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Isolation, Screening, and Identification of Pectin Degrading Bacteria from Soil

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KEYWORDS

Pectin, Pectinase, Screening, Pectin degrading bacteria.

ABSTRACT:

Pectin, a complex polysaccharide, undergoes degradation facilitated by the enzyme pectinase, marking a significant focus in enzymology. The heightened interest in pectinase production stems from its extensive applications in bioremediation and various industrial sectors, including food, pharmaceuticals, and agriculture. Microorganisms and plants serve as the primary sources of pectinase enzymes, with microbial pectinases gaining preference in industries due to economic considerations. This study aimed to isolate, screen, and identify pectin-degrading bacteria from diverse soil samples. The confirmation of pectinase production by bacterial isolates was achieved through the utilization of a pectinase screening agar medium (PSAM). Notably, four pectin-degrading bacterial strains were successfully isolated from soil samples collected from the Thoseghar hilly area in the Satara district. These strains include Enterobacter cloaceae amol2223, Enterobacter cloaceae Nazneen23, Acinetobacter junii Asmita23, and Bacillus vallismortis Amol12.

Introduction

Pectin is a heteroglycan i.e., made up of more than one kind of monosaccharide unit. It is plenty in herbaceous parts of telluric plants. It plays a crucial role in the plant cell wall, particularly in the cell plate, constituting the middle lamella. The function of pectin includes cementing the primary cell walls of adjacent cells together (Shilpa et al., 2021). Pectic materials are made up of protopectins, pectinic acids, and pectin. The major chain of pectin polysaccharide is partly methyl-esterified 1,4-D-galactomannan. Demethylation of pectin forms pectic acid (pectate) also known as polygalacturonic acid (Rokade et al., 2015). Methyl esterification or acylation of D-galacturonic acid monomers forms linear polymers of pectin known as homogalacturonans. If 75% of carboxylic acids in homogalacturonans are methylated then it is known as pectin, if less than 75% of carboxylic acids are methylated then these materials are known as pectic acids. In case no carboxylic acid is methyl-esterified then it is called polygalacturonic acid (Tabssum et al., 2018).

Enzymes are proteins in nature and act as biocatalysts that speed up the reactions in the body like a breakdown of complex materials (carbohydrates and proteins) into simpler molecules without being involved in the reaction. Enzymes are highly specific in nature and have a great efficient activity that upgrades the use of enzymes in different industries like food, pharmaceutical, and chemical industries (Shilpa et al., 2021). Most plant pathogens including different microorganisms produce various kinds of enzymes to attack target plant cells which helps them to enter host tissues. These enzymes degrade cells and due to this expansion of plant pathogens in the plant tissues carried out (Aaisha et al.2016). Pectinase is a group of enzymes that acts on complex materials like pectin to degrade them into simpler molecules like sugars and other useful substances. Pectinase enzymes are commonly hydrolases that help to break down bonds between complex sugar molecules, lyases that catalyse reactions by breaking chemical bonds by means other than oxidation, and esterase that splits esters into acid and alcohol. Pectinases may be of two types-acidic and alkaline. The acidic pectinases are used in the juices and beverages industries for clarification and purification of juices and wines. Alkaline pectinases are employed in the wastewater treatment of raw vegetables (Tabssum et al.2018).

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Among various pectinases, extracellular bacterial pectinases stand out as the most suitable and significant compared to those derived from other organisms such as plants, animals, viruses, and fungi. Notably, pectinases produced extracellularly by Bacillus and Cocci species are particularly in high demand and represent a focal point in biotechnological studies. The utilization of extracellular pectinases extends across various industries, including food, pharmaceuticals, and paper, contributing to approximately 10% of the total enzyme production (Aaisha et al., 2016).

Microorganisms, chosen for the production of pectinase enzymes, offer distinct advantages as they are less susceptible to environmental factors. Furthermore, microbes can be subjected to environmental and genetic modifications to enhance enzyme production (Torimiro et al., 2013). When pectinase enzymes act on pectin sugar molecules, they lead to a reduction in viscosity and the formation of clusters, simplifying processes like filtration or centrifugation. Consequently, the juice becomes clearer, presenting a more intense taste and color (Haile et al., 2022). The putative microbes isolated from the soil are mesophilic, generally tolerant to stress and produce enzymes in a broader range of growth parameters. Hence, pectinase-producing microbes were isolated from the soil and the identification was performed.

