Isolation, screening, and molecular identification of pectinase producers from fruits, vegetables, and soil samples

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Received: 1 April 2022
Revised: 28 May 2022
Accepted: 30 May 2022
Published: 21 September 2022

Egyptian Pharmaceutical Journal 2022, 21:302–311

Background and objective
In this work, isolation, identification (morphological and chemical), and molecular characterization were done of local isolates of some pectinase-producing microorganisms such as bacteria, actinomycetes, fungi, and yeast.
Materials and methods
A total of 22 local bacterial isolates were obtained from various sources and were assayed for pectinolytic activity after optimization of conditions for pectinase production. Isolate no. 19 showed the highest pectinase-specific activity (6.73 U/ml) on glucose-supplemented medium, whereas isolate no. 5 gave the lowest pectinase productivity (3.21 U/ml). The identification of isolate no. 19 revealed that it belonged to the genus Bacillus based on morphological and biochemical characteristics. Based on molecular identification (16 S rRNA technique), isolate no. 19 was named *Bacillus* sp. strain NRBANKI-4 (with 99% similarity), with Gene Bank accession number OM540351.
Results and conclusion
A total of 14 local actinomycete isolates were obtained from soil samples. Isolate no. 13 showed the highest pectinase-specific activity (6.48 U/ml), whereas sample no. 10 gave the lowest pectinase-specific activity (3.07 U/ml). Based on molecular identification (16 S rRNA technique), isolate no. 13 was named *Streptomyces* sp. KP 12 (90.63% similarity), with Gene Bank accession number OM403596. A total of 10 fungal isolates were obtained from crop waste soil. Isolate no. 2 gave the highest pectinase productivity (21.20 U/ml). Based on molecular identification (internal transcribed spacer-PCR technique), isolate no. 2 was named *Aspergillus niger* F8121 (99.47% similarity), with Gene Bank accession number OM392061. Following the same trend, 10 yeast isolates were isolated from crop waste soil. The isolate that gave the highest pectinase productivity was no. 7, which gave 22.03 U/ml. The isolate that gave the lowest was no. 9 (20.74 U/ml). Isolate no. 7 was named *Pichia barkeri* Y1 (90.91% similarity), with Gene Bank accession number OM392066.

Keywords:
identification, isolation, microorganisms, pectinase producers

Egypt Pharmaceut J 21:302–311

1687-4315

Introduction

Pectinases are one of the essential group of enzymes in the field of biotechnology and have applications in various industries, including food, textile, paper industries, and waste management [1]. Pectinase enzymes present a high-priced category of microbial enzymes with many potential applications in various food and oil industries, with an estimated market share of $41.4 billion by 2020 [2], whereas the estimated value of sales of all industrial enzymes in 1995 was $1 billion, of which some $75 million was assessed for pectinases [3].

Pectinases were some of the first enzymes to be used in homes. Their commercial application was first observed in 1930 for the preparation of wines and fruit juices. Only in the 1960s did the chemical nature of plant tissues become apparent, and with this knowledge, scientists began to use a greater range of enzymes more efficiently. As a result, pectinases are today one of the upcoming enzymes in the commercial sector. Primarily, these enzymes are responsible for the degradation of the long and complex molecules called pectins that occur as structural polysaccharides in the middle lamella and the primary cell walls of young plant cells. Pectinases are now an integral part of fruit juice and textile industries and have various biotechnological applications.

Microbial pectinases are the leading enzymes of the industrial sector. They are being used extensively for various industrial applications like wine industries,

 DOI: 10.4103/epj.epj_39_22

food industries, paper industries for bleaching of pulp and wastepaper recycling [4], in the processing of fruit–vegetables, tea–coffee fermentation, animal feed, extraction of vegetable oil, and scouring of plant fibers, and new applications are still coming up. However, the main consideration is titer and/or activity of the enzyme to make the process cost-effective [5]. Production of pectinases has been reported by many workers and cost-effective substrates have also been used, but still the production cost is high due to either low activity or instability of enzyme for longer duration [6]. So, activity or stability of enzyme further decreases its cost for industrial application. Therefore, during enzymatic treatment to reduce the cost of industrial processes, it becomes necessary to use active enzymes to give additional advantage to the strain. Further research should be concentrated for increasing the activity and stability of enzyme to reduce the cost of the enzyme for their efficient industrial use [7,8]. So, new microbes with high extracellular pectinase activity, stability for a longer period, along with their cost-effective production have been the focus of recent research [9]. Immobilization and remobilization of pectinases into cost-effective material can have great potential in the clarification of beverages for making the process more cost-effective [6]. More research is also needed to discover strains producing pectinase in combination with other enzymes and the specific combination that is required for drastically decreasing the production cost for industrial application [5].

The main objective of this work was to get rid of harmful bacterial substances and turn them into useful substances, in addition to producing bacterial strains that could produce high-value pectinase enzymes for application in many food industries and textile industries.

Materials and methods

Local isolates of some pectinase-producing microorganisms such as bacteria, actinomycetes, fungi, and yeast were used as follows.

