Isolation And Characterization Of Pectinase Producing Bacteria From Avocado Peel Wastes For Application In Juice Clarification

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Abstract

Background: Pectinase is an enzyme of bacterial origin that has been used to degrade pectin polysaccharide materials in various industries. This study aims to isolate and screen pectinase-producing bacteria from avocado peel wastes and to study the produced pectinase enzyme's application in fruit juice extraction and clarification.

Results: First, four different bacterial strains were isolated from avocado peel waste samples through the primary screening method. These isolates were further screened for pectinase production capability by employing the secondary screening method. Two bacterial isolates (W3 and R5) which had higher pectinase activities were then identified to be *Lysinibacillus macrolides* (W3) and *Serratia marcescens* (R5) respectively. The analysis of pectinase synthesis and application in fruit juice clarification was performed using one of the bacterial strains, *Serratia marcescens* (R5). As a result, the maximum crude pectinase enzyme activity from *Serratia marcescens* (R5) was found in 72 hours of incubation time, at the optimum temperature of 35^{o}C, pH 8, using 1% pectin substrate as carbon sources. This bacterial strain's pectinase enzyme was partially isolated using ammonium sulfate and dialysis. In comparison to crude pectinase (33.68 U/mg), the partially distilled pectinase had a high basic enzyme activity after dialysis (47.32 U/mg). Finally, the percentage yield and clarity of apple, lemon, and mango fruit juices were investigated using both crude and partially filtered pectinase. As a result, for lemon fruit juice processing, the partially filtered pectinase enzyme had the highest percentage yield and clarity of 86.67% and 96.67% percent, respectively.

Conclusions: According to this article, the pectinase enzyme isolated from *Serratia marcescens* has the potential to clarify fruit juices. Further research should focus on a comprehensive evaluation of this enzyme to ensure and improve the efficiency of the bacteria and pectinase enzyme it produces for possible use in the fruit industry and other applications.

Introduction

Enzymes are biological catalysts that help chemical reactions take place under a variety of physicochemical conditions. All enzymes are protein in nature but each has a unique function to perform. Enzymes were first identified in the mid-nineteenth century. Takamine Jokichi was the first to recognize the technical potential of cultivated enzymes and to commercialize them. He was primarily interested in fungal enzymes, but 20 years later, Boidin and Effront in France pioneered the synthesis of bacterial enzymes **[35].**

Most of the traditional manufacturing sectors use a variety of chemicals that are hazardous to the environment. Microorganisms on the other hand can generate a variety of extracellular enzymes that are both environmentally friendly and have functional properties that are similar to their chemical counterparts. Now that people around the world are more aware of the effects of emissions, public

pressure is mounting on businesses and governments to adopt environmentally friendly technologies **[14].**

Industrial enzyme technology today is reliant on microbial sources such as bacteria and yeasts. These microorganisms are important in the production of pectinase enzymes, which are used in biotechnological processes that use pectin as a carbon source **[15].** Pectins are substances found in higher plants with a higher molecular mass. They are a component of the plant's cell wall and middle lamella, as well as a very thin extracellular layer that connects the young cells. The pectin substances are complex colloidal acid polysaccharides with a long galacturonic acid pillar chain and glycoside bonds linking them together. Around 17 different monosaccharides and at least seven different polysaccharides were found in these pectin substances **[30].** Other sugars, such as D-Glucuronic acid, L-Fucose, D-Glucose, D-Mannose, and D-Xylose, are present in the side chains of pectin compounds, according to Anand Nighojkar **[7]**. Pectic acid, Pectinic acid, Pectin, and Protopectin are the four types of pectin substances used as a substrate in pectinase processing, according to the American Chemical Society. The solubility of certain pectic substances in water was one of the most relevant criteria used to identify those **[18].**

Microbial pectinase accounts for 25% of the global food and industrial enzyme market, with market growth occurring regularly. Pectinase is an enzyme that is used in the extraction of fruit juice, clarification of juice, refining of vegetable fibers, degumming of natural fibers, and wastewater treatment [22]. It also speeds up tea fermentation and eliminates the foam-forming property of instant tea powder by destroying the pectin present in tea powder. They're also employed in the process of removing the mucilaginous layer from coffee beans **[32].**

Even though pectinase is used in a variety of industries, the majority of pectinase is produced by fungal organisms. For the degradation of pectic compounds, demand for pectinase has increased in a variety of industrial sectors **[30].** A bacterial source of pectinase is an alternative to meeting this demand for pectinase. Pectinases degrade pectin, resulting in a decrease in viscosity and the development of clusters, which makes separation by centrifugation or filtration easier. As a result, the juice has a clearer appearance and a more intense taste and color **[3].** However, the effective use of pectinolytic enzymes in fruit juice clarification is dependent on the substrate, which contains varying levels of pectin and Pectinases, resulting in better juice extraction and clarification **[45].**

Pectinases are used in both acidic and alkaline environments and are especially useful in the food and textile industries **[38].** To achieve optimal fastened activity with enzymes, application studies with pectinases are underway in global research fields. Pectinase has a wide range of applications due to rising global demand, and some of these enzyme applications are shown in the diagram below in **Fig. 1.** Because of the value of the pectinase enzyme, the main goals of this research were to Isolation and Characterization pectinase - producing bacteria from avocado peel wastes for fruit juice clarification.

Result

Isolation of Bacteria from Avocado Peel

Serial dilution, pour plating, and streaking isolation methods were used to isolate potential bacteria from avocado peel wastes. To obtain the pure isolate, the isolate was sub-cultured into a new growth medium **[Figure 2].** The four pure isolates were obtained after extensive isolation techniques. To make it easier to distinguish between them, the pure isolates were labeled W3, W32, R5, and Y31 **[Figure 3].**

Primary screening

There was a strong (hydrolysis) zone among all four bacterial isolates, indicating the existence of pectinase activities. The diameter of each hydrolysis zone was calculated to determine the potential bacterial isolate. Isolate R5 measured the largest diameter around the colony at 20.54 ± 1.32 **[Table 1].**

Table 1
Primary Screening of pectinase producing bacterial isolates using zone of inhibition methods

S. No.	Isolate code	Clear Zone Diameter (in mm)
1	W3	14.08 ± 0.75
2	W32	10.24 ± 0.54
3	Y31	12.86 ± 1.80
4	R5	20.54 ± 1.32
*SD represented standard deviation		

Secondary screening

The activities of crude pectinase were measured in the secondary screening. The selected isolates from the primary screening method were subjected to fermentation media and their behaviors were assessed to be further screened. 0.4ml of the liquid sample was taken to pectinase activates by using phosphate buffer after 24 hours of incubation in the production media. Isolate R5 had the highest pectinase activity of 5.41 ± 0.14U/ml, while isolate Y31 had the lowest pectinase activity of 2.49 ± 0.23 **[Table 2].**

Table 2
Secondary screening of crude Pectinase activities in unit/ml

S. No.	Isolate code	Pectinase activities	Relative activities
1	W3	3.09 ± 0.17	57.12
2	Y31	2.49 ± 0.23	46.02
3	R5	5.41 ± 0.14	100
* Values is mean ± standard deviation of replicates			

Morphological and biochemical Identification of Bacterial isolates

Microscopically, isolates R5, and Y31 was found to be Gram-negative bacteria, while isolate W3 was found to be Gram-positive bacteria using Bergey's Manual of Determinative Bacteriology. The isolates were tentatively described as *Bacillus species, Serratia spp.*, and *Erwinia species***[Table 3]***using* ABIS-online software.

