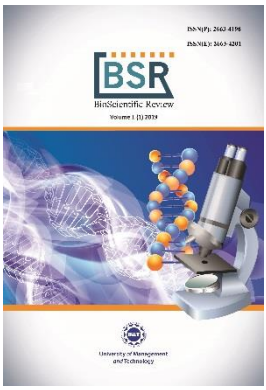

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Isolation and Screening of Pectinolytic Bacterial Strains from Rotten Apples from Lahore, Pakistan

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Abstract

Pectinases are pectin degrading enzymes predominantly used as biocatalysts in various industries such as wine extraction, fruit juice extraction, and making of paper pulp. Large scale production of pectinases using biological systems (bacteria, fungi, plants) is a common method used in the industry. In the current study, different bacterial isolates obtained from rotten apples were used for pectinase production and their pectinolytic activity was investigated. Five bacterial strains were isolated on the growth medium containing 0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.2% NH_4Cl, 0.5% NaCl, 1% Pectin, 1.5% Agar, 1mM $CaCl_2$, and 10mM $MgSO_4$. The isolates of five samples A, B, C, D and E were then biochemically characterized as *Serratia marcescens*, *Klebseilla pneumoniea*, *Pseudomonas aeruginosa* and *Escherichia coli*, respectively. They were also identified at the molecular level through 16S rRNA gene sequencing.

Keywords: bacterial isolation, DNS assay, pectin, pectinase, pectinolytic activity, zymography, 16S rRNA sequencing

1. Introduction

Biologically, enzymes are active compounds involved in the regulation of many chemical reactions happening in living tissues [1]. Structurally, pectin is a significant part of the plant cell wall. Naturally, pectin is a polymeric material and it contains carbohydrates esterified with methanol. In the middle lamella, it is present in an excessive concentration where it works as an establishing substance between adjacent cells. Structurally and functionally, pectin is the most multiplex polysaccharide in the plant cell wall [2]. Pectin consists of α-1, 4-linked residues of D-galacturonic acid [3]. Pectin was first isolated and described by Henri Braconot in 1825. Pectin is mainly extracted from citrus food and it is produced at commercial level as a white to light brown powder. Pectin is an important polysaccharide of plant cell wall which permits plant growth. Pectin is broken down through the enzyme pectinase during fruit ripening, in that process the fruit becomes pulpy and cells are separated from each other due to the breakdown of the middle lamellae [4].

Pectin is naturally produced as a secondary product in sunflower oil and sugar manufacturing industries because it is a non-toxic and amorphous carbohydrate present in the cell wall of plant tissue. Pectin is ecofriendly and biodegradable. Therefore, it is cheap and present in large quantities. In the food industry, pectin is frequently utilized as a gelling and stabilizing agent [5]. This study aims to isolate and screen pectinase producing bacterial isolates obtained from rotten apples.

2. Materials and Methods

2.1. Place of Work

Experimental work was done in the laboratory of the Institute of Molecular Biology and Biotechnology (IMBB), University of Lahore.

2.2. Collection of Sample

Samples of rotten apple were collected from the fruit market of Lahore, Punjab, Pakistan. They were stored at 4°C until further processing.

2.3. Isolation of Bacterial Strains

2.3.1. Serial dilution. Bacterial strains were isolated using the serial dilution technique in selective media (minimal media) containing pectin as a sole carbon source. The collected rotten apple sample was serially diluted in autoclaved distilled water. Five test tubes were labelled as TA, TB, TC, TD, and TE, respectively. 5ml of autoclaved dH_2O was taken in TA. In residual test tubes, 4.5mL of autoclaved dH_2O was taken. Then, about 1g of apple sample was blended in TA. From TA, 0.5ml of test blended water was taken and mixed into TB. From TB, 0.5ml of sample was taken and mixed into TC and similarly, other dilutions were prepared. The test tubes were placed at 4°C until the media was prepared.

2.3.2. Preparation of minimal media. Minimal media was prepared using Na_2HPO_4 (0.65%), KH_2PO_4 (0.3%), NaCl (0.5%), NH_4Cl (0.2%), 1M $CaCl_2$ (100µl), 1M $MgSO_4$ (1 ml), pectin (1%), and agar (1.5%).

