Characterization of novel pectinase isolated from *Bacillus halotolerant sp.* LC3 from avocado peel

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Abstract
With an increasing demand of enzymes in different industrial sectors including textile, food, pharmaceutical, paper and pulp industry, researchers have explored various different natural sources of enzyme including different microbes (fungi, yeast and bacteria) and plants. Pectinases are the enzymes which have more applications in processing of plant fiber and treatment of pectin wastewater. Here pectinolytic bacterium was isolated from avocado peel using repeated streaking method. By using 16S rRNA sequencing, Bacillus halotolerance species was identified as pectinase producing bacteria. These bacteria showed its best activity at 40^{0}C optimized temperature,optimized time of incubation as 72 hours, optimum pH 8 and optimum substrate concentration as 1.5% pectin.

Our results displayed that sucrose, yeast extract and NaCl were best sources of carbon, nitrogen and metal ions for bacterial growth. Pectinase protein was extracted by using different saturation level of ammonium sulphate and then dialysis was performed. For determining the size of protein, SDS PAGE was performed. Our research work was to investigatethose bacteria that were producing pectinolytic effect from avocado peel.

Keywords: Pectinase, avocado, pectin.

Introduction

Pectin is a most complex structure of polysaccharides that takes play an important role in strength,support and structure go plant tissues as a part of the middle lamella[11]. Shaikh et al[15] investigated that pectinase or pectinolytic enzymes hydrolyze particular substances and they account for 25% of all food enzyme sales worldwide. Pectin comprises of different subunit bonded together covalently. Homogalacturonan is main part of pectin chain composed of -D-galacturonate units linked by glycosidic bonds also known as pectinsmooth region[13]. If D xylose is attached to linear chain of homogalacturonan, then it will be called xylogalacturonan. When galactose arabinose and L rhamnose are being inserted in chain, then it will become rhamnogalacturonan I and finally with the addition of borate and L- Aceric acid, rhamnogalacturonan I will be converted to rhamnogalacturonan II. Pectin is being degraded by pectinase enzyme. Pectinases have three major catagories that act on pectin smooth regions or on pectin hairy regions. Protopectinases enzymes degrade the insoluble protopectin to produce highly polymerized soluble pectin. Esterases enzymes catalyze the de- esterification process of pectin by the removal of methyl esters. They include pectin methyl esterases PME and pectin acetyl esterases PAE. Depolymerases enzymes undergo hydrolytic cleavage ofthe 1 → 4 glycosidic bonds that are present in D -galacturonic acid components of the substancesthat contain pectin l[10].

Pectinase enzyme from different microbial sources like bacteria, fungus, yeast have been reported. These pectinolytic microbes are majorly produced by oranges, strawberries, mango, coffee pulp, wheat bran, citrus peel, apple peel, potato, tomato and banana peel etc.[3,14] We can also produce pectinolytic bacteria from avocado peel[4]. In Pakistan, the widely grown avocado trees are Hass and Lula. Hass and Lula grafted varieties have successfully been grown in Sargodha, Lahore, Sukkur and even in Charsadda in Khyber Pakhtunkhwa. Hass variety is the export variety[1].

Material and Methods

Laboratory facilities: All the research work was done at Research Laboratory of Lahore College for Women University, Department of Biotechnology. The laboratory is well equipped with all necessary equipment's and instrument.

Collection of samples: Avocado peel was washed with distilled water for 3-4 times. After washing, peels were dried off, crushed and stored at 4^{0}C until further processing.

Serial dilution: Serial dilution was done in order to isolate the bacteria. The avocado peel was serially diluted in autoclaved distilled water. The test tubes were labelled as 10X,20X, 30X, 40X, 50X and 60X respectively. 4.5ml of distilled water was taken in all test tubes. 0.5g of crushed avocado peel sample was added in test tube labelled as 10X. Take aliquot 0.5ml from 10X test tube and add it in 20X. Similarly take aliquot of same volume from 20X and add in 30X and so on. The prepared serial dilution was kept at 4^{0}C until further use.

Preparation of pectin agar media: In 120ml distilled water, 0.12g beef extract, 0.24g yeast extract, 0.6g peptone, 0.6g NaCl, 0.48g pectin and 2.0g agar were added for media preparation.