Table 3
Morphological and biochemical characterization of bacterial isolates

Characteristic	Bacterial isolates		
	R5	**W3**	**Y31**
Morphological features			
Color	Creamy red	White	Yellow
Shape	Rod	Rod	Long rod
Surface	Smooth	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque
Gram staining	Negative	Positive	Negative
Biochemical characteristic			
Sucrose hydrolysis	Positive	Positive	Positive
Lactose hydrolysis	Negative	Negative	Positive
Glucose hydrolysis	Positive	Positive	Positive
Indole test	Negative	Negative	Negative
Methyl red test	Negative	Positive	Positive
Urease test	Negative	Negative	Positive
Voges - Proskauer test	Positive	Negative	Positive
Casein hydrolysis	Negative	Positive	Negative
Citrate utilization test	Positive	Negative	Negative
H_2S test	Negative	Negative	Positive
Catalase test	Positive	Positive	Positive
Similarity of bacteria	*Serratia spp*	*Bacillus spp*	*Erwinia spp*

Molecular Identification of Isolates.

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using genomic DNA from selected bacterial isolates as templates. The genomic DNA and PCR products were analyzed using agarose gel electrophoresis, as shown in **Fig. 4.** To obtain the right sequences, the PCR products were filtered and sequenced. Each isolate's sequences were uploaded to the NCBI database and compared to previously published sequences. The closest neighbors of the isolates **R5-MN932109.1**Serratia marcescens and **W3-MN932110.1** ***Lysinibacillus macrolides*** are queried using NCBI BLAST (HTTP://www.ncbi.nlm.nih.gov/Blast). The nucleotide *Serratia marcescens* strain (MN508465.1) and

Serratia marcescens C1 (GU220796.1)16S ribosomal RNA gene sequences from R5 quest (mBLAST, NCBI) showed 99 percent homology. **Figure 5**, shows how the phylogenic tree of *Serratia marcescens* was built and the sequences of W3 search (mBLAST, NCBI) showed 86% similar to the nucleotide of the *Lysinibacillus sp.* N121 (JQ900521.1) and 85% similar to the nucleotide of *Lysinibacillus macrolides* strain SS-86(KX959971.1) the phylogenetic tree of *Lysinibacillus macrolides* shown in **Fig. 6**.

Production of pectinase and its optimization

The development of a broad hydrolysis zone on pectin agar plates was used to characterize the screened pectinolytic bacterial isolates. Fermentation and quantitative screening of isolate R5-*Serratia marcescens* were performed, with pectinase activity being measured. After 48 hours of fermentation, the maximum pectinase activity was 5.41 ± 0.14U/ml.

Effect of fermentation time on pectinase production

To get the most pectinase production from isolate R5-*Serratia marcescens*, we selected five incubation times (hours) that were used. Pectinase activity was increased gradually over 24 hours of incubation time until optimum pectinase activity was achieved. After the optimum incubation period, the pectinase activities began to decrease. In this analysis, the optimum fermentation period for pectinase development was found to be 72 hours, and the highest pectinase activities were found to be 6.86 ± 0.32U/ml of released glucose when pectin was used as a substrate. Then similar 72 hours incubation time high relative Pectinase activates in 95%.

Effect of pH on pectinase production

The effect of different pH of fermentation medium on pectinase production by bacterial isolate R5-*Serratia marcescens* was studied. As the result of this study indicated in pH, the pectinase production could be affected due to the variations of pH medium. The pectinase activities were increased up to the optimum pH and then decreased activity after the optimum pH value. The production medium which was adjusted at pH 8 produced maximum pectinase activity (8.84 ± 0.34 U/ml) followed by pH 7 (6.66 ± 0.73U/ml).

Effect of temperature on pectinase production

The effects of temperature on pectinase production by bacterial isolate R5-*Serratia marcescens* indicated that maximum pectinase activity was obtained at 35^0C. As the result showed, pectinase activity was increased as temperature increases and slightly declined after an optimal temperature of 35^0C. However, pectinase activity was not completely lost even if the temperature increased to 50^0C. Although the maximum pectinase activity was recorded as 7.76 ± 0.56U/ml of glucose released at 35^0C, pectinase activities above the optimal temperature were greater than the pectinase activity below the optimal temperature.

Effect of substrate concentration on pectinase production

The effect of substrate concentration on pectinase production by bacterial isolate R5-*Serratia marcescens* showed antagonistic effects after 1% of pectin. As the result observed in **Table 4**, pectinase activity increased with increasing the concentration of substrate (pectin) up to the optimal concentration of pectin and declines after the optimal substrate concentration.

Table 4
Effect of substrate concentration on pectinase production in isolate R5 (*Serratia marcescens)*

Substrate Concentration (%)	Pectinase activities (U/ml)	Relative Activities (%)
0.25	4.99 ± 0.30	56.00
0.5	7.53 ± 0.30	84.51
0.75	7.92 ± 0.31	88.66
1	8.91 ± 0.23	100
1.25	8.22 ± 0.46	92.56
1.5	7.94 ± 0.80	88.72

Purification of pectinase and determine protein concentration

Pectinase precipitation is an essential method of concentration and an ideal step in the purification process. One of the most well-known and widely used methods of purifying and concentrating pectinase, especially at the laboratory scale, is salting-out proteins, particularly with the use of ammonium sulfate. Pectinase was isolated from isolate R5-*Serratia marcescens* and subjected to various saturation levels of ammonium sulfate varying from 30–90 percent. The pectinase activities and protein content partially purified by ammonium sulfate are presented in **Table 5.**

Table 5
Purification profile of pectinase production in isolate R5 (*Serratia marcescens)*

Purification	Total Volume (ml)	Total activity (U/ml)	Total Protein (mg)	Specific activity (U/mg)
Crude pectinase	200	1935	57.45	33.68
Ammonium Sulphate (70%)	75	773.85	20.1	38.5
Dialysis	40	473.28	10	47.32
*Total activity = enzymes activity x total volume,				
*Total protein = protein x total volume				
*Specific activity = total enzyme activity/ total protein content.				

Application of Pectinases in Fruit Juice clarification and yield

This study tried to determine the effect of pectinase on the volume of juice, juice yield, and juice clarity in terms of transmittance by using apple, lemon, and mango fruits. The experiments were carried out with crude pectinase, partially purified and water as control. According to the results shown in **Table 6**, the volume of lemon juice was enhanced by three folds from control to crude pectinase (10–13 ml) and two folds increment from purified pectinase (13-14.5ml). The same experiment was performed by using mango fruit. Here, pectinase and water treatment for 1hours resulted in two folds enhancement in increasing the volume of mango juice from control to crude pectinase enzyme (8-10ml) and increased almost three folds from crude pectinase to purified pectinase (10-13.5ml).

Table 6
Application of pectinase Vs juice yield

Fruits (15ml)	Characteristics	Crude Pectinase	Purified Pectinase	Control
Apple	Volume (ml)	11	14	8
	Yield %	73.33%	93.33%	53.33%
Lemon	Volume (ml)	13	14.5	10
	Yield %	86.67%	96.67%	66.67%
Mango	Volume (ml)	10	13.5	8
	Yield %	66.67%	90%	53.33%

The same experiment was performed to express the yield of juice in terms of percentages. As the above table indicates, the percentage of juice yield was improved starting from control to purified pectinase **(Table 6).** The yield variation of apple juice between control and crude pectinase observed was 20%. In the same trend, the 20% of apple juice yield variation was recorded between crude pectinase and purified pectinase. Therefore, apple fruit showed consistent variation from control to crude and crude to purified pectinase. The yield of lemon juice variation between control and crude pectinase was 20%, but the variation between crude pectinase and purified pectinase was recorded as 10%. Therefore, the variations of lemon juice yield from control to purified pectinase were not consistent. In mango juice, yield also showed 13.34% of variation from control to crude pectinase and 23.33% of juice yield variation was recorded between crude pectinase and purified pectinase. The maximum yield of juice treated by purified pectinase among the three fruits was recorded in lemon fruit as 96.67% followed by apple juice which was recorded as 93.33% and 90% yield of juice was recorded as the minimum amount in mango fruit.