Materials and methods

A) Sample collection

The four different soil samples were collected from the Thoseghar hilly area of Satara district (Maharashtra, India). These samples were collected from soil belonging to Strawberry, Jamun, Rice, and Organic manure fields. The soil was acquired with the help of a sterile spatula, kept in sterile plastic bags, and processed in the laboratory.

B) Isolation of bacterial colonies from soil samples

Aseptically, 1g of soil sample was taken and subjected to serial dilution using sterile distilled water, ranging from 10^{-1} to 10^{-6}. Subsequently, 0.1mL of the diluted samples from tubes with dilutions 10^{-5} and 10^{-6} was taken and spread evenly on sterile nutrient agar plates. These plates were then incubated at 37°C for 24 hours. Following the incubation period, a mixed culture of colonies was observed on the plates for all four soil samples. To facilitate the purification of isolates, morphologically distinct colonies were selected and streaked onto additional sterile nutrient agar plates. These plates were once again incubated at 37°C for 24 hours (Shilpa et al., 2021).

C) Screening of Pectin Degrading Bacteria

For further confirmation of Pectin degrading bacteria, different isolates from nutrient agar plates were streaked on selective media of pectin-degrading bacteria i.e., PSAM (Pectinase Screening Agar Medium) (Rokade et al.2015) and kept that streaked PSAM plates at 37°C for 24 hrs in an incubator. PSAM (gm/ Lit) contained sodium nitrate 2g; potassium chloride 0.5g; magnesium sulphate 0.5g; dipotassium hydrogen phosphate1gm; tryptone 0.5g; pectin 10g; agar 30g (Bibi et al.2018). After incubation, plates were examined for bacterial colonies. The strains that could use pectin as a source of carbon grew on PSAM media and were chosen as a positive culture. The isolated colonies obtained on sterile PSAM plates were poured with iodine solution. The clear zone of hydrolysis after the iodine treatment indicated pectin degrading bacteria (Bharadwaj et al.2019).

D) Identification of Pectin Degrading Bacteria

The putative pectin degrading bacteria were identified based on morphological characteristics and biochemical tests (Aaisha et al.2016). Morphological Tests such as size, shape, color, margin, elevation, opacity, and consistency were examined. Gram staining and motility of bacterial strains were performed for the identification of pectin-degrading bacteria. Different biochemical tests like catalase, oxidase, IMViC, N_2 reduction, H_2S production, and urease activity were done. Carbohydrate fermentation tests for glucose, lactose, fructose, sucrose, and xylose were performed for the identification of pectin-degrading bacteria.

E) Molecular identification of the bacteria

The bacterial genomic DNA isolation followed the protocol outlined by Turzhanova et al. (2018). After drying the pellet overnight, 25 µl of DNAse-free distilled water was added for DNA dissolution, left at room temperature for 2 hours. Electrophoresis was conducted on 1% agarose gel in TBE buffer. The resulting gel underwent staining with a 0.5 µg/ml ethidium bromide solution for 10 minutes and visualization using a gel documentation system (Biorad, Inc. USA). DNA sample quality was assessed through nanodrop analysis, and the samples were stored at -20 °C. The bacterial 16S rRNA gene amplification was carried out using the universal primers 16S27F (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16S1492R

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(5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Weisburg et al.1991). The total PCR mixture of 25 µl was prepared for each reaction, comprising 1 µl of DNA template (100 ng/µl), 1 µl each of the primers, 0.33 µl of Taq polymerase (3 units/µl), 0.5 µl of dNTPs, 2.5 µl of 10× PCR buffer with 25 mM MgCl2, and 18.67 µl of Milli Q water. The PCR reactions were conducted in an ABS Thermal Cycler, 3rd Generation, following these conditions: pre-denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 40 sec, extension at 72 °C for 7 min, and a final extension for 4 min at 60 °C. Subsequently, the amplicons were electrophoresed on a 1.5% (w/v) agarose gel stained with ethidium bromide (0.5 µg/ml) for visual examination under UV light. Purified amplicons were then subjected to cycle sequencing using BDT v3.1 and subsequently sequenced on an ABI 3500XL Genetic Analyzer.