Bacterial and actinomycete alkaline pectinase

Pectinase screening agar medium

Pectinase screening agar medium (PSAM) medium was used as a basic medium for screening of pectinase-producing bacteria and actinomycetes [10].

Pectinase production medium

This medium is used for pectinase production and prepared according to the method described by Tripathi *et al.* [11].

Collection, isolation, and screening of pectinase-producing bacterial and actinomycete local isolates

Bacterial and actinomycete isolates were isolated from agricultural crop wastes using the serial dilution method [12]. Bacterial isolates were screened for pectinase production on PSAM and incubated at 37°C for 24 h. Pectinolytic activity was observed by visualizing a zone of clearance around the colonies, which prove the capability to utilize pectin as a source of carbon and thus produce pectinase enzyme. This was performed after flooding the plates with 1% CTAB solution and incubated for 15 min at 37°C. Approximately 49 colonies from serially diluted plates with different morphologies were selected, and purified colonies were maintained on PSAM and stored at 4°C [7].

Pectinase activity assay for bacterial and actinomycetes isolates

Pectinase enzyme production: the pure bacterial and actinomycete cultures were inoculated on LB medium and incubated at 37°C in a rotary shaker at 2.69 g overnight. The fresh overnight culture was used as an inoculum for enhanced enzyme production. Five milliliters of mother inoculum was cultured in 100 ml of LB medium, from which 5% of inoculum concentration (0.5 at 600 OD) was transferred aseptically into the pectinase production medium with pH 7.2 and 1% pectin. The inoculated medium was incubated at 37°C for 48 h by the shake flask fermentation method at 4.79 g. An aliquot of 10 ml of the culture suspension was centrifuged at 2990.65 g for 15 min, and cell-free extract was subjected to enzyme assay [13].

Pectinase activity assay dinitrosalicylic acid (DNS): estimation of enzyme activity using the DNS assay was done [14].

Molecular identification of pectinolytic bacteria

Strains grown on basal medium enriched with chicken pectin's were identified with a molecular procedure, which included extraction of total DNA, partial amplification of 16 S rRNA, and finally sequence analysis. All molecular experiments were conducted according to the instructions described on the kits by the supplier.

DNA extraction and amplification of 16S rRNA genes: the total genomic DNA was extracted using NucleoSpin Tissue Kit (Macherey-Nagel, Fisher Scientific, USA). 16 S rDNA gene sequence was amplified from genomic DNA using universal primers (Integrated DNA Technology, India) (Table 1).

Table 1 Oligonucleotide universal primers used for 16S rDNA PCR amplification and sequencing

Primer	Sequence (5ì →3ì)	Size of amplicon (bp)
16 S rDNA-27F	5'AGAGTTTGATCATGGCTCAG-3'	~1500 bp
16 S rDNA-1492R	5'-GGTTACCTTGTTACGACTT-3'	

PCR amplification reactions were carried out in a 20-µl reaction volume, which contained 1× PCR buffer, 0.2 mM each dNTPs, 2.5 mM $MgCl_2$, 1 U of Ampli-Taq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5 pM of forward and reverse primers, and 2 µl of template DNA. PCR amplifications were conducted for 35 cycles according to the following procedure: initial denaturation at 95°C for 5 min, final denaturation of 95°C for 30 s, annealing at 50°C for 40 s, and final extension at 72°C for 60 s using T100-Thermal cycler (Bio-Rad). Almost 5 µl of the PCR products was analyzed by 1.2% agarose gel in 0.5× TBE buffer at 75 V for 1–2 h.

Sequencing and sequence comparison: sequencing analysis was performed on a 1600-bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator, version 3.1 cycle sequencing kit. The 16 S rRNA sequences were aligned and compared with other 16 S rRNA in the GenBank using the NCBI Basic Local Alignment Search Tool (BLASTn) program (http://www.ncbi.nlm.nih.gov/BLAST) and submitted to GenBank for accession numbers.

Phylogenetic analysis: the sequences of 16 S rRNA were aligned using the Bio-edit program after deleting the regions containing ambiguous nucleotides. The phylogenetic trees were constructed by neighbor-joining statistical method using MEGA X (https://www.megasoftware.net/dload_win_gui). To determine the stability of phylogenetic tree, the sequence data were sampled 1000 times for bootstrap analysis using MEGA X with 50% cutoff [15].

Fungal and yeast acidic pectinase

Isolation of pectinase-producing fungi and yeast

Fungal and yeast isolates were isolated on PDA and YPG agar media, respectively. Isolates that produce pectinase enzyme were assayed using modified pectin agar medium. The composition of modified pectin agar medium is Citrus Pectin-10 g, $(NH_4)_2HPO_4$ 3 g, K_2HPO_4 2.0, $MgSO_4$ 0.1 g, agar 20 g, distilled water-1000 ml, and pH 5.5. Streptomycin (100 mg/ml) was added to this medium composition to restrict bacterial growth [16].

Pectinase activity assay for fungi and yeast isolates

Pectinase enzyme production: 25 ml of the sterilized modified pectin agar medium was inoculated with 1 ml of inoculum and kept at 30°C for 72 h in a shaking incubator at 160 rpm. After the fermented broth was centrifuged for 15–20 min at 4306.54 g, the clear supernatant was used for the determination of pectinase activity [17].