Effect of pectinase on juice clarification

The effect of pectinase on juice clarity was determined by the transmittances of the clarified juice in terms of percentage **(Fig. 7)**. As shown in three different fruits [apple, lemon, and mango] were subjected to crude and purified pectinase by taking water as control, the maximum clarity of juice was obtained was 95.82% of transmittance for lemon fruit. The transmittance of lemon juice treated with purified pectinase was greater by 16.83% of transmittance compared to the result obtained for crude pectinase, which was recorded as 78.99% of transmittance of juice clarity.

Discussion

In primary screening, hydrolysis of isolate R5 is almost identical to those of Hitha **[16]**, who obtained 22.8mm and 20.5mm, respectively, in their previous study reported by Varghese **[46].** The hydrolysis zone of isolate (W3), in particular, was linked to the last tomato isolate **[29].** Similarly, the results of each isolate's secondary screening method are more similar to the previous investigation from decomposing Orange Peels mentioned by Siddiqui **[39].** Pectinase-producing bacteria were isolated from coffee pulp, according to Oumer **[31]** and the results of the secondary screening method were lower than isolate R5. Biologists have used a series of biochemical tests to distinguish closely related bacteria in the detection of bacteria; Bergey's Manual of Determinative Bacteriology **[10]** and ABIS-online software were used to identify pectinolytic bacteria based on their morphological and biochemical characteristics, http://www.tgw1916.net/bacteria_abis.html. **[46].** In molecular identification, the neighbor-joining method was used to build the phylogenic tree based on the 16S rRNA gene sequences of isolate R5 and W3 and associated nucleotide sequences **[26].**

Molecular characterization of pectinase production with Serratia species was investigated by different researchers. *Serratia rubidaea* (E9.HM585373) was isolated from tomato fruits and characterized using the 16S rRNA method, this bacterial species found to be producing polygalacturonase at a temperature of 40^{o}C as reported by Abd-alla **[2].** About 20 enzyme-producing bacterial strains were isolated from

municipal solid waste using the 16S rRNA method. Among those bacterial strains, most of the strains were *Bacillus species*. The only two bacterial strains *Serratia marcescens* (MH194203) and *Lysinibacillus species* (MH194187) were related to this finding **[36].** Similarly, *Serratia oryzae* strain S32 (SOZ00000000.1) was isolated from lake water and proved that bacterial strain can produce pectinase as investigated by Hugouvieux **[17].**

Pectinase-producing bacillus species have been identified from fruit wastes and agricultural wastes at the biochemical and molecular level by different researchers. According to Rehman **[34]** investigation, Oumer **[31]** study about pectinase producing microbes from the coffee pulp processing area; they identified various bacillus species in molecular method and they reported that *Bacillus subtilis* Btk27 has the highest potential of pectinase production as compare with the other strains. **Mahalik [26]** reported that *Lysinibacillus species* isolated from estuaries of Odisha and molecularly identified for cellulase production. *Lysinibacilli* obtained from fruits and vegetable waste, this species was identified at the molecular level and applied in Protease production as reported by **Ahmad [5].** Only one possible R5 (*Serratia marcescens*) bacteria were selected for further optimization analysis.

The pectinase, which was developed in just 24 hours of incubation, was found to be two times more potent than the 2.43 U/ml pectinase obtained by *Bacillus sonorensis* in the same incubation period by **Mohandas [28].** The enzyme activity results obtained after 72 hours were similar to those obtained by **Jayani [19].** Who obtained 7.88l/ml of pectinase activity after 72 hours of fermentation time using *Bacillus sphaericus*.

This study finding shows that this bacterial isolate R5-*Serratia marcescens* require alkaline condition in pectinase production processes **[13].** According to the investigations by **Mohandas [28]**, the highest pectinase activities 2.43 U/ml were recorded at alkaline pH 8 by *Bacillus sonorensis*. According to Sohail and Latif **[43]**, the optimal polygalacturonase production of *Bacillus mojavensis* was at pH 8.0. *Streptomyces species* require the slightly alkaline condition of pH 8.5 for maximum pectinase production.

Temperature is one of the most important parameters essential for the success of pectinase production. According to Kothari **[25]**, the maximum polygalacturonase activities were produced at a temperature of 35^{o}C by *Erwinia carotovora*. In the same bacterial species (*Erwinia spp)*, the highest pectinase (polygalacturonase and pectin lysate) activity was recorded at the temperature of 35^{o}C **[40].** Other recent investigations indicated that the highest amount of pectinase produced by *Enterobacter tabaci* NR1466677 was at the optimal temperature of 35^{o}C **[29].**

In this study, various concentrations of pectin substrate were subjected to a fermentation medium to produce pectinase as a carbon source for bacterial growth. In this current study, the pectinase activity data seem to be comparable to works of Darah **[11]** in which they obtained maximum polygalacturonase activity at 1% of pectin concentration for *Enterobacter aerogenes NBO2*. Some bacterial species such as *Enterobacter tabaci NR14667* could produce the highest pectinase activity at 0.3% of pectin

concentration as reported **[29].** More recently, maximum pectinase activity at 2% of citrus pectin concentration was also recorded for *Chryseobacterium indologenes strain SD* **[21].**

From crude to dialyzed pectinase, the unique activities of pectinase increased. With 10 mg of protein concentration, the maximal activity was 47.32U/mg, suggesting that the protein molecules separated by ammonium mainly contained the enzyme pectinase and that the proportion of protein other than pectinase was higher in the crude form of the enzyme **[8].** Purification steps also resulted in the removal of interfering materials found in the crude cell-free sample, allowing for increased enzyme activity **[44].** From application pectinase, the volume of juice treated with both crude and purified pectinase was varied with the type of fruits used in the process. As the results indicate, the highest juice volume was obtained from pectinase-treated lemon fruit which might be due to the presence of solubility of pectin in lemon fruit **[41].** Similarly, the volume of juice variation from fruit to fruit that treated with pectinase was investigated by using different fruits such as strawberry juice (7–10 ml), Grape juice (15-21.5ml), apple juice (12-18.5ml), peach apple juice (12-10.5ml), chary apple juice (8-10ml) and orange juice (9-10ml) **[12].** The result of juice clarity treated with crude and purified pectinase were 1.99% and 18.82% of transmittance greater than the previous report by Maktouf **[27]** respectively, which was 77% of transmittance for pectinase using lemon fruit as raw material for juice clarification. The result of mango juice transmittance obtained in this current experiment was happened to closely relate to the report made by Kumar **[25]**, who obtained 92.5% of transmittance after 150 minutes of incubation time. The effect of pectinase on juice clarification was also applied in apple fruit. According to Yuan **[47]**, the clarification of apple juice treated with pectinase was increased by 71.8% of transmittance. In their experiment, the clarification of apple juice has increased to 84 % of transmittance.