2.3.3. Composition of luria broth liquid medium. LB broth (5ml) was taken in five different test tubes labelled A, B, C, D, and E respectively and autoclaved. After autoclaving, the broth was chilled off at room temperature and then distinct colonies from pure culture were picked, moved into these test tubes and were put in shaking incubator at 37°C for 24 hours. After the growth in LB broth, the bacterial culture was centrifuged for five minutes at 10000rpm to get bacterial cell pellets for DNA extraction. The culture from the LB broth was centrifuged and later on used for the preparation of glycerol stock and for the identification of microbes.

2.4. Identification and Characterization of Bacterial Species

2.4.1. Gram's staining

Gram's staining of bacterial cultures was performed following the technique described by [6, 7].

2.5. Characterization of Bacterial Species

2.5.1. Biochemical tests. Biochemical tests were performed to identify bacterial species based on their biochemical activities, intracellular metabolic action and other properties specific for different bacterial species. On the basis of the particular attributes of bacterial species, we effortlessly strived to determine our desired species. Distinctive tests performed were catalase test [8], coagulase test, indole test, urease test, and SIM (Sulphide Indole Motility) test [9].

2.5.2. Extraction of genomic DNA. Genomic DNA was extracted from bacterial cultures using the method described by Bazzicalupo M. and Fancelli S. [10].

2.5.3. Gel electrophoresis. Genomic DNA was run on gel electrophoresis following the protocol published by Lee PY et al. [11].

Table 1. Sequence, Length and T_m of Universal Primers

Primers	Sequence	Length	T_m
Forward	AGRGTTYGATYMTGGCTCAG	20	54.3
Reverse	TACGGYTACCTTGTTACGACTT	22	58.4

Table 2. PCR Conditions

Steps	Temperature	Time
Initial Denaturation	94°C	4min
30 Cycles	94°C	30 sec
	52°C	30 sec
	72°C	2 min
Final Extension	72°C	10 min
Store	4°C	∞

2.6. Polymerase Chain Reaction (PCR)

2.6.1. Reagents and materials for PCR. The reagents and materials utilized included16S rRNA gene universal primers (forward and reverse), dNTPs, Taq polymerase, Taq reaction buffer, template DNA, dH_2O, $MgCl_2$, 2mL Eppendorf, PCR tubes, water bath, micropipettes, microtips, gloves, PCR thermal cycler and gel electrophoresis.

2.6.2. 16S rRNA gene sequence (Universal Primers). Both forward and reverse primers (16S rRNA gene sequence) were utilized to amplify DNA or the gene of interest. The sequence, length and T_m (melting temperature) of universal primers are given below in Table 1. PCR conditions are given in Table 2.

2.6.3. 16S rRNA gene sequencing analysis. The amplified DNA samples were sent to Macrogen, Korea for the confirmation of bacterial species.

3. Results

3.1. Isolation of Bacterial Strains from the Sample

3.1.1. Growth on petri plates. Serially diluted samples formed mixed bacterial colonies on petri plates containing minimal media.

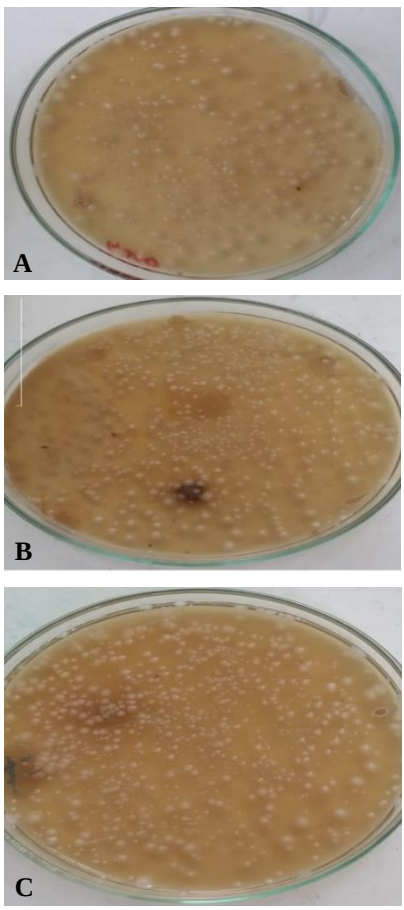


Figure 1. Mixed bacterial colonies of diluted samples T3 (A), T4 (B), and T5 (C)

3.1.2. Pure culture of bacterial colonies. Different colors of bacterial colonies appeared on all six portions of the partitioned petri plate.