Pectinase activity assay, DNS assay: pectinase activity was determined using the substrate citrus pectin. The reaction mixture contained 1 ml of pectin (1%) prepared in sodium acetate buffer (0.1 M; pH 5.5), and 1 ml of crude enzyme was incubated at 50°C in water bath for 30 min. After incubation, 1 ml of DNS reagent was added and kept for 5 min in boiling water bath followed by addition of 7 ml of distilled water. A blank was also run parallel in which 1 ml of distilled water was added instead of the enzyme extract. The reducing sugars were determined by the Miller [18] method. The absorbance was recorded at 540 nm using a spectrophotometer. Galacturonic acid was used as a standard. One unit of enzyme activity was defined as the 'amount of enzyme that required to release one micromole of galacturonic acid under the standard assay conditions' [17].

Molecular identification of pectinolytic fungi and yeast using internal transcribed spacer technique

DNA extraction and purification: DNA was extracted from high pectinase-producing isolates and purified according to DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany).

PCR of internal transcribed spacer primers (molecular characterization using internal transcribed spacer)

Molecular identification of *Fusarium spp.* cultures was carried out based on conserved ribosomal internal transcribed spacer (ITS) region. We amplified the ITS regions between the small nuclear 18 S rDNA and large nuclear 28 S rDNA, including 5.8 S rDNA using universal primer pairs ITS1 (5'-TCCGT AGGTGAACCTGCGG-3') and ITS4 (5'-TCCT CCGCTTATTGATATGC-3'). Amplification was performed on a Thermal Cycler (Bio-Rad T100) with 25 µl of reaction mixture, containing 2.5 µl of 10× buffer (10 mM Tris-HCl, pH 8.8), 2.5 mM $MgCl_2$, 2 mM each of dNTP, 25 pmol/ml primer

(each of ITS1, ITS4, ITS5 and ITS2), 1 U of Taq DNA Polymerase, and 60–100 ng of genomic DNA. The amplification cycle consists of an initial denaturation at 95°C for 2 min followed by 35 cycles at 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 10 min. Amplified PCR products were separated on an agarose gel (2% w/v) in 1× TAE buffer at 75 V for 150 min and photographed under Gel Doc XR+ Gel Documentation System Gene ruler ladder 100 bp. Thermo Scientific Gene Ruler 100 bp DNA Ladder (Fermentas) was used as a size standard. They were then eluted by Min-Elute PCR purification kit (Qiagen). DNA from All isolates were subjected to automated DNA sequencing and subsequently used for sequencing.

ITS data analysis: the ITS nucleotide sequences for each isolate were determined on both strands for each of the isolates and were aligned for comparison. Most sequence comparisons were carried out using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST) analysis, which aligns two or more homologs to detect the presence of one or more ambiguous regions within the segments under comparison. Phylogenetic tree was created using the online version of MAFFT (https:// mafft.cbrc.jp/ alignment/ server) based on UPGMA (unweighted pair group method for arithmetic analysis). The alignments were further edited manually in MEGA v. 6.0 and deposited in Tree BASE (http: //treebase.org).

Results

First: collection, isolation, and assay of pectinase-producing bacterial and actinomycetes local isolates.

Bacterial alkaline pectinase production

Collection, isolation, and assay of pectinase-producing bacterial

A total of 22 bacterial isolates were isolated from the agricultural crop waste soil at National Research Center (NRC). They were purified after plating on PSAM to know their capability to degrade pectin [12]. The isolates showed clearance of zones around their growth on PSAM. These zones could be observed only after plates were flooded with 1% CTAB solution for 15 min at 37°C [7]. They showed several levels of production capabilities using pectin substrate in the basic medium, and the enzyme assay was carried out at 37°C, 48 h, shaker 4.79 g, pH 7.2, 1% pectin, and 5% of inoculum (0.5 at 600 OD) concentration [13] (Table 2).

A total of 22 bacterial isolates obtained from serially diluted soil samples were assayed for pectinase production. Pectinase activities of different 22 bacterial isolates were assayed, and the results showed a broad range of productivity, that is, 3.21–7.12 U/ml, as shown in Fig. 1. Of 22 bacteria grown on the screening medium, only one isolate gave high productivity. The highest pectinase-producing isolates were numbers 19, 11, 17, 22, 3, 7, 1, and 9, with activities of 6.73, 6.23, 5.87, 5.85, 5.85, 5.84, 5.78, and 5.75 U/ml, respectively. The lowest isolates were no. 5, which gave 3.21 U/ml.

As an indicator for pectinase activity, relative growth production was calculated for each bacterial isolate (Table 2). The results showed different relative growth productions (C/G) among the isolates, ranging from 36.0 to 10.11. The most active isolates were 21, 8, 10, 6, 15, 1, and 22, which had 36.00, 32.11, 28, 25.00, 25.00, 21.77, and 12.56 in relative growth production (C/G), respectively. Isolate no. 14 showed fewer active isolates, with 10.11 in relative growth production (C/G).