F) Sequencing

The successful PCR amplicons (2.5 µL) underwent cleanup to degrade the remaining primers by adding Shrimp Alkaline Phosphatase (SAP) (0.5 µL) and Exo I (0.25 µL), followed by incubation at 37°C for 45 min. Subsequently, an additional incubation at 80 °C for 15 min was performed to inactivate ExoSAP. The cleaned-up amplicons were then advanced for cycle sequencing reactions using the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA, USA) with a 16-fold dilution. The same primers utilized in the PCR reaction were employed during the cycle sequencing. The thermal cycle conditions comprised an initial 5-step period of 2 min at 96 °C, followed by 35 cycles of 30 sec at 96 °C, 15 sec at 55 °C, and 4 min at 60 °C. The cycle-sequenced products underwent ethanol precipitation using the following master mix (MM) components: MM I (NFW 10 µL + 125 mM EDTA 2 µL) and MM II (3M NaOAc, pH 4.6, 2 µL + Absolute Ethanol 50 µL). The sequencing reaction mixture consisted of Ready Reaction Mix (2.5X, 0.5 µL), Dilution Buffer (5X, 1.7 µL), Primer (1 pm, 2 µL), Template DNA (5-20 ng, 1 µL), and NFW (4.8 µL), resulting in a final volume of 10 µL. Ethanol-precipitated product templates were dissolved in HiDi formamide (12 µL) with one minute of spinning. Following this, the products were snap-chilled, followed by denaturation at 95 °C for 3 min. The snap-chilled products were bidirectionally sequenced in the ABI 3130 Genetic Analyzer using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA).

BLAST

Basic Local Alignment Search Tool (BLAST) finds regions of similarity between homologous sequences. The program compares nucleotide sequences to sequence databases and calculates the statistical significance.

Phylogenetic Analysis

For the phylogenetic analysis, up to 10 closest-neighbor sequences belonging to different taxa from amongst the top 1000 hits with the highest similarity in the search results were retrieved from the database and aligned using the MUSCLE aligner (Edgar et al.2004).

A phylogenetic tree was used to visually depict this relationship to investigate how closely related groups of species are to one another evolutionarily. Each branch of a phylogenetic tree indicates a taxon that is being compared within the tree. Taxa that have evolved from their common ancestor are represented by branches that start at a single node, also known as a node representing a point of divergence. A common root to the tree reflects the most ancestral taxon from which all taxa within the tree are likely to have evolved. Trees can be rooted or unrooted.

Typically, when determining the evolutionary distance between two animals, the length of the branch is what is important. Therefore, discussing the topology of the tree in terms of branch lengths rather than their vertical arrangement is more pertinent. The horizontal lines, which are branches, show how different evolutionary lineages have evolved. The amount of genetic alteration increases with the length of the horizontal branch. The numbers at the nodes are the bootstrap percentage values, which show how often the branches were repeated with the same configuration during the iterations. As a result, a higher bootstrap number indicates a higher level of confidence in the branch.

Results

Isolation of bacteria

Collected soil samples from rice, organic manure, strawberry, and jamun fields were spread on nutrient agar. After the incubation period, a mixed culture of different bacterial colonies was observed on nutrient agar plates. Colonies from mixed cultures were selected for further studies. 15 different bacterial isolates were found.

Screening of pectin-degrading bacteria

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The isolated and bacterial isolates were streaked on selective media (PSAM) for pectin degradation. From 15 different bacterial cultures, four cultures showed growth on selective media plates namely J2, J4 (isolated from jamun field), O1 (isolated from organic manure), and S2 (isolated from strawberry field) as shown in Figure 1. Four bacterial isolates O1, S2, J2, and J4 showed positive results in pectinase degrading (Figure 2). These isolates were named O1-AN1, S2-AN2, J4-AN4, and J2-AN5. Cultural, morphological, and biochemical characteristics of AN1, AN2, AN3, and AN4 are shown in Table 1 and Table 2 respectively.