Conclusion

The use of pectinolytic bacteria to produce pectinase and the use of pectinase in fruit juice clarification was found to be an effective method. Since fruits contain a high amount of pectin, the extraction of fruit juice has historically resulted in cloudy, unappealing color and high viscosity. Several attempts have been made in the past to produce pectinase using bacterial strains as an alternative technology for juice clarification. This study aimed to isolate and screen pectinase-producing bacteria from avocado waste for juice clarification. Avocado wastes were found to contain four distinct bacterial strains. On an agar plate assay, three bacterial strains displayed pectinolytic activity. Submerged fermentation was used to screen the pectinolytic bacterial strain for pectinase activity, and isolate R5 was found to have a higher pectinase enzyme-producing capacity. The strain was classified as Serratia species based on morphological and biochemical characteristics and was later identified as *Serratia marcescens* using 16S rDNA sequences. Fermentation time, pH, and temperature, substrate concentration, were all tested on this isolate. At 72 hours, pH-8, 35^{o}C, and 1% substrate concentration, the best optimum pectinase activity was 8.91U/ml. Ammonium sulfate and dialyzes were used to purify the pectinase enzyme. From crude to dialyzed pectinase, the basic activity of refined pectinase was increased by 0.3 fold and used to clarify fruit juice. For the lemon juice, the maximum clarity of treated juice reported was 96.67 percent. The

pectinase enzyme isolated from *Serratia marcescens* has a promising potential to explain fruit juices, according to this report. Further research should concentrate on a thorough evaluation of this enzyme to ensure and enhance the efficiency of the bacteria and pectinase enzyme it generates for potential use in the fruit industry and potential application.

Materials And Methods

Sample collection:

The avocado peel wastes were collected from the juice processing site of ECOPIA PLC. This private limited company is located behind leger general hospital, Addis Ababa, Ethiopia. The samples were transferred into sterilized plastic bags and brought to the microbiology laboratory. The sample containing bags were closed and stored in a 4°C refrigerator until the time of the analysis.

Serial dilutions:

One gram (1g) of the avocado sample was suspended in 9mL of sterilized distilled water and was properly mixed. The mixtures of 1g avocado and 9mL of distilled water were serially diluted 10^{-1} to 10^{-6}**[34].**

Media preparation

The media for bacterial growth was yeast extract pectin agar, which was prepared by dissolving 1 % yeast extract, 1 % pectin, 0.5 % sodium chloride, 1.5 % agar, and 100mL sterilized distilled water as directed by the manufacturer **[21].** A pH meter was used to set the pH of the medium to 7. The media was sterilized for 15 minutes at 121^0C. After sterilization, about 25mL of sterilized nutrient agar media was poured onto sterilized Petri plates and allowed to solidify at room temperature in a microbiological hood.

Isolation and purification

For isolation of bacteria, 200µL aliquots of samples from the 10^{-2}, 10^{-4}, and 10^{-6} dilutions were inoculated and spread onto pectin agar medium plates using a metal spreader which has been sterilized by dipping in 95% ethanol and flaming. An un-inoculated culture medium was kept as a control. The plates were incubated at 30^0C for 24 hours in an incubator. The growth of bacterial colonies was observed after 24 hours of incubation time. The next task was purification and preservation of the culture. The individual colonies which have similar character and size were isolated from cultured plates and transferred to new agar plates to obtain pure colony by repeated streaking method. The purity of the test isolate was assessed using colony morphology and microscopy; pure colonies of bacteria were preserved with 20% of glycerol and stored at -80^0C for further study **[37].**

Primary screening of pectinase producing bacteria isolates

All pure colonies from freshly activated plates were transferred to new pectin agar media and incubated at 30^0C for 48 hours. At the end of incubation time, 0.3% of Congo red solution was flooded onto the Petri

dishes and left for 10 minutes. This solution formed a clear zone around the colonies which indicates that bacterial isolates have abilities to produce pectinase and the diameter of clear zones is proportional to the bacteria's relative pectinase production capacity **[9].**

Secondary screening of pectinase producing bacteria isolates

The bacterial Isolates showing maximum clear zone on primary screening media were considered as the highest pectinase producer **[34].** Those bacterial Isolates which have a higher clear zone were subjected to submerged fermentation for pectinase production using the same medium as primary screening but without agar. The freshly cultured bacterial isolates 0.2ml in yeast extract broth were inoculated on 99.8ml of sterilized production media in 250mL of the flask and incubated at 30^0C on a rotary shaker at 125rpm for 48 hours. At the end of incubation time, the media was transferred to test tubes and centrifuged at 10,000rpm for 10 minutes. The supernatant was used as crude enzymes to evaluate the efficiency of bacterial isolates on the production of pectinase activities by using sodium acetate buffer pH6.8 **[1, 14].**

Determination of pectinase activity

Pectinase activity was determined by measuring the amount of released reducing sugar under assay conditions or by enzymatic degradation of pectin as described Nelson-Somogyi methods. The procedure was started by mixing 1 ml of substrate solution that was prepared by Phosphate buffer (pH 7) and 0.4 mL of clear supernatant enzyme in test tubes. This mixed solution was incubated at 40^oC in the water bath for 40 minutes. 0.3mL of Somogyi copper reagent was added to the mixture. The test tubes were incubated in a boiling water bath for 10 minutes. After incubation times, the tubes were cooled to room temperature and 0.3mL of the Nelson arsenomolybdate reagent was added. The solution was cooled to room temperature and the absorbance of this solution was measure at 540 nm after centrifuged at 10,000rpm for 10 minutes by taken supernatant as the enzyme. The amount of released glucose per milliliter per minute was calculated from the standard curves by using D-glucose (10–100 micromoles). One unit of pectinase activities was defined as the amount of glucose released in the term of μmol of reducing sugar per ml per minute under Standard assay conditions **[21].** The pectinase activity was calculated by using the following formula **[23].**

$$\text{Pectinase Activities } (\mu\text{mol/ml/min} = \frac{\mathbf{RG(\mu moL/mL)} \times \mathbf{TVA(mL)}}{\mathbf{T(min)xVEA(mL)}}$$

Where **RG** is released glucose obtained from D-glucose standard curve

TVA is the total volume of assay

T is the Incubation time

VEA is the volume of enzyme used to assay

Morphological and Biochemical test for Identification of bacterial Isolates

A single colony grown on pectin agar medium was smeared on a glass slide and heat-fixed. The smear was covered with crystal violet and washed with sterilized distilled water. After that, the slide was flooded with Gram's iodine solution and washed again by sterilized distilled water. To remove the color, the slide was decolorized with 95% (V/V) ethyl alcohol. Then, the slide was washed with sterilized distilled water and counterstained with Safranin solution. Finally, the smeared slide was air-dried and observed under the microscope. The following various biochemical tests: Carbohydrate fermentation tests, Indole test, Methyl red (MR) test, Vegas-Proskauer (VP) test, Citrate utilization test, Hydrogen sulfide production test, Catalase test, and Urease test were used for identification of bacterial isolates **[4].**

Molecular Identification and PCR amplification of screened isolates

The potential isolates which were screened and selected on the primary and secondary screening method (R5 and W3) were identified using molecular techniques. The genomic DNA of the isolates was extracted by using the Bacterial Genomic DNA extraction kit according to the manufacturer protocol (QIAGEN, QIAamp DNA Mini Kit) with some modification. The PCR mixture consisted of 10 μl the master mix containing 10x Taq buffer, 10mM dNTPs, 25mM of MgCl**2**, 1U of Taq DNA polymerase), 1μl of forwarding primer, 1μl of Reverse primer, 2 μl of Genomic DNA, and 6μl of PCR grade water were added and the PCR amplification was done. The total time duration for amplification was 2:35. The PCR products were visualized on 0.8 % agarose gel stained with DNA-safe stain. Finally, the PCR products were sent to be sequenced, the obtained sequence data were analyzed using the basic local alignment search tool (BLAST) software (http://www.ncbi.nlm.nih.gov/blast) against the 16S ribosomal RNA sequence database and the mega X software was used to generate the phylogenetic tree from the national center for biotechnology information **[26].**