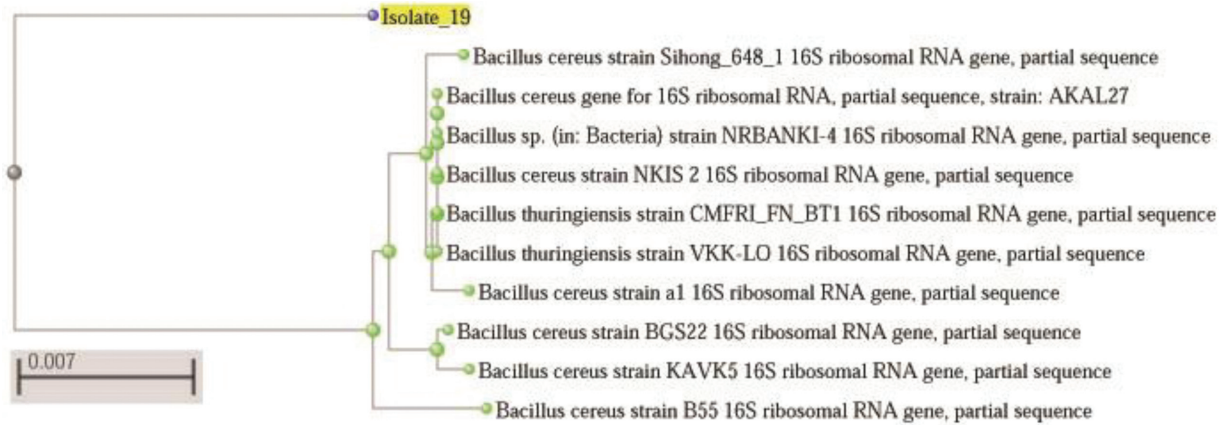
The results in Table 2 illustrate that there is no correlation between relative growth production (C/

Table 2 Pectinase-specific activity (U/ml), source, total protein, and relative growth production of 22 pectinase-producing local bacterial isolates

Isolate no.	Isolate code	Source of isolate	Pectinase-specific activity (U/ml)	Relative growth production (C/G) *
1	Bp2	Apple	5.78	21.77
2	Bp5	Soil	4.78	11.77
3	Bp6	Soil	5.85	11.11
4	Bp10	Banana	5.84	15.00
5	Bp12	Soil	3.21	16.00
6	Bp16	Soil	5.72	25.00
7	Bp17	Soil	5.48	17.25
8	Bp19	Banana	5.19	32.11
9	Bp22	Orange	5.75	15.00
10	Bp23	Mango	5.39	28.00
11	Bp24	Soil	6.23	18.77
12	Bp27	Mango	5.38	18.77
13	Bp28	Soil	4.77	15.00
14	Bp32	Tomato	4.38	10.11
15	Bp34	Soil	5.35	25.00
16	Bp36	Mango	5.10	18.77
17	Bp37	Carrot	5.87	18.77
18	Bp39	Apple	5.15	11.77
19	Bp43	Soil	6.73↑**	15.00
20	Bp45	Soil	5.18	18.44
21	Bp47	Soil	5.12	36.00
22	Bp48	Soil	5.85	12.56

*(C) lyses zone area, (mm^2); (G) colony growth area (mm^2). **The highest bacterial strain that produces the enzyme pectinase.

Figure 1



Phylogenetic analysis based on 16S rRNA sequences shows that the highest pectin productivity bacterial isolate was no. 19, which was named *Bacillus* sp.

G) and the pectinase-specific activity. The results showed that isolates 21 and 19 produced 36.00 and 15.00 as relative growth production (C/G), whereas isolate 19 was more efficient in pectinase activity (6.73 U/ml) than isolate 21 (5.12 U/ml).

Actinomycetes alkaline pectinase production

A total of 14 actinomycete isolates were isolated from agricultural crop waste soil at the NRC and purified after plating on PSAM to know their capability to degrade pectin [12]. Isolates were assayed for pectinase-specific activity. They showed several levels of production capabilities using pectin as a substrate in the basic medium, and the enzyme assay was carried out at 37°C, 48 h, shaker 4.79 g, pH 7.2, 1% pectin, and 5% of inoculum (0.5 at 600 OD) concentration [13] (Table 3).

Table 3 Pectinase-specific activity (U/ml) and source of 14 pectinase-producing actinomycete local isolates

Serial number	Isolate	Source	Pectinase-specific activity (U/ml)
1	Ap1	Soil	4.38
2	Ap2	Soil	3.19
3	Ap3	Soil	4.46
4	Ap4	Soil	5.34
5	Ap5	Soil	4.23
6	Ap6	Soil	5.58
7	Ap7	Soil	4.17
8	Ap8	Soil	3.66
9	Ap9	Soil	3.99
10	Ap10	Soil	3.07
11	Ap11	Soil	4.78
12	Ap12	Soil	5.73
13	Ap13	Soil	6.48
14	Ap14	Soil	5.68

A total of 14 actinomycete isolates obtained from serially diluted samples were assayed for pectinase-specific activity. Pectinase activities of different 14 actinomycete isolates were assayed, and the results showed a broad range of productivity, 3.07–6.48 U/ml, as shown in Table 3. Of 14 actinomycete isolates grown, only one isolate gave the high productivity. The highest activity was shown by isolate no. 13, which showed 6.48 U/ml. The lowest activity was by isolate no. 10, which showed 3.07 U/ml.