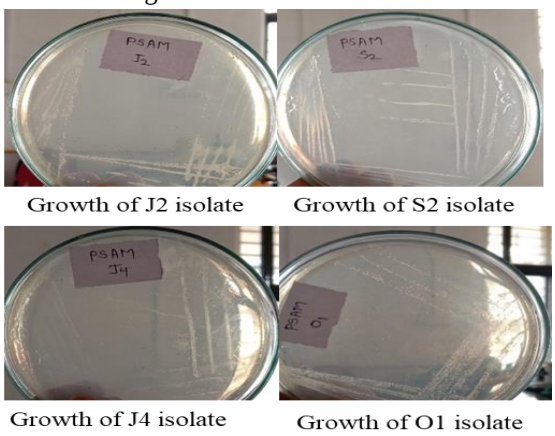

Figure 1: Four bacterial isolates obtained after primary screening on PSAM

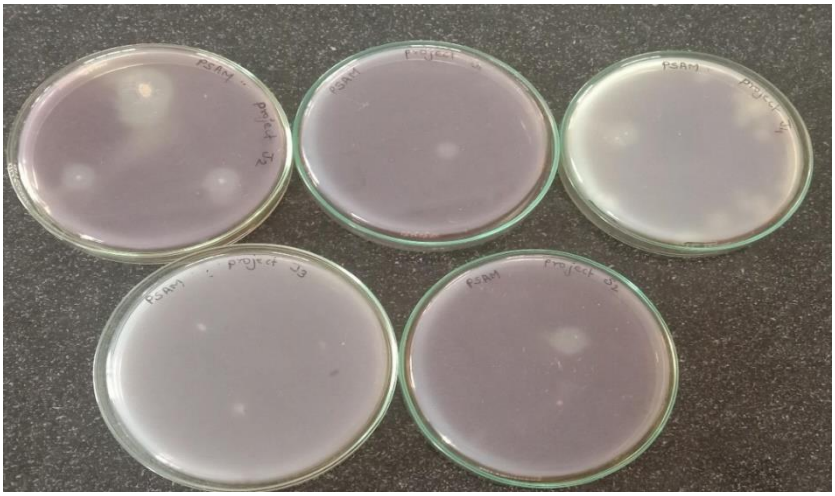

Figure 2: PSAM plates showing clear zones around bacterial colonies

Table 1: Cultural and Morphological characteristics

Characteristics	**Cultural code of bacterial isolates**			
	AN1	**AN2**	**AN4**	**AN5**
Size	2 mm	2 mm	1 mm	2 mm
Shape	Circular	Circular	Circular	Circular
Color	White	White	White	Yellow
Margin	Irregular	Entire	Entire	Entire

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Elevation	Raised	Raised	Raised	Raised
Opacity	Transparent	Opaque	Transparent	Opaque
Consistency	Smooth	Smooth	Smooth	Smooth
Gram nature	Gram negative	Gram-negative	Gram-negative	Gram-positive
Motility	Motile	Motile	Non-motile	Motile

Table 2: Biochemical characteristics of bacterial isolates

	AN1	**AN2**	**AN4**	**AN5**
Carbohydrate fermentation test				
Glucose	+	+	-	+
Fructose	+	+	-	+
Sucrose	+	+	-	+
Lactose	-	-	-	-
Xylose	+	+	-	+
Enzymes test				
Catalase	+	+	+	+
Oxidase	-	-	-	-
Urease	-	-	-	-
IMViC Test				
Indole	-	-	-	-
MR	-	-	+	-
VP	+	+	-	+
Citrate	+	+	Variable	+
N_2 reduction	+	+	-	+
H_2S production	-	-	-	-

16S RNA Sequencing

Four different types of bacterial isolates were identified by using 16S RNA sequencing. Following are the results of 16S RNA sequencing (figure 3 to figure 6).

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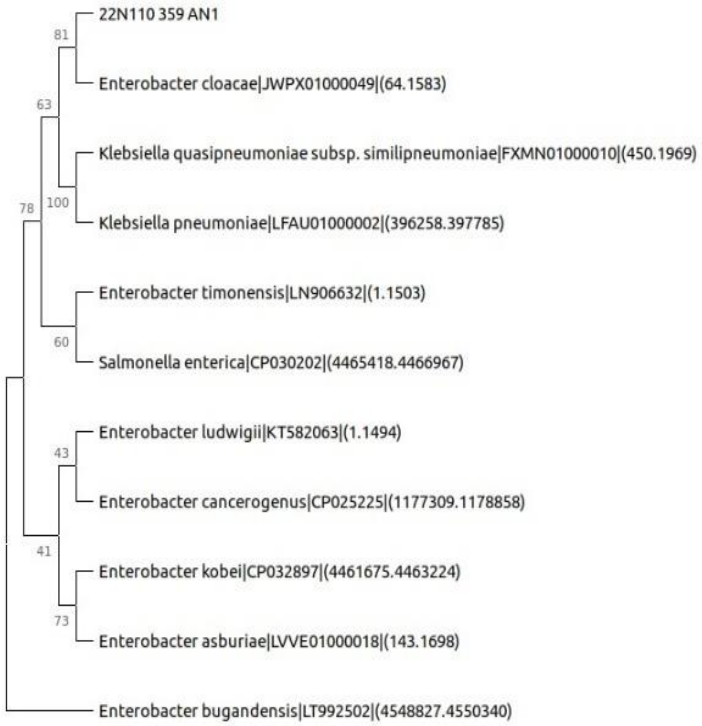

Figure 3: Phylogenetic tree based on 16S rRNA gene sequences showing the position of AN1 – Enterobacter cloaceae amol2223 (Accession Number: OR512552) bacterial strain.