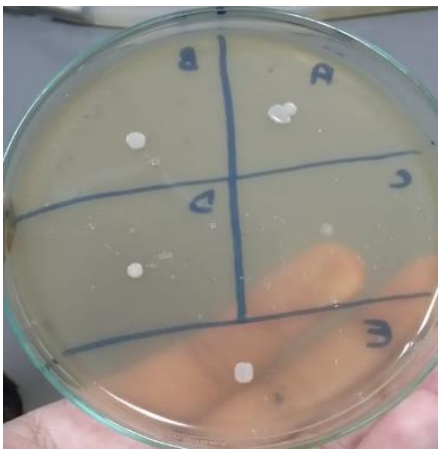
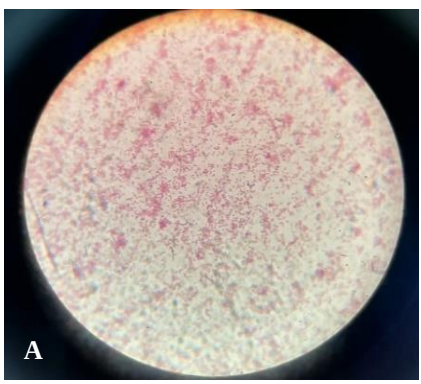


Figure 2. Pure culture of bacteria

3.2. Morphological and Biochemical Characteristics of Bacterial Strains

3.2.1. Gram staining. The prepared slides of five different bacterial strains were observed under microscope at 100X. Gram staining of all strains (*Serratia marcescens, Klebseilla pneumoniae, Pseudomonas aeruginosa* and *Eschericehia coli*) showed that samples A, B, C, D, and E had gram negative rod shaped bacteria.



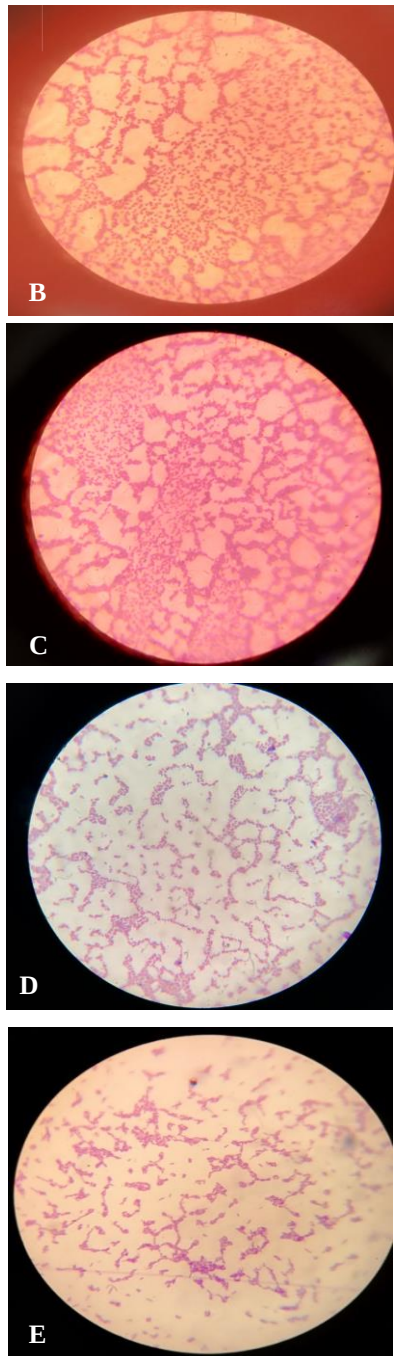


Figure 3. Gram staining of bacterial isolates

3.3. Biochemical Characterization of Bacterial Isolates

3.3.1. Catalase and coagulase test. All five isolated bacterial strains showed positive results for catalase test which proved their catalytic activity. On the other hand, none of the clump formations on glass slide showed coagulase negative results for all five strains.