Second: molecular identification of pectinolytic bacteria and actinomycetes using 16S rRNA alignment in GenBank (BLAST).

Detection of the PCR products

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1× TBE buffer at 95 V. A 100-bp DNA ladder was used as a molecular size standard. PCR products were visualized on UV light and photographed using a Gel Documentation System (Bio-Rad 2000).

Purification of PCR products

Amplified products for all PCR were purified using EZ-10 spin column PCR product purification. PCR reaction mixture was transferred to a 1.5-ml microfuge tube, and three volumes was added of binding buffer 1. After that, the mixture solution was transferred to the EZ-10 column and left to stand at room temperature for 2 min. After the centrifugation, 750 µl of wash solution was added to the column and centrifuged at 11 962.60 g for 2 min. After repeated washing, 11 962.60 g was spun for an additional minute to remove any residual wash solution. The column was

transferred into a clean 1.5-ml microfuge tube and 50 µl of elution buffer was added, and incubated at room temperature for 2 min, and the purified DNA was stored at −20°C.

Sequencing analyses of PCR products

PCR products were sent to FazaPazhouh Co. for sequencing using forward and reverse primers in an ABI 3730×l DNA analyzer.

Nucleotide sequencing analysis

For bacteria: nine strongest isolates in terms of growth (1, 3, 5, 7, 9, 11, 17, 19, and 22) were selected in this test. Six of them (1, 3, 7, 9, 17, and 19) were characterized by 16 S rRNA via amplification and sequencing. The amplified product was purified, and its nucleotide sequence was determined by GATC company using ABI 3730×l DNA sequencer by forward and reverse primers. The sequences were aligned using Clustal X program, and the phylogenetic tree was booted by MEGA3 software. This sequence was compared with other related sequences available in GenBank.

For actinomycetes: DNA extracted from the highest pectinase-producing actinomycetes (no. 13) was used in this test. Sequence similarity search was performed using the NCBI BLASTn online tool (http://ncbi.nlm.nih.gov/BLAST/) against the nucleotide collection (nr/nt) database.

Molecular identification of bacteria isolate

The amplified products of two highest pectin enzyme-producing isolates (no. 19 and no. 11) were purified, and its nucleotide sequence was determined by GATC Company using ABI 3730xl DNA sequencer by forward and reverse primers. The sequences were aligned using Clustal X program and the phylogenetic tree was booted by MEGA3 software. This sequence was compared with other related sequences available in GenBank. Two bacterial strains that have high pectinase-producing activity were obtained as follows:

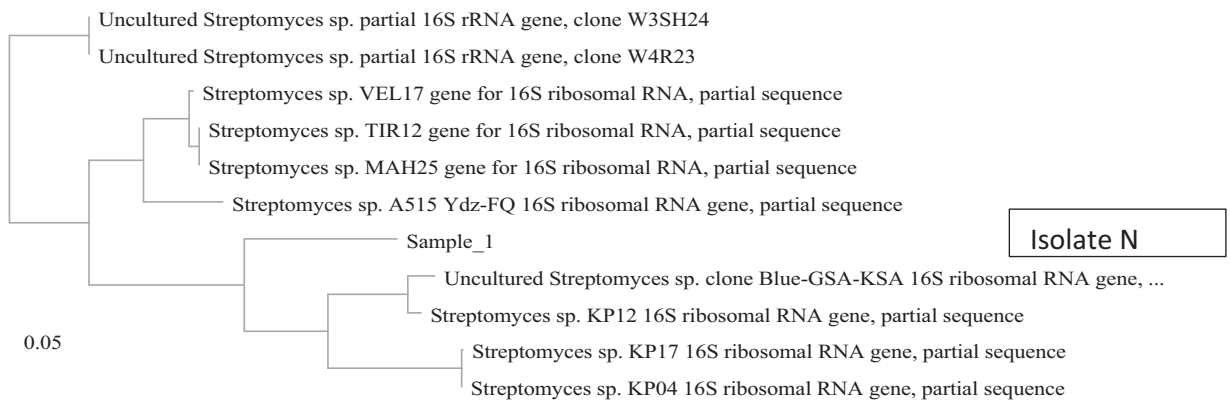
Alignment in GenBank (BLAST) indicated that the isolate no. 19 16 S rRNA was 99% similar with 16 S rRNA *Bacillus* sp. strain NRBANKI-4. Phylogenetic analysis based on 16 S rRNA sequences showed that the isolate no. 19 is closely related to *Bacillus* sp.; therefore, it was named *Bacillus not* sp. strain NRBANKI-4 (as shown in Fig. 1) and used for further studies.

