rRNA gene of isolates A1 and A2:**

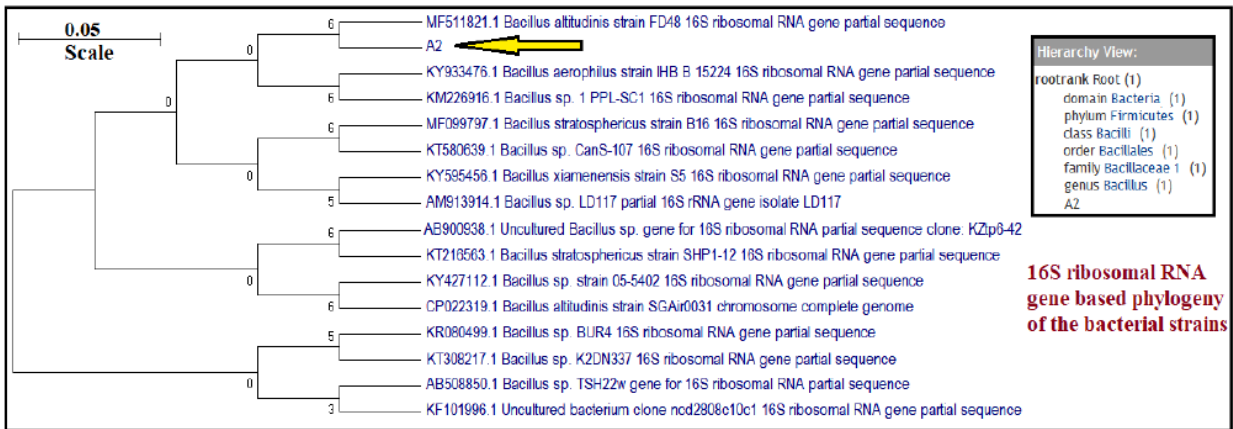
The characterization of A1 isolated from different vegetable sample sources (Table 2) exhibited uniform phenotypic properties. Further, the physiological tests revealed the production of detectable enzymes such as lipases, proteases but not amylase, phosphatase, gelatinase and chitinase. The nucleotide sequences of the amplicons were saved as FASTA files and run with NCBI nucleotide database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and the sequences with a highly significant E-value score (0.00) were retrieved. The result displayed shows the query gene sequence of A1 isolate has 99% sequence identity to the available 16S rRNA gene sequences of *Bacillus pumilus*. Similarly, the Characterization of A2 isolated from different vegetable sample sources (Table 2) exhibited uniform phenotypic properties. Further, physiological tests revealed the production of detectable enzymes such as proteases, amylase, gelatinase but not lipases,

phosphatase and chitinase. The isolate A2 gene sequence has 99% sequence identity to the available 16S rRNA gene sequences of *Bacillus altitudinis*. For predicting the phylogenetic position of isolated strains, the phylogenetic tree view of isolates with their closely related type and non-type strains were analyzed using RDP and MEGA software tools. The analyzed nucleotide sequences of 16S rRNA isolates were deposited (Accession numbers: MF678618 and MF678619) in NCBI Genbank [https://www.ncbi.nlm.nih.gov/genbank/] database. The resulting phylogenetic tree was depicted in figure 4 for A1 isolate and fig 5 for A2 isolate respectively.



**Figure 4:** Phylogenetic tree view (Neighbor Joining method) of isolates A1

The phylogenetic tree was constructed using the Neighbour-Joining method while the distances were computed using the Jukes-Cantor method. The scale bar represents estimated sequence divergence.



**Figure 5:** Phylogenetic tree view (Neighbor Joining method) of isolates A2

The phylogenetic tree was constructed using the Neighbour-Joining method while the distances were computed using the Jukes-Cantor method. The scale bar represents estimated sequence divergence.

## DISCUSSION

The genus *Bacillus* contains numerous industrially important species and approximately half of the present commercially produced of bulk enzymes derives from strain of *Bacillus* [20]. Different species of the *Bacillus* (Firmicutes) are known to secrete anti-microbial substances; hence, the existence of species belonging to *Bacillus* inhibited the growth of bacteria from other class [21]. Recently, emphasis has been laid on extremophiles for their potential use in the production of extremozymes which can be function optimally at extreme alkaline pH values and high temperatures [22]. The enormous interest in proteases and lipases is reflected by a rapidly growing number of review articles and monographs covering molecular biology, biochemical characterization, three-dimensional structures and biotechnological applications of proteases and lipases from prokaryotic and eukaryotic origins [23].