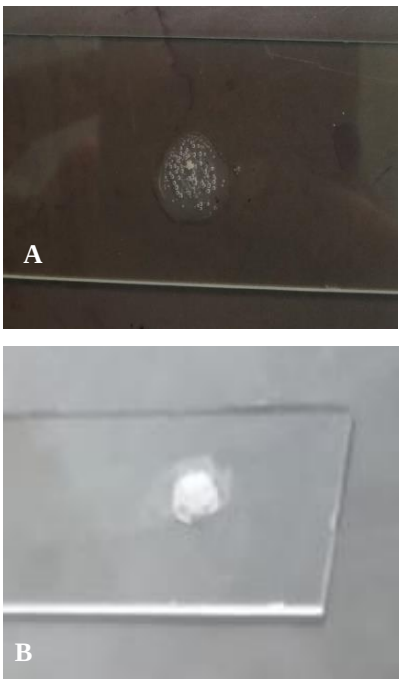


Figure 4. Catalase test (A) positive; coagulase test (B) negative

3.3.2. Indole test. A few drops of Kovac's reagent were added in five different test tubes containing five different bacterial cultures. A red color ring was observed in *E. coli* test tube which showed positive results for indole test. However, colored ring was not formed in the remaining four test tubes which indicated the presence of *S. marcescens, K. pneumoniae and P. saeruginosa.*

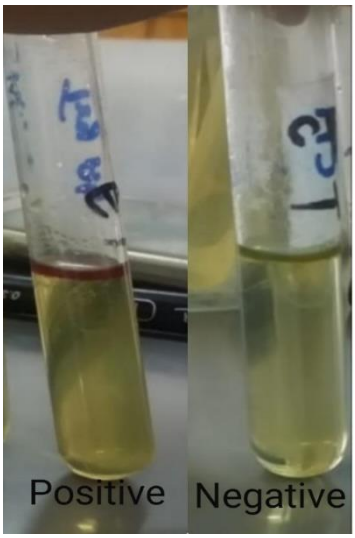


Figure 5. Results of indole test

3.3.3. Sulfide Indole Motility (SIM) Test. SIM test was checked by the inoculum of pure bacterial culture in test tubes incubated for three days. No black color was formed in the medium or no H_2S gas appeared in test tubes but motility was observed in all bacterial species including *S. marcescens, P. aeruginosa, E. coli* and *Serratia marcescens* except *K. pneumonia* which was non-motile.

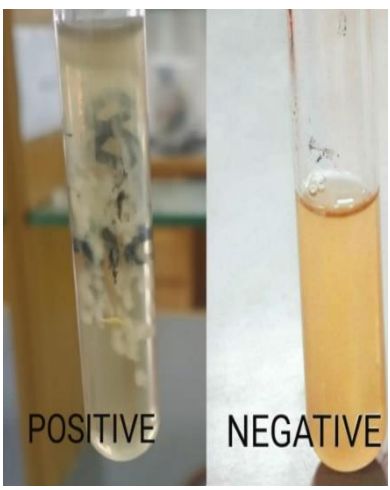


Figure 6. Results of H_2S test

3.3.4. Urease test. The color of urea broth in A, B, and E test tube strains (*S. marcescens* and *K. pneumoniae)* changed into pink which showed a positive result. No change in color of urea broth was observed in C and D test tube strains (*E. coli* and *P. aeruginosa).*