Molecular identification of actinomycete isolate: nucleotide sequencing of 16 S rRNA gene of actinomycete isolate no. 13 indicated that it contained an open reading frame of 1428 nucleotides. Nucleotide sequencing was performed using the BLAST program. Nucleotide alignment of 16 S rRNA gene of the actinomycete isolate no. 13 in GenBank (BLAST) showed 90.63% similarity with 16 S rRNA gene of *Streptomyces* KP 12. Nucleotides alignment was performed using the BLAST program.

Phylogenetic tree showed that the actinomycete isolate no. 13 was found in the same group and closely related to *Streptomyces* KP depending on 16 S rRNA gene sequence. The isolate no. 13 was named *Streptomyces*, and phylogenetic tree was constructed with neighbor joining method, as the results shown in Fig. 2.

Third: fungal and yeast acidic pectinase.

Figure 2



Phylogenetic tree of *Streptomyces* KP(actinomycetes) based on 16S rRNA gene sequences of isolate (N)7 constructed by using neighbor joining method.

Collection, isolation, and assay of pectinase-producing fungus isolates

Ten fungal isolates were isolated from agricultural crop waste soil at the NRC and purified after assaying for pectinase-specific activity to know their capability to degrade pectin [16]. They showed several levels of production capabilities using pectin as the substrate in the basic medium, and the enzyme assay was carried out at 37°C, 120 h, shaker 4.79 g, pH 5, 1% pectin, and 5% of inoculum (0.5 at 600 OD) concentration, as shown in Table 4.

Ten fungus isolates obtained from serially diluted samples were assayed for pectinase production. Pectinase activities of different fungus isolates were assayed, and the results showed a broad range of productivity, as shown in Table 3. Of 10 fungal isolates, only one isolate gave the highest productivity. The highest productivity isolate was no. 2, which showed 21.20 U/ml. The lowest productivity isolate was no. 5, which gave 1.36 U/ml.

Collection, isolation, and assay of pectinase-producing yeast isolates

Ten yeast isolates were isolated from agricultural crop waste soil at the NRC and purified after assaying for pectinase-specific activity to know their capability to degrade pectin [16]. They showed several levels of production capabilities using pectin as the substrate in the basic medium, and the enzyme assay was carried out at 37°C, 120 h, shaker 4.79 g, pH 5, 1% pectin, and 5% of inoculum (0.5 at 600 OD) concentration, as shown in Table 5.

Ten yeast isolates obtained from serially diluted samples were assayed for pectinase production. Pectinase activities of different yeast isolates were assayed, and the results showed a broad range of productivity, from 1.36 to 22.03 U/ml, as shown in Table 4. Of 10 yeast isolates, only one isolate gave the highest productivity. The highest productivity isolate was no. 7, which gave 22.03 U/ml. The lowest productivity isolate was no. 5, which gave 1.36 U/ml.

Molecular identification of fungal isolate (no. 2F) and yeast isolate (no. 7Y) using internal transcribed spacer region gene alignment in GenBank (BLAST)

The ITS region of the rDNA was amplified by PCR with previously described universal primers: ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) [19]. The PCR amplification of ITS1 and 4 of the two

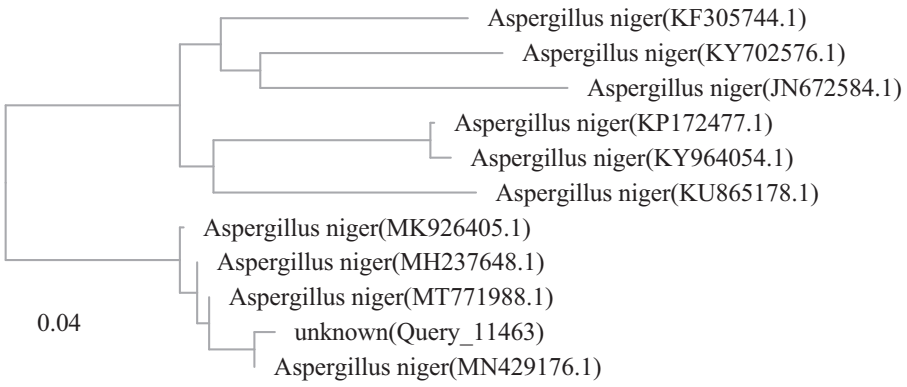
Table 4 Pectinase-specific activity (U/ml) and source of local 10 pectinase-producing fungus isolates

Serial number	Isolate	Source	Pectinase-specific activity (U/ml)
1	Fp12	Soil	20.70
2	Fp13	Soil	21.20↑
3	Fp14	Soil	17.54
4	Fp15	Soil	18.91
5	Fp16	Soil	1.36
6	Fp17	Soil	20.64
7	Fp18	Soil	21.16
8	Fp19	Soil	20.44
9	Fp20	Soil	21.19
10	Fp21	Soil	18.17

Table 5 Pectinase-specific activity (U/ml) and source of local yeast pectinase-producing fungus isolate