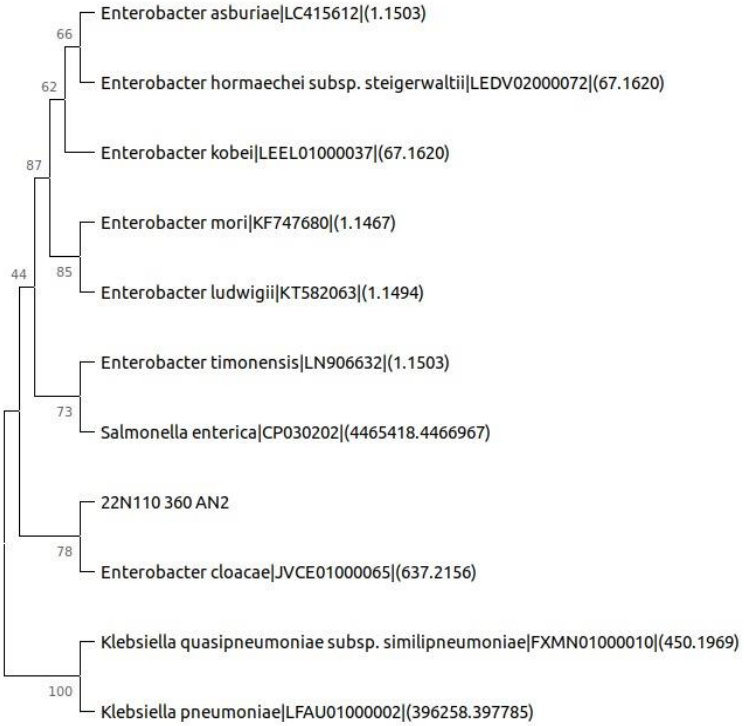

Figure 4: Phylogenetic tree based on 16S rRNA gene sequences showing the position of AN2 – Enterobacter cloaceae Nazneen23 (Accession Number: OR518411).
bacterial strain

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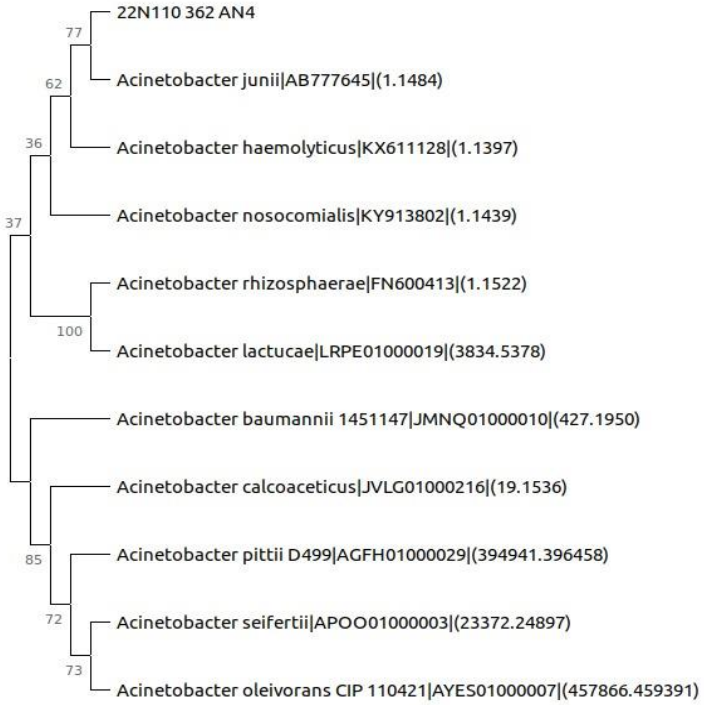

Figure 5: Phylogenetic tree based on 16S rRNA gene sequences showing the position of AN4- Acinetobacter junii Asmita23 (Accession Number: OR523591) bacterial strain.