Production of crude pectinase by submerged fermentation

The production of pectinase by submerged fermentation was conducted as described by Ajit Kumar and Rita Sharma **[6].** The bacterial isolate identified as a potential candidate from primary and secondary screening methods was selected for the production of this crude enzyme. About 2mL of the overnight bacterial culture was inoculated to a pre-sterilized fermentation medium that contained 100ml of 1% yeast pectin media in a 250 ml volume conical flask to maintain pH 8 **[21].** And incubated at 30^0C using a rotary shaker at 125 rpm for 48 hours. At the end of the closed fermentation time, the production medium was centrifuged at 10,000 rpm for 10 minutes. The clear supernatant was used as crude polygalacturonase to evaluate polygalacturonase activities **[42].**

Optimization of pectinase production

The production of pectinase was optimized by using four parameters namely, fermentation time, temperature, pH, and substrate concentration. The relative activity of each parameter was calculated as the percentage by using the following formula:-

$$\text{Relative Activity} = \frac{AS}{MS} \times 100$$

Where **As** = the activities of sample in micromole per milliliter.

Ms = the maximum activities of sample in micromole per milliliter

Optimization of fermentation time on pectinase production

To examine the influences of fermentation time on enzyme production by bacterial isolate, the production media was prepared by constant pH 7 and 1% substrate concentration. The production media was sterilized at 121^{o}C for 15 minutes. This sterilized production media was inoculated with 2 ml of overnight culture bacteria and incubated at a temperature of 30^{0}C using a rotary shaker with 125 rpm for 120 hours and the activities of polygalacturonase were assayed in 24 hours intervals **[41].**

Optimization of pH on pectinase production

To investigate the influences of pH variation on enzyme production by bacterial isolate, the pH level of production media was adjusted to a pH5 to pH10 using 0.1M sodium acetate and 0.1M Sodium hydroxide **[35]** with a few modifications. The production media was sterilized at 121^{o}C for 15 minutes and inoculation with 2ml of overnight bacterial cultured bacteria. This inoculated media was incubated at 30^{o}C using a rotary shaker with 125 rpm for 72 hours after which the enzyme activities were assayed.

Optimization of temperature on pectinase production

To investigate the influences of temperature variation on enzyme production using bacterial isolate, temperatures maintained for the production of the enzyme were 25, 30, 35, 40, 45, and 50^{o}C **[24].** The production media (pH 8) was inoculated with 2 ml of overnight cultured bacteria and incubated using a rotary shaker at 125 rpm for 72 hours. After the end of the fermentation times, the enzyme activities were assayed.

Optimization of substrate concentration on pectinase production

To study the influences of substrate concentration on enzyme production using the bacterial isolate, the enzyme was produced by various concentrations of substrates (pectin). The concentrations of substrate maintained for enzyme production were 0.25%, 0.5%, 0.75%, 1%, 1.25%, and 1.5 % **[20].** The sterilized production media (pH 8) was inoculated with 2 ml of overnight cultured bacteria and incubated at 30^{o}C using a rotary shaker at 125 rpm for 72 hours. After the end of the fermentation time, the enzyme activities were assayed.

Purification of pectinase by Ammonium sulfate precipitation

The crude enzyme was partially purified by using Ammonium sulfate precipitation methods as described by Ramalingam **[33].** To avoid the denaturation of enzymes, all purification steps were carried out in a cold environment using an ice bath and temperatures of 40°C. The addition of four different saturation levels of ammonium sulfate (30 percent, 50 percent, 70 percent, and 90 percent) precipitated about 150 milliliters of crude enzyme. Any of the precipitated enzyme proteins was then dissolved in sodium phosphate buffer and dialyzed using a dialysis membrane after completing all of the above steps **[24].**

Application of Pectinases in Fruit Juice clarification

The purified pectinase was applied in the fruit juice-making process to test the clarity of the juice. Three different fruits which have the sign of physical damages (Lemon, Mango, and apple fruits) were bought from a fruit market (Akaki) and brought to microbiology laboratories for juice preparation.

Juice preparation

Lemon, Mango, and apple fruits were washed carefully and chopped into smaller sizes on a side with a sharp knife. Twenty grams (15 g) of each of the chopped fruits were weighed into separate beakers. Those chopped were treated with crude pectinase, purified pectinase, and untreated samples kept as controls in which the enzyme was replaced by distilled water. The controls and enzyme-treated samples were incubated in a water bath at 40°C for 1hour, and the activity was stopped by cooling in an ice bath. Then, each juice was filtered through filter paper before measuring the volume and juice yield. The filtered fruit juice after enzymatic treatments was pasteurized at 60 ºC for 20 min. The juice yield was estimated as a percentage of the weight of the juice obtained according to the method of Rai **[32].** The formula is:

$$\text{juice yield}[\%] = \frac{weight\ of\ juice}{weight\ of\ fruits} x100$$

Clarification of juice

Using a UV spectrophotometer and the **[38]**, procedure, the clarity of each fruit juice was measured in terms of percentages of transmittances. After heating at 40°C to inactivate any natural fruit enzymes or microbes present, around 8ml of each fruit juice was taken and cooled in a water bath before adding pectinase enzyme product. In 8ml of fruit juice, the same amount of enzymes (2ml) was added. The samples were incubated for 4 hours before being heated for 3 minutes at 40°C. The juice was centrifuged for 20 minutes at 3000 rpm, the supernatant was filtered out with filter paper, and the clarity of the juice was calculated by measuring the absorbance at 660 nm with a UV spectrophotometer, distilled water was used as a blank, and the clarity was expressed in percentages **[16].**

Abbreviations

- °C - Celsius
- g - Gram
- ml - milliliter
- mg - milligram
- rpm - revolutions per minute
- µmol - micromoles
- µL - microliters
- nm - Nanomolar
- min - minutes
- U - Units
- PCR - *Polymerase chain reaction*
- BLAST - Basic Local Alignment Search Tool
- DNA - Deoxyribonucleic Acid

Declarations

Ethics Approval and Consent to Participate

There was no human or animal participation in the research, and no data was collected.

Consent for Publication

All of the writers agreed to the publication of their work.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The genome sequence data for R5 and W3is available in the GenBank repository under the project of Addis Ababa Science and Technology University (Website: https://www.ncbi.nlm.nih.gov/nuccore/MN932109.1 andhttps://www.ncbi.nlm.nih.gov/nuccore/1796387721)

Competing Interests

The authors declare that they have no competing interests.

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Contributions

All authors have carried out the avocado peel samples were collected from the dumping site of ECOPIA PL randomly from a decayed avocado waste using a pre-sterilized spatula. MT & SH carried out the isolation of potential bacteria. SH & CM carried out the identification of bacteria. All authors are contributed by the final confirmation of potential Pectinase-producing bacteria and manuscript revisions. All authors have approved the final version of the manuscript and agree to be held accountable for the content therein.