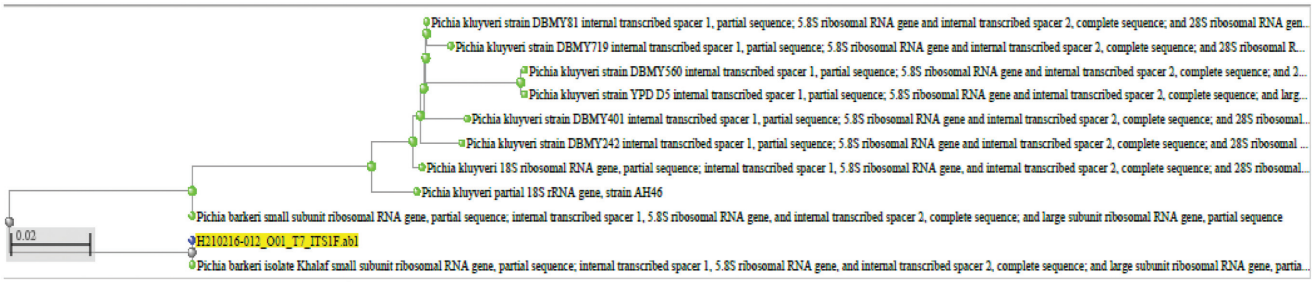
Serial no.	Isolate	Pectinase-specific activity (U/ml)
1	Yp1	21.52
2	Yp2	21.64
3	Yp3	21.88
4	Yp4	21.75
5	Yp5	21.14
6	Yp6	21.69
7	Yp7	22.03↑
8	Yp8	21.26
9	Yp9	20.74
10	Yp10	21.27

Figure 3



Phylogenetic tree of *Aspergillus niger* 2F using neighbor joining method.

Figure 4



Phylogenetic tree of *Pichia barkeri* Y1 isolate using neighbor joining method.

isolates fungi and yeast yielded ~863 and 1138 bp, respectively, as follows:

Molecular identification of fungi isolate: nucleotide alignment of the ITS region of the highest pectinase-producing local fungal isolate Ep13 (no. 2 F) in GenBank (BLAST) showed 99.47% similarity with ITS of *Aspergillus niger* strain F8121. The nucleotide alignment was performed using BLAST program, as shown in Fig. 3.

Molecular identification of yeast isolate: nucleotide alignment of ITS region of yeast isolate (no. 7Y) in GenBank (BLAST) showed 90.91% similarity with ITS of *Pichia barkeri* strain. The nucleotide alignment was performed using BLAST program, as shown in Fig. 4.

Discussion

Pectin is a major component of the primary cell wall of all plants. Pectin substances are colloidal polysaccharides, with galacturonic acid backbone linked by α (1–4) linkage. Pectinases degrade complex pectin to mono-galacturonic acid molecules.

Collection, isolation, and assay of pectinase-producing bacterial and actinomycete local isolates

Pectinase has a wide variety of applications, such as in fruit juice clarification in fruit processing industry. *Bacillus* sp., *Erwinia* sp., and *Pseudomonas* sp. are major producers. Local bacterial and actinomycete isolates have shown clearance of zones around their growth on PSAM. A total of 22 bacterial isolates obtained from serially diluted soil samples were assayed for pectinase production. Pectinase activities of 22 different bacterial isolates were assayed, and the results showed a broad range of productivity from 3.21 to 7.12 U/ml, as shown in Fig. 1. Of 22 bacteria grown on screening medium, only one isolate gave the highest productivity. The isolates with higher pectinase productivity were 19, 11, 17, 22, 3, 7, 1, and 9, with activities of 6.73, 6.23, 5.87, 5.85, 5.85, 5.84, 5.78, and 5.75 U/ml, respectively. The lowest activity was shown by isolate no. 5, which gave 3.21 U/ml. These results agree with the results obtained by Tabssum and Ali [20]. They found clearance zones of hydrolysis produced by the local Bacillus strains, which ranged from 0.9 to 2.6 mm^2 in diameter. Enzyme production units were 59.0, 57.0, 58.0, and 58.0 µg/ml/min by the bacterial isolates designated as BCTL-SL-197. Moreover, Mitra and Roy [21] demonstrated that when they used 16 S rRNA gene technique, strain 2479 was amplified using Bacillus-specific primers and obtained a 1465-bp amplified product. Comparison of 16 S rDNA region (1465 bp) of the isolate 2479 by Ribosomal Database Project II – sequence match showed greatest similarity with genus Bacillus sp. JDM-2-1 (Accession No. EF584539). Phylogenetic analysis involved the identification of homologous sequences, their multiple alignment, and phylogenetic reconstruction, and the graphical representation of the inferred tree was done in Phylogeny tree package. Phylogenetic tree showed strain 2479 had 100% similarity with *Bacillus cereus* group. Following the same trend, these results agree with the results reported by Aunstrup [22] and Solaiman *et al.* [23]. These results may be due to incompatibility or differences in growth conditions, for example, depth of agar layer can affect the diffusion of enzyme.

A total of 14 actinomycete isolates obtained from serially diluted samples were assayed for pectinase-specific activity. Pectinase activities of different 14 actinomycete isolates were assayed, and the results showed a broad range of productivity, 3.07–6.48 U/ml, as shown in Table 3. Of the 14 actinomycete isolates grown, only one isolate gave high productivity. The highest productivity isolate was no. 13, which gave 6.48 U/ml. The lowest productivity isolate was no. 10, which gave 3.07 U/ml. These results agree with the results obtained by Atala *et al.* [24]. They isolated and evaluated pectinase activity of local Iraqi *Pseudomonas* sp. isolates.