Isolation and Purification: The growth of bacterial colonies was observed after 24hrs of incubation at 37^{0}C. The

individual colonies from culture plate were picked by a sterilized loop and streaking was done on new pectin agar media. The pure culture was obtained after repeated streaking method. The morphological characteristics of pure isolate was observed.

Primary screening of pectinase producing bacteria: The overnight pure culture was transferred to new pectin agar media incubated at 37^0C for 24hrs. After incubation, 5ml of congo red solution was flooded in culture plate containing single colonies and was observed after 10 minutes. Congo red solution formed a clear zone around bacterial colonies indicating the presence of pectinolytic bacteria.

Secondary screening of pectinase producing bacteria: The bacterial isolates that showed maximum clear zone, were considered for the highest pectinase producing bacteria. These bacterial isolates were further processed for submerged fermentation. The media components for submerged fermentation are same as primary media but without agar. The loopful of bacterial colony was inoculated in fermentation media and was incubated at 37^0C for 24hrs in shaking incubator at 125 rpm. After incubation, the media was transferred to centrifuge tubes and centrifuged at 10,000 rpm for 10 minutes. The supernatant pallet was used as crude enzyme to determine the activity of pectinase producing bacteria using DNS method.

Determination of pectinase activity: Pectinase activity was determined by DNS method. The procedure started by mixing 900µl of pectin substrate prepared in phosphate buffer of pH 7.0 in 100µl of crude enzyme. Incubate the sample in water bath for 10 minutes at 50^0C. After incubation, add 100µl crude enzyme and 2000µl DNS reagent, incubate for 10 minutes at 92^0C. Observe the color change. The solution was cooled to room temperature and OD was measured at 540nm. The amount of released sugar was calculated by D- glucose standard curve. 1 unit of pectinase activity is defined as glucose released in terms of µmol reducing sugar per ml per minute under standard assay conditions. The enzyme activity was calculated by using following formula:

$$\text{Pectinase activity} = \frac{\text{Released glucose in } \mu\text{mol ml}^{-1} \text{ x total volume of assay in ml}}{\text{Incubation time x volume of enzyme used in assay}}$$

Identification of Bacterial specie: The identification of bacteria was done by Gram staining and by 16S rRNA sequencing. On the basis of shape, size, elevation, opacity morphological characteristics were determined.

Optimization of pectinase producing bacteria: Characterization of pectinase producing bacteria was checked at different times of incubation, effect of temperature, effect of substrate concentration, effect of different metal ions, carbon sources, nitrogen sources and effect of pH.

SDS PAGE analysis: Sodium dodecyl sulphate polyacrylamide electrophorosis was performed to identify the size of pectinase protein produced by pectinolytic bacteria. As the size of pectinase protein varies from 35 to 48KDa, so polyacrylamide gel used was 12%.

Partial purification of pectinase by ammonium sulphate precipitation: Salting-out protein was the preferred concentration method. Pectinase enzyme that was isolated from isolated bacteria, was then subjected to various saturation levels of ammonium sulfate varying from 30–90%. The mixture was stirred and then centrifuged for 10000 rpm for 15 minutes. Precipitates produced after centrifugation was then dissolved in phosphate buffer 0.1M at pH 7.0. The precipitate were then placed in dialysis bag that was submerged in sizeable amount of buffer in order to remove salt.

Statistical analysis: Statistics analysis was done by using IBM SBSS software of version 20. All results were performed in triplicate and data was expressed as Mean ±Standard deviation.

Results

Isolation of bacteria from avocado peel: Isolation was done by serial dilution, pour plating method and by streaking, bacterial pure isolates were produced.

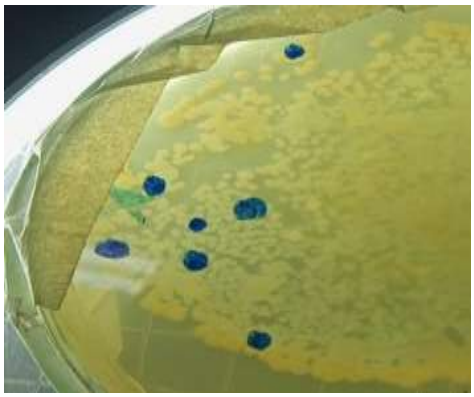

Figure 1: Bacterial colonies produced on pectin agar media after 24h of incubation.