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Figures

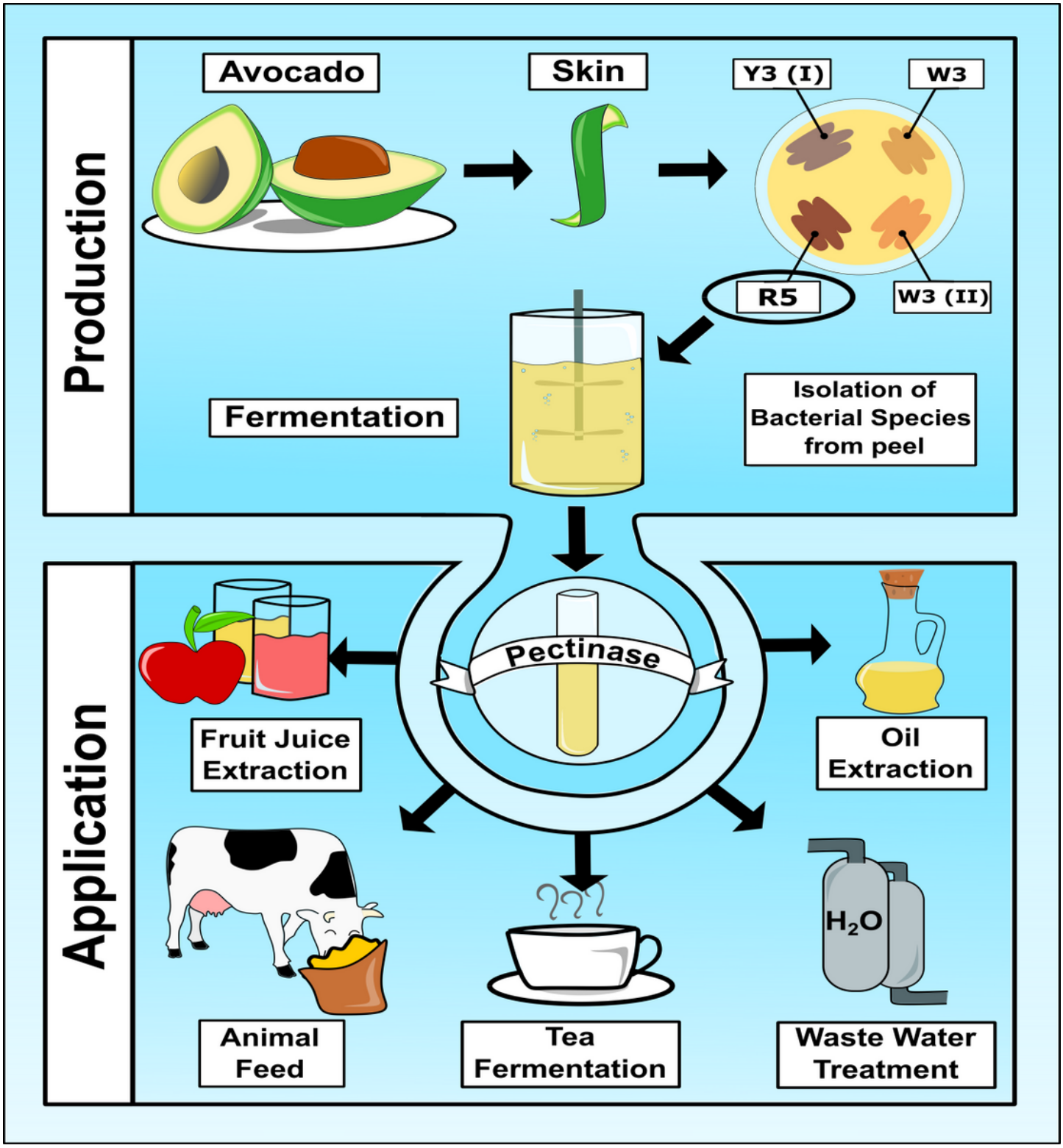


Figure 1

Isolation of pectinase bacteria, Production, and Application of Pectinase

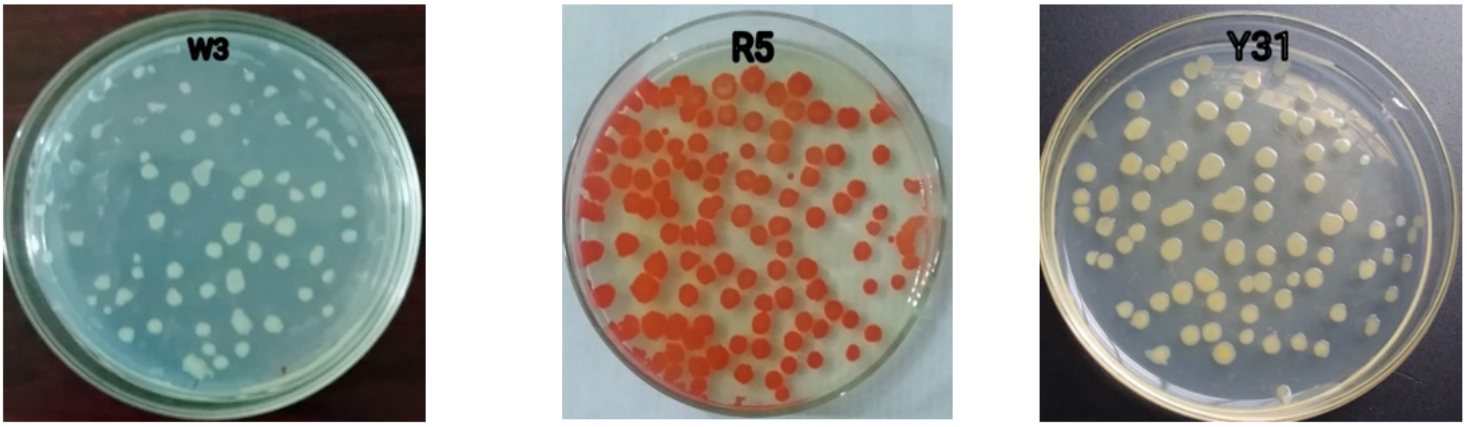


Figure 2

Primary screening of isolates on pectin agar medium

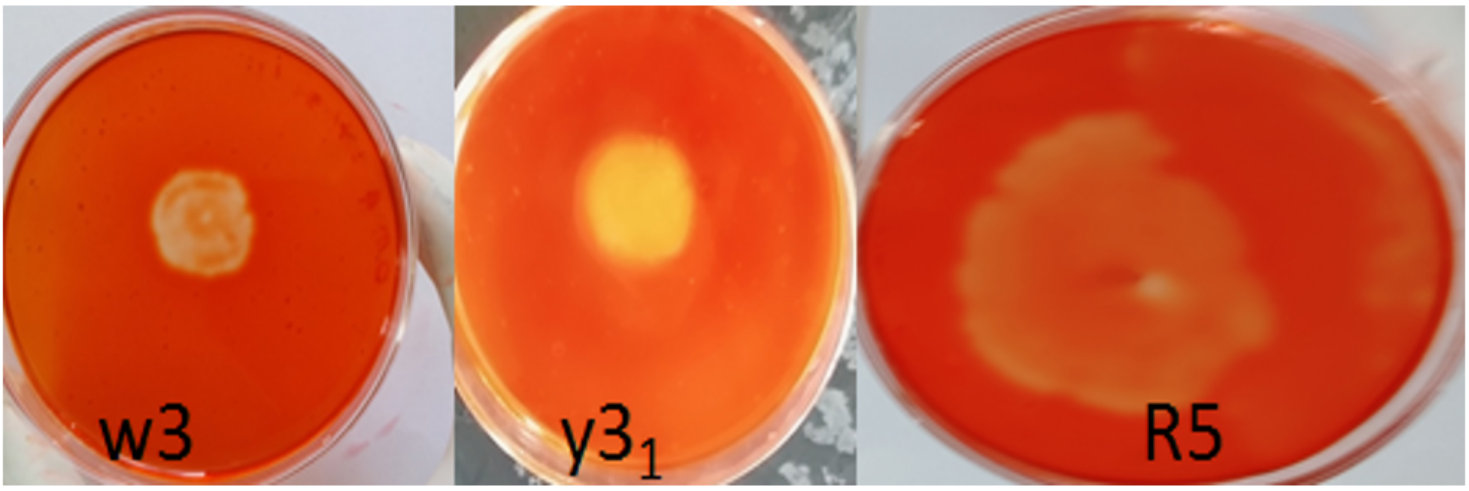
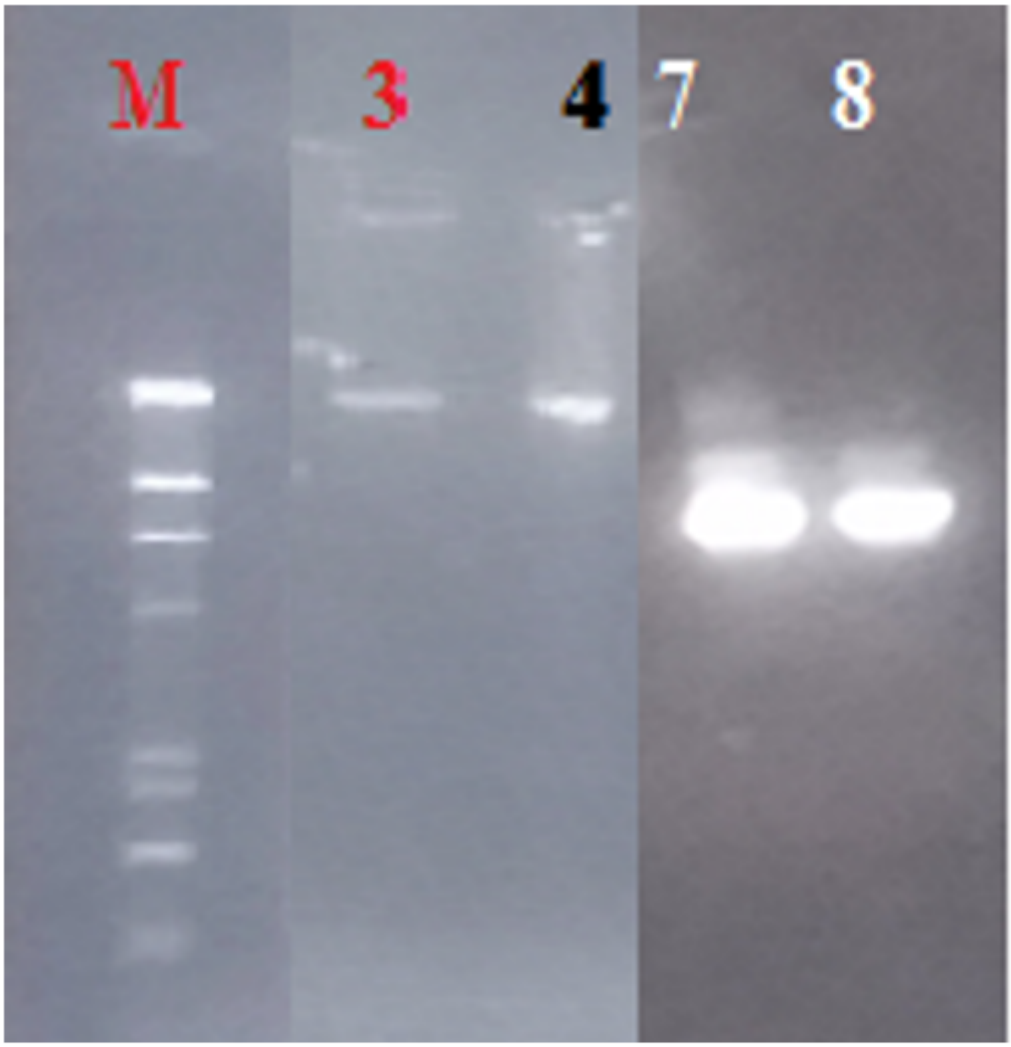


Figure 3