Molecular identification of bacteria and actinomycete isolates

Alignment in GenBank (BLAST) indicated that isolate no. 19 16 S rRNA was 99% similar to 16 S rRNA *Bacillus* sp. strain NRBANKI-4. Phylogenetic analysis based on 16 S rRNA sequences showed that the isolate no. 19 was closely related to *Bacillus* sp.; therefore, it was named *Bacillus* sp. strain NRBANKI-4 and used for further studies.

Phylogenetic tree showed that the actinomycete isolate no. 13 was found in the same group and closely related to *Streptomyces* KP depending on 16 S rRNA gene sequence. Therefore, the actinomycete isolate no. 13 in this study was named *Streptomyces*, and phylogenetic tree was constructed with neighbor joining method. In 2022 Atala *et al*. [24], demonstrated in their work that molecular identification was also performed using analysis of 16 s rRNA. The characteristic band of about 1487 bp was obtained. The sequencing analysis was performed and revealed that the isolate is very close to *Streptomyces cinereoruber*. The obtained sequence, 405 bp, was deposited in GenBank under accession, MT907291.1. Following the same trend, these results agree with Abdel-Aziz *et al*. [25]. They found that phylogenetic analysis based on rRNA sequences showed that local bacterial isolates are closely related to *Klebsiella oxytoca N22*.

Collection, isolation, and assay of pectinase-producing fungal and yeast acidic pectinase

Ten fungus isolates obtained from serially diluted samples were assayed for pectinase production. Pectinase activities of different fungus isolates were assayed, and the results showed a broad range of productivity, as shown in Table 3. Of 10 fungal isolates, only one isolate gave high productivity. The highest productivity isolate was no. 2, which gave 21.20 U/ml. The lowest productivity isolate was no. 5, which gave 1.36 U/ml.Ten yeast isolates obtained from serially diluted samples were assayed for pectinase-producing bacteria. Pectinase activities of different yeast isolates were assayed, and the results showed a broad range of productivity, from 1.36 to 22.03 U/ml, as shown in Table 4. Of 10 yeast isolates, only one isolate gave the highest productivity. The highest productivity isolate was no. 7, which gave 22.03 U/ml. The lowest productivity isolate was no. 9, which gave 20.74 U/ml.

Molecular identification of fungal isolate (no. 2F) and yeast isolate (no. 7Y) using internal transcribed spacer region gene alignment in GenBank (BLAST)

Nucleotide alignment of the ITS region of the highest pectinase-producing fungal isolate Ep13 (no. 2 F) in GenBank (BLAST) showed 99.47% similarity with ITS of *A. niger* strain F8121. The nucleotide alignment was performed using BLAST program, as shown in Fig. 3. In 2006, Villa-Carvajal *et al*. [26] used ITS1 and ITS2 to differentiate species in the genus Pichia. At same trend, Jeyaram *et al*. [27] used molecular identification technique on 163 yeast isolates by analysis of the restriction digestion pattern generated from PCR amplified ITS region along with 5.8 S rRNA gene (ITS1-5.8S-ITS2). Nine groups of yeast were identified as *Saccharomyces cerevisiae*, *Pichia anomala*, *Trichosporon* sp., *Candida tropicalis*, *Pichia guilliermondii*, *Candida parapsilosis*, *Torulaspora delbrueckii*, *Pichia fabianii*, and *Candida montana.*

Nucleotide alignment of ITS region of the yeast isolate (no. 7Y) in GenBank (BLAST) showed 90.91% similarity with ITS of *P. barkeri* strain. The nucleotide alignment was performed using BLAST program, as shown in Fig. 4. These results agree with the results obtained by Atala *et al*. [24]. They used sequence analyses of ITS region ITS technique to identify 135 isolates of *Aspergillus keratitis* performed by nucleotide- nucleotide BLAST analysis followed by the initial identification of the isolates based on conidial and colony morphology. The sequence analysis revealed several unusual species that were never reported in eye infections such as *Aspergillus tamrii*, *Aspergillus tubingensis*, *Aspergillus brasiliensis*, *Aspergillus nomius*, *Aspergillus pseudonomius*, *Aspergillus sydowii*, and *Eurotium amstelodami*.

Conclusion

This work was focused on isolation, screening, and identification of some local isolates of bacteria, actinomycetes, fungi, and yeast pectinase producers from different sources such as soil and rotting fruits and vegetables. Four highly productive pectinase producers were obtained: a bacterial strain, which was *Bacillus* sp. strain NRBANKI-4 (isolate no. 19), an actinomycete strain, which was *Streptomyces* sp. KP 12 (isolate no. 13); one fugal strain, which was *A. niger* strain F8121 (isolate no. 2); and finally, one yeast strain, which was *P. barkeri* Y1 (isolate no. 7).

Financial support and sponsorship

The financial funding for this work was provided by the twelfth research plan projects at the National Research Center (Project No.12020115) in the period from 2019–2022.

Conflicts of interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the

manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

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