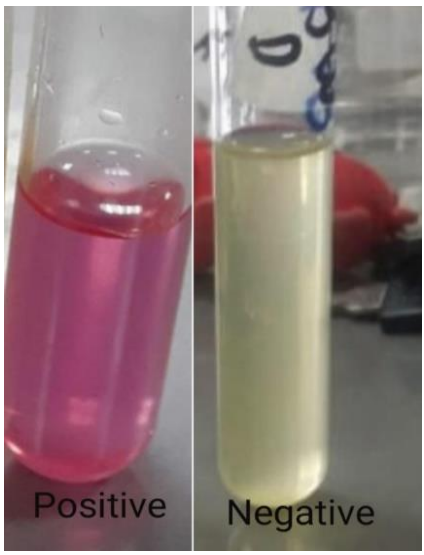


Figure 7. Results of Urease test

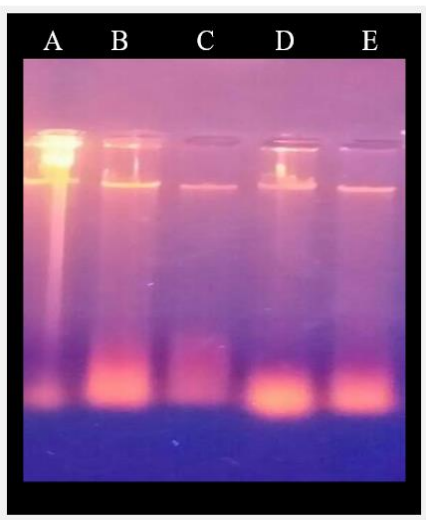


Figure 8. Genomic DNA bands of different isolates on agarose gel

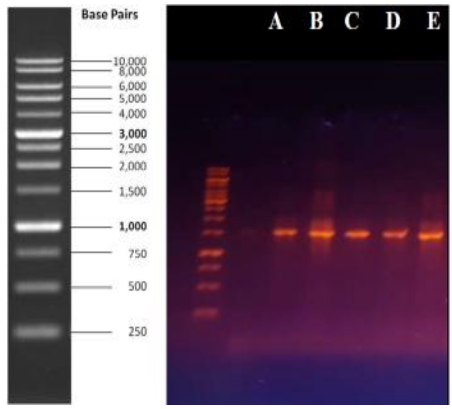


Figure 9. Agarose gel electrophoresis of amplified DNA

3.4. Confirmation of Genomic DNA by Gel Electrophoresis

Gel electrophoresis was performed to confirm the genomic DNA isolated from bacterial strains. After running the gel, it was observed under UV light. Clear bands were observed under UV light which indicated that genomic DNA was successfully isolated from bacterial isolates.

3.5. Confirmation of Amplified DNA by Gel Electrophoresis

PCR (using universal primers) was performed to amplify the DNA isolated from different strains. The amplified DNA was then again run on agarose gel to confirm the amplification of the genomic DNA. The appearance of bands indicated the successful amplification of DNA.

3.6.Sequence of Biochemically Identified Bacterial Strains

After confirmation, DNA amplicons were sent to Korea for sequencing. The sequences were then analyzed on the NCBI database. The resulting FASTA sequences were then applied on the Blast Nucleotide filter of NCBI and bacterial strains were confirmed.

Table 3. Morphological and Biochemical Characteristics of Bacterial Isolates

#	Biochemical Test	Observation				
		Serratia marcescens	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*E.coli*	*Serratia marcescens*
1	Catalase	+ve	+ve	+ve	+ve	+ve
2	Coagulase	-ve	-ve	-ve	-ve	
3	Indole	-ve	-ve	-ve	+ve	-ve
4	Urease	+ve	+ve	-ve	-ve	+ve
5	H_2S	-ve	-ve	-ve	-ve	-ve
Morphological characteristics						
6	Shape	Rods	Rods	Rods	Rods	Rods
7	Gram	-ve	-ve	-ve	-ve	-ve
8	Motility	+ve	-ve	+ve	+ve	+ve

Table 4. NCBI Blast Result of the Sequence of the Strain *Serratia marcescens*

Description	Max score	Total score	Query cover	E value	Ident	Accession
Serratia marcescens strain 135 16S ribosomal RNA gene, partial sequence	1439	1439	93%	0.0	90%	MF399276.1
Serratia marcescens strain 97 16S ribosomal RNA gene, partial sequence	1439	1439	93%	0.0	90%	MF399275.1
Serratia marcescens strain 160 16S ribosomal RNA gene, partial sequence	1439	1439	93%	0.0	90%	MF399274.1
Serratia marcescens strain 93 16S ribosomal RNA gene, partial sequence	1439	1439	93%	0.0	90%	MF399273.1
Serratia marcescens strain 78 16S ribosomal RNA gene, partial sequence	1439	1439	93%	0.0	90%	MF399272.1
Serratia marcescens strain UMH2, complete genome.	1439	9933	93%	0.0	90%	CP018924.1