Secondary screening of isolates on pectin agar medium



M- DNA marker
3-4 - Genomic DNA of isolate R5 & W3
7-8- PCR product of R5 &W3 DNA respectively

Figure 4

Agarose gel electrophoresis (DNA extracted from bacterial isolates R5 and W3)

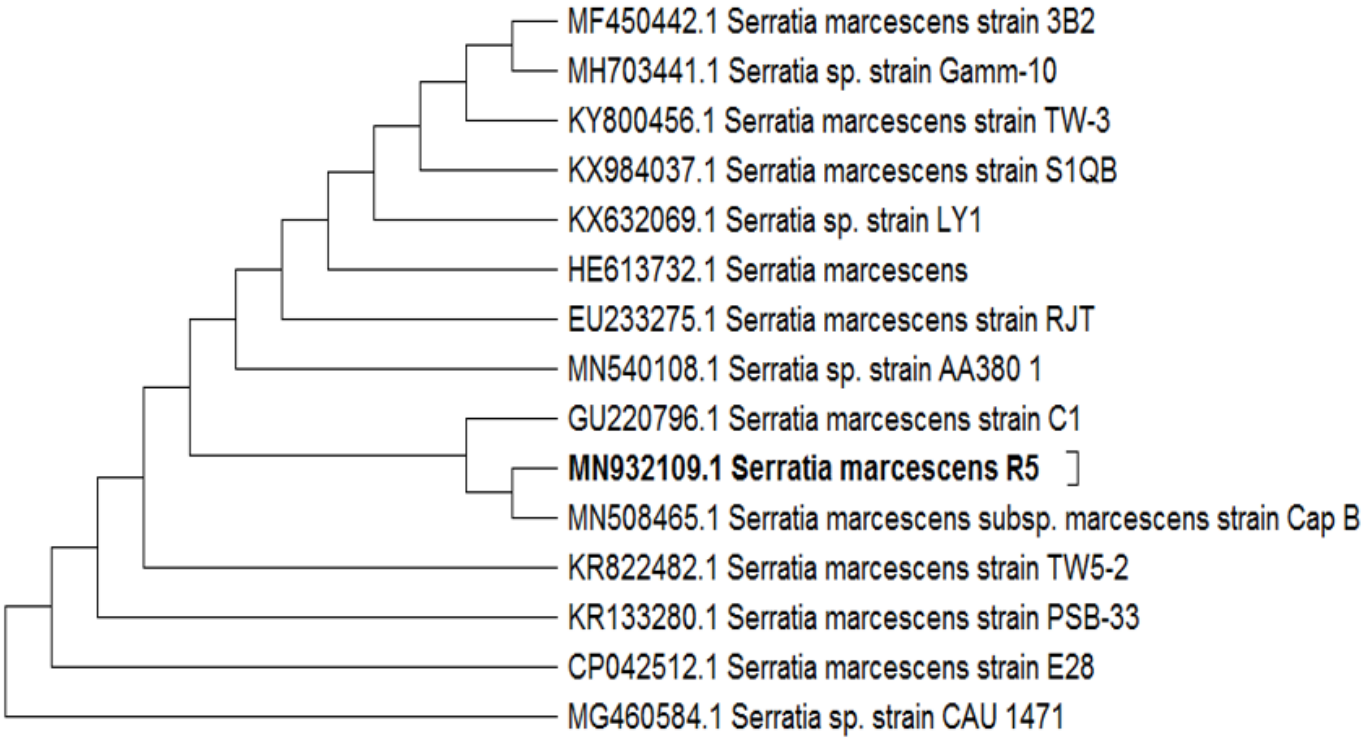


Figure 5

Phylogenetic tree constructed based on 16S rRNA gene sequences of Serratia marcescens _R5 with other Serratia species obtained from GenBank database.