Primary screening of pectinolytic bacteria: The screening of pectinolytic bacteria was done by using Grams' iodine solution on pectin agar plates. The congo red was flooded on pectin agar media containing different duplicates of pure isolate. The zones of hydrolysis are shown in figure 2.

Morphological results of bacterial colony: The results of the morphological analysis revealed several characteristics of bacterial colonies. Table 1 showed lists of morphological features of the bacteria that produce pectinase from 40X and 60X dilutions. Different colors including white and creamy yellow are produced by some colonies. The colonies had circular, irregular and elevated shapes.

Effect of temperature: Characterization was done on different temperature ranges in which the highest extracellular activity was shown at 40 ºC which was U/ml 5.829 and highest intracellular activity was at 45 ºC which was 1.531U/ml. The detailed values are mentioned in table 2 and are represented by the figure 3.

Time of incubation: Characterization was done on different time of incubation in which the highest extracellular activity was shown at 72h which was 5.706 U/ml and highest intracellular activity was at 48h which was 0.631U/ml. The detailed values are mentioned in table 3 and are represented by the figure 4.

Effect of different carbon sources: Characterization was done by using different sources of carbon where the highest extracellular activity was shown of sucrose which was 23.232U/ml and highest intracellular activity was of starch which was 0.764U/ml. The detailed values are mentioned in table 4 and are represented by the figure 5.

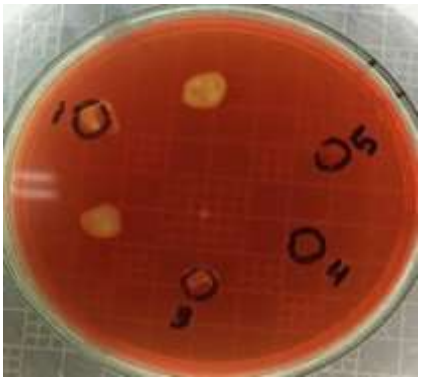

Figure 2: The pectinolytic bacteria showed clear zone of hydrolysis on pectin agar plates.

Table 1
Colony morphology of isolated bacteria from 60X dilution

S.N.	Features	Bacteria 1.1	Bacteria 1.2	Bacteria 1.3
1	Size	0.1mm	0.2mm	0.3mm
2	Shape	Rod	Round	Round
4	Color	White	Greenish	White
5	Elevated	Raised	Raised	Raised
6	Consistency	Sticky and thick	Sticky and thick	Sticky and thick
7	Odour	Null	Null	Null
8	Opacity	Opaque	Translucent	Translucent

Table 2
The pectinolytic bacteria at different temperature showing its extracellular and intracellular activity.

S.N.	Temperature	Extracellular O.D at 540nm	Extracellular enzyme activity (U/ml)	Intracellular O.D at 540nm	Intracellular enzyme activity (U/ml)
1	25°C	0.921	2.163	0.221	0.213
2	30°C	1.788	4.728	0.346	0.312
3	35°C	2.846	5.436	0.374	0.469
4	40°C	2.869	5.892	0.543	1.362
5	45°C	1.463	3.692	0.478	1.531

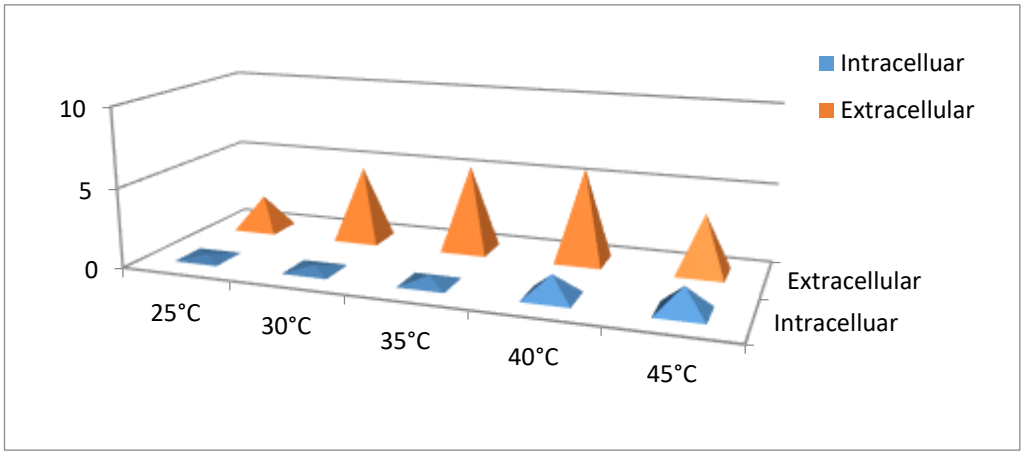

Figure 3: Graphical representation of different temperature ranges. In these ranges, bacteria at 40ºC show high enzyme activity as compared to other ranges.