Table 5. NCBI Blast Result of the Sequence of the Strain *Klebseilla pneumoniea*

Description	Max score	Total score	Query cover	E value	Ident	Accession
Klebseilla sp. enrichment culture clone SRC DSB14 16S ribosomal RNA gene, partial sequence	1439	1439	56%	0.0	93%	GU374044.1
Uncultured Klebseilla sp. clone F5feb.9 16S ribosomal RNA gene, partial sequence	1439	1439	56%	0.0	93%	GQ415978.1
Klebseilla sp.090305 16S ribosomal RNA gene, partial sequence	1439	1439	56%	0.0	93%	EF522816.1
Klebseilla pneumoniea subsp. pneumoniea strain CAU3407 16S ribosomal RNA gene, partial sequence	1437	1437	56%	0.0	93%	MF429125.1
Klebseilla sp. strain CAU1120 16S ribosomal RNA gene, partial sequence	1437	1437	56%	0.0	93%	MF429119.1
Klebseilla pneumoniea strain CN1, complete genome	1437	11397	56%	0.0	93%	CP015382.1

Table 6. NCBI Blast Result of the Sequence of the Strain *Pseudomonas aeruginosa*

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pseudomonas aeruginosa partial 16S ribosomal RNA gene, isolate AAll-9	2109	2109	86%	0.0	95%	LN558613.1
Pseudomonas aeruginosa strain kasamber11 16S ribosomal RNA gene, partial sequence	2073	2073	88%	0.0	94%	KY549647.1
Pseudomonas aeruginosa strain R-15 16S ribosomal RNA gene, partial sequence	2073	2073	88%	0.0	94%	KX756232.1
Pseudomonas aeruginosa strain BRPO3 16S ribosomal RNA gene, partial sequence	2073	2073	88%	0.0	94%	KX664101.1
Pseudomonas aeruginosa strain PBS 16S ribosomal RNA gene, partial sequence	2073	2073	88%	0.0	94%	KX180920.1
Pseudomonas aeruginosa strain HB135 16S ribosomal RNA gene, partial sequence	2073	2073	88%	0.0	94%	KJ829356.1

Table 7. NCBI Blast Result of the Sequence of the Strain *Escherichia coli*

Description	Max score	Total score	Query cover	E value	Ident	Acceession
Escherichia coli strain H17 chromosome	1827	12710	70%	0.0	95%	CP021193.1
Escherichia coli strain CCUG 70745 chromosome	1827	12792	70%	0.0	95%	CP023258.1
Escherichia coli strain DA33137 chromosome, complete genome	1827	12659	70%	0.0	95%	CP029579.1
Escherichia coli strain DA33133 chromosome, complete genome	1827	12625	70%	0.0	95%	CP029574.1
Escherichia coli strain RM10466 chromosome, complete genome	1827	12620	70%	0.0	95%	CP028381.1
Escherichia coli strain ECCRA-119 chromosome, complete genome	1827	12736	70%	0.0	95%	CP029242.1

Table 8. NCBI Blast Result of the Sequence of the Strain *Serratia marcescens*

Description	Max score	Total score	Query cover	E value	Ident	Accession
Serratia sp.XL-10 16S ribosomal RNA gene, partial sequence	1267	1267	89%	0.0	88%	KP262344.1
Serratia sp. JD8-16S ribosomal RNA gene, partial sequence	1266	1266	89%	0.0	88%	KJ191397.1
Serratia marcescens subsp. marcescens strain TSS40 16S ribosomal RNA gene, partial sequence	1262	1262	89%	0.0	88%	MF620086.1
Serratia marcescens strain XG1S-2 16S ribosomal RNA gene, partial sequence	1262	1262	89%	0.0	88%	MF716688.1
Serratia marcescens strain WZ025 16S ribosomal gene, partial sequence	1262	1262	89%	0.0	88%	MF193900.1
Serratia marcescens subsp. marcescens strain X301 16S ribosomal RNA gene, partial sequence	1262	1262	89%	0.0	88%	KU240492.1