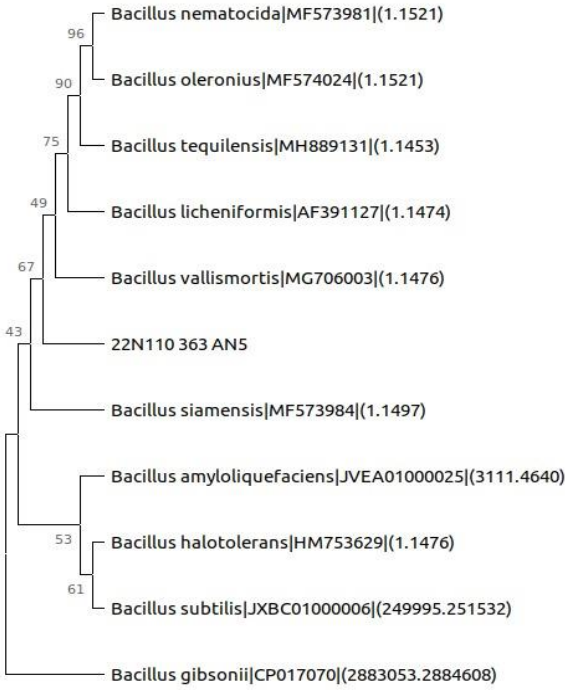

Figure 6: Phylogenetic tree based on 16S rRNA gene sequences showing the position of AN5- Bacillus vallismortis Amol12 (Accession Number: OR523588).
bacterial strain

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Discussion

Pectinase is widely used in many industries as it is cheap in production price to enrich the extraction of coffee, fruits, pulp, and paper. Mostly soil is a rich source of pectinase-producing microorganisms (Shilpa et al.2021). In this study, from four soil samples we isolated a mixed culture of bacteria on nutrient agar and from that total of 15 morphologically different colonies were taken from four plates and named according to sample initials. Then these different colonies were streaked on selective media PSAM for primary screening. Out of 15 bacterial isolates, 4 showed growths on selective media. Rice field soil sample isolates showed no growth on selective media, which means bacterial isolates from that soil sample cannot degrade pectin. For secondary screening, an iodine solution was poured on the colonies on PSAM, and a zone of clearance around all 4 different colonies was observed (figure 2). These 4 isolates are considered positive bacterial isolates that degrade pectin. This secondary screening confirms that isolated colonies degrade pectin by producing a pectinase enzyme.

All four isolates were further studied for their morphological and biochemical characteristics. In morphological characteristics, all bacterial colonies are circular in shape and entire in margins. Colour of the most colonies is white except AN5 which is yellow in color. The size of most colonies ranges from 0.1mm but the AN4 isolate showed pinpoint colonies 1mm in size. All these isolates were characterized based on Gram staining and different biochemical tests (Table 1&2). Further on molecular level identification by using Sanger sequencing, PCR amplification, and phylogenetic analysis; the identified bacterial isolates are AN1- *Enterobacter cloaceae* (OR512522), AN2- *Enterobacter cloaceae* (OR518411), AN4- *Acinetobacter junii* (OR523591) & AN5- *Bacillus vallismortis* (OR523588) shown in figure 3-6.

The soil from the hilly region of the Satara district was found to be a good source of different microorganisms that degrade pectin. Screening of these microbial strains from soil samples may help in the supply of increasing demand for pectinase enzyme by large-scale production industries as secondary screening shows positive results for pectin degradation. Plant waste biodegradation also will be performed by using enzymes produced by these microorganisms.

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Author contribution

AJ designed the study, analyzed data, and reviewed the manuscript. NI and AD conducted experiments and wrote the manuscript.

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Data Availability

16S rRNA Sequencing data were deposited into the GenBank database under accession numbers OR512522, OR518411, OR523591, and OR523588 and are available at the following URL: https://www.ncbi.nlm.nih.gov/nuccore/OR512522, https://www.ncbi.nlm.nih.gov/nuccore/OR518411, https://www.ncbi.nlm.nih.gov/nuccore/OR523591, https://www.ncbi.nlm.nih.gov/nuccore/OR523588 respectively.

Declarations

Ethical approval

This research article does not contain any studies with human participants or animals performed by any of the authors.

Competing interest

The authors declare no competing interest.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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