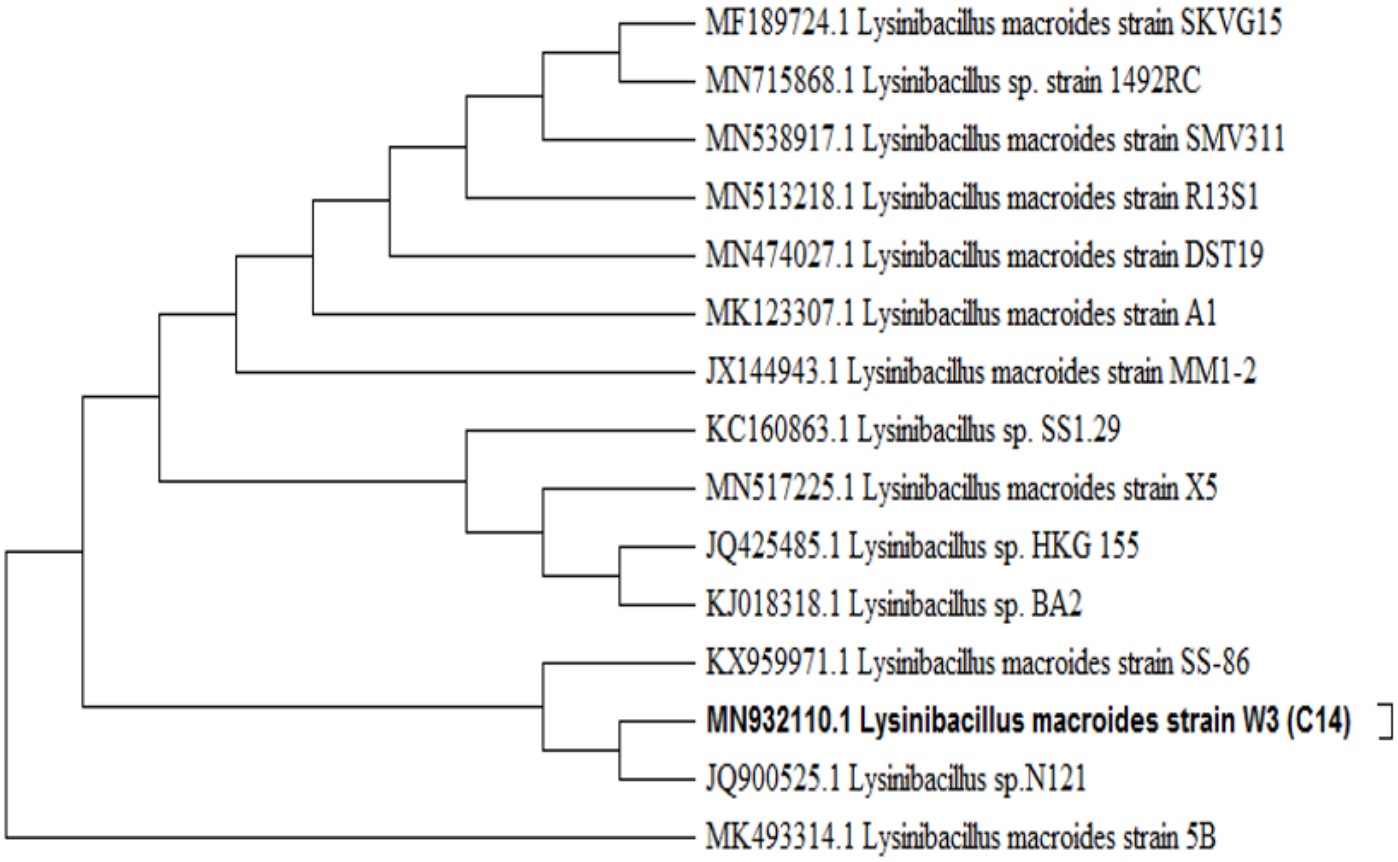


Figure 6

Phylogenetic tree constructed based on 16S rRNA gene sequences of Lysinibacillus macroides_W3 with other Lysinibacillus species obtained from Gene Bank database.

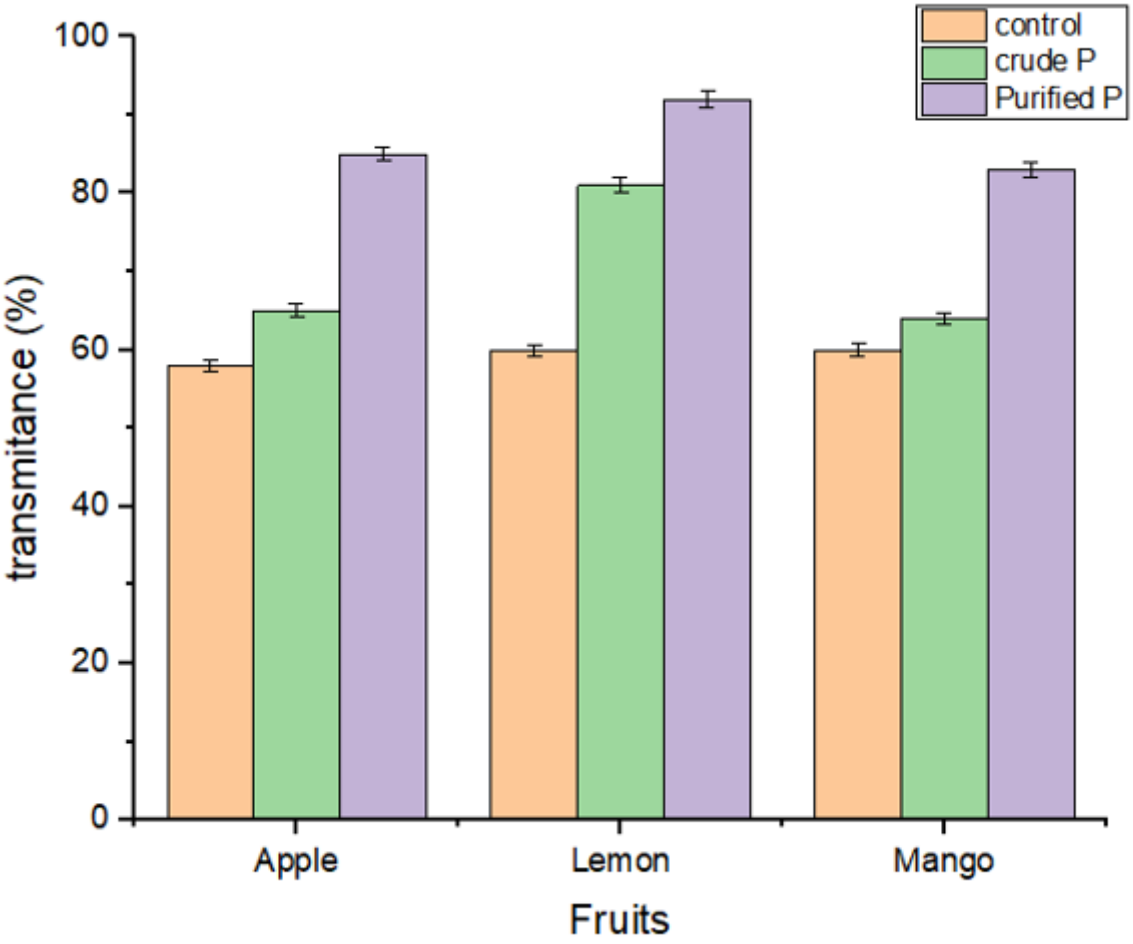


Figure 7

Effect of pectinase on juice clarity

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