Among the several proteases and lipases described in literature, only the enzymes belonging to few species have been demonstrated to have high stability and biosynthetic capabilities to allow routine use in organic reactions and therefore they might be considered as industrially relevant enzymes [24]. This study was carried out to identify the microbial genomic diversity of cultivable proteolytic and lipolytic organisms and 16S rRNA gene sequencing to characterize the microbial communities which are isolated from vegetable market waste tanks. The bacterial cultures were named as A1-A2 and their growth characteristics were studied in detail. The results displayed shows the query gene sequence of A1 isolate has 99% sequence identity to the available 16S rRNA gene sequences of *Bacillus pumilus*. The isolate A2 gene sequence has 99% sequence identity to the available 16S rRNA gene sequences of *Bacillus altitudinis*. Previous report documents that, most of the commercially available proteases and lipases are alkaline and biosynthesized mainly by bacteria such as *Bacillus*, *Pseudomonas*, *Clostridium* species and some fungi were also reported to produce these enzymes which are used for several industrial purposes [25].

Proteases are ubiquitous in occurrence, found in all living organisms and are essential for cell growth, development and differentiation. These extracellular proteases have high commercial value and find multiple applications in various industrial sectors [26]. Several *Bacillus* species, such as *Bacillus licheniformis*, *B.firmus*, *B.alcalophilus*, *B.amyloliquefaciens*, *B.proteolyticus*, *B.subtilis*, *B.thuringiensis*, *B.cereus*, *B. sterothermophilus*, *B. mojavensis* and *B. megaterium* are previously documented to produce proteases. These are the most important group of industrial enzymes and account for nearly 60% of the total enzyme that are commercially produced in large scale biotechnological companies [27]. Proteases are important components in biopharmaceutical products such as contact-lens enzyme cleaners and enzymatic deriders [28]. These proteolytic enzymes offer a selective debridement, supporting the natural healing process in successful local management of skin ulcerations by efficient removal of the necrotic material [28]. Alkaline serine proteases of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in food industries, medicinal formulations and resolution of amino acid mixtures [29].

Lipases are produced by all biological systems including animals, plants and microbial communities.

In comparing with animal and plant lipases, microbial lipases are interesting and can be produced relatively inexpensively by fermentation process in large quantities [30]. The bacterial lipolytic enzymes (e.g. lipases and esterases) were classified by Jaeger & Arpigny based on comparison of their amino acid sequences and by basic biological properties in the year 1999. As a result, bacterial lipolytic enzymes were classified into eight families. Family I, which is sub-divided into six sub-families, called as 'true' lipases: *Pseudomonas lipases*; lipases from Gram positive bacteria, such as *Bacillus* and *Staphylococcus*; and other lipases from *Propionibacterium* and *Streptomyces species* [31].

Largely bacterial lipases are mainly glycoproteins, but a few extracellular lipases are lipoproteins, such as Staphylococcal lipases. Most of the bacterial lipases are constitutive, non-specific in their substrate specificity and a few being thermo stable [32]. More than 60 lipases have been identified, purified and characterized till date, which originate only from natural resources such as animals, plants and microbes (wild type or genetically engineered) [33]. Besides being lipolytic, lipases also have the ability to possess esterolytic activity. They have a diverse substrate range, highly specific as enantio selective and chemo-regio catalysts [34]. These lipases have been used for the degradation of wastewater contaminants and oil spills. The treatment processes involves the massive cultivation of lipase-producing microbial strains in the effluents from microbial treatment [35].

## CONCLUSION

The present research reveals significant findings which establish that market vegetable wastes possessing cultivable microbial communities belonging to proteolytic and lipolytic organisms. These organisms are getting adapted to extreme environment, thereby producing enzymes such as proteases and lipases which can be used successfully for commercial purposes. Further, our characterized isolates were having protein and lipid hydrolysis profiles, these bacteria could be useful in industries such as textile, leather, detergents, pharmaceutical process, and bioremediation. This study also provides an experimental initiation and new potential biotechnological applications of the enzymes from the bacterial community.

### Conflicts of interest:

The author declares that no conflict of interest.

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