4. Discussion

Pectinolytic or pectic enzymes are normally delivered through microorganisms. *Aspergillus niger* is the most commonly used fungus for the production of pectinolytic enzymes at industrial level. Pectinase enzyme isolated through various microbial sources do not only have diverse biochemical and physical properties but their mode of action is also extraordinary. Polygalacturonases extracted from fungi and yeast have the ideal pH (3.5-5.5) and temperature varies from 30°-50°C. Since fruit juices are treated at a higher temperature (up to 70°C), so thermostable pectinases are more appropriate enzymes at commercial level [3, 12]. Exopolygalacturonases, made generally by bacterial species, are basic in nature and easy to reap as well as steady and dynamic at high temperature [3]. Among different microscopic organisms, *Pseudomonas*, *Clostridium* and various types of *Bacillus* are well reported in literature for the production of pectinase enzymes [13]. Pakistan expends a lot of foreign exchange to import pectinase enzymes. With the specific end goal of sparing cash and fulfilling the national economic interests, there is a need to find novel microbes to upgrade the safe production of pectinase enzymes. Jayani *et al*. (2005) reported that *Staphylococcus gallinarum* created critical measures of exopolygalacturonases (2.8 U/µl), whereas *Klebsiella veriicola* and *Klebseilla pneumoniae* created 2.0 U/µl and 1.6 U/µl exopolygalacturonases, respectively. These qualities are comparable with those of effectively revealed bacterial and fungal species that produce various levels of pectinase enzymes [3]. Several studies also reported the production of exopolygalacturonase from bacterial species (*Klebsiella* and *Staphylococcus*) isolated from rotten vegetables and fruits. Novel strains from plants, particularly *S. gallinarum*, can be utilized to create polygalacturonase at commercial level.

In the current study, five bacterial strains were isolated from rotten apples collected from the fruit market of Lahore, Punjab, Pakistan. The biochemical and morphological characteristics showed that the isolates were *S. marcescens, K. pneumoniea*, *P. aeruginosa* and *E. coli*. Their identification was further confirmed by 16S rRNA gene sequencing. Latif and Sohail (2012) also worked on *Klebsiella sp*. and they identified the strain through biochemical tests and then further analyzed it through 16S rRNA gene sequencing [14]. Ausubel and Cramton (2010) extracted genomic DNA from the competent strains of *Pseudomonas* and *Bacillus spp*. through CTAB method. These isolates were then grown in modified MS media at 37°C for 24 hours in shaking condition for the production of extracellular pectinase [15]. Reetha *et al*. (2014) reported that *Pseudomonas fluorescens* produced a higher level of pectinase enzyme as compared to *Bacillus subtilis* [16].

Soreas et al. (2001) worked on bacterial isolates which were purified and subjected to pectinolytic activity after growing on agar medium containing pectin for 24 hours at 37°C. Pectinase activity of pectinolytic strains such as *S. marcescens, K. pneumoniea, E. coli, and P. aeruginosa* was resolved by growing them on the minimal essential media containing 1% pectin as the source of carbon. A small pectin concentration was utilized to avoid the stress created by a higher pectin concentration that hinders the activity of pectinase [17].

The current research reported *S. marcescens* as a novel pectinase producing isolate because *S. marcescens* has never been reported to produce pectinase.

During the cloning of protease, Ohara *et al*. (1989) obtained a clone of *E. coli* which could produce serine protease from *Serratia* spp. [18]. Begum *et al*. (2012) observed the lipase activity of *S. marcescens* by cultivating them in Rhodamine B agar medium [19]. Likewise, Bach *et al.* (2012) also studied the production of keratinolytic protease from *S. marcescens* [20]. So, the major achievement of this research was to screen the most efficient pectinase producing strain *S. marcescens* using rotten apples.

5. Conclusion

Pectinase is an enzyme used to catalyze the pectic substance through depolymerization response. Pectinase is used in food processing industries where the elimination of pectin is essential in the manufacturing of fruit juice, tea, coffee and the extraction of vegetable oil. In this study, different pectinolytic microbial strains were isolated from rotten apples on the MS medium and were identified as *Serratia marcescens, Klebseilla pneumoniea, Pseudomonas aeruginosa* and *Escherichia coli* respectively after different biochemical tests and 16S rRNA sequencing. All four strains showed pectinolytic activity.

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