Table 3
The pectinolytic bacteria at different time of incubation showing its extracellular and intracellular activity.

S.N.	Time of incubation	Extracellular O.D at 540nm	Extracellular enzyme activity (U/ml)	Intracellular O.D at 540nm	Intracellular enzyme activity (U/ml)
1	24 hrs	3.198	3.918	0.423	0.583
2	48 hrs	3.291	4.203	0.574	0.631
3	72 hrs	4.212	6.706	0.311	0.354
4	96 hrs	2.904	4.32	0.315	0.369

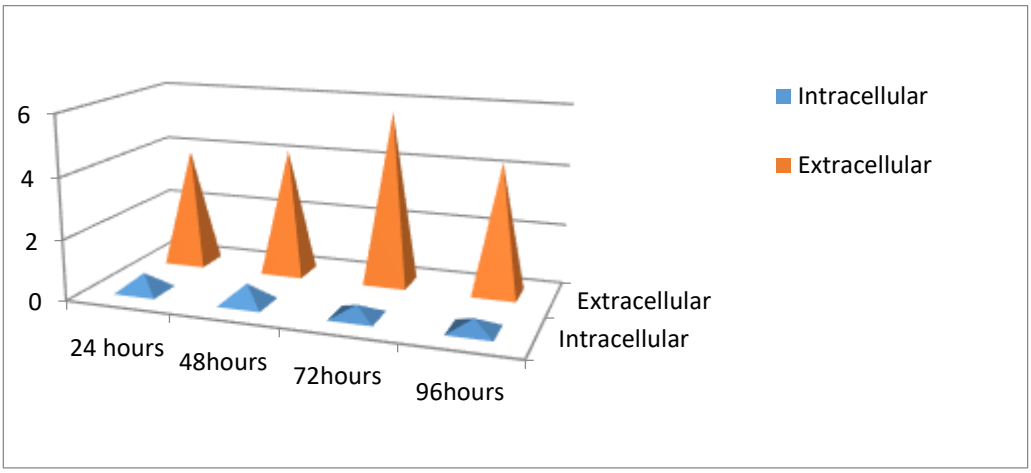

Figure 4: Graphical representation of different time of incubation. These bacteria at 72 hours show high enzyme activity as compared to other periods.

Table 4
The pectinolytic bacteria grown on different Carbon sources showing its extracellular and intracellular activity.

S.N.	Carbon sources	Extracellular O.D at 540nm	Extracellular enzyme activity (U/ml)	Intracellular O.D at 540nm	Intracellular enzyme activity (U/ml)
1	Sucrose	5.928	23.232	0.487	0.623
2	Starch	4.269	7.857	0.597	0.764
3	Maltose	4.581	5.304	0.464	0.579
4	Glucose	3.75	20.808	0.346	0.464
5	Lactose	2.01	8.427	0.597	0.646

Effect of different nitrogen sources: Characterization was done by using different sources of nitrogen where the highest extracellular activity was shown in yeast extract which was 5.886 U/ml and highest intracellular activity was observed in peptone which was 1.537U/ml. The detailed values are mentioned in table 5 and are represented by the figure 6.

Effect of different metal ions: Characterization was also done by using different sources of metal ions where the highest extracellular activity was shown in sodium chloride which was 6.928U/ml and highest intracellular activity was shown in calcium chloride which was 0.675U/ml. The detailed values are mentioned in table 6 and are represented by the figure 7.

Effect of different substrate concentration: Characterizations was also done by using different substrate concentration where the highest extracellular activity was shown at 1.5% substrate concentration which was 9.753U/ml and highest intracellular activity was also at 1.5% substrate concentration which was 0.583U/ml. The detailed values are mentioned in table 7 and are represented by the figure 8.

Effect of different pH: Characterization was done on different pH ranges in which higher extracellular .activity was shown at pH of .8.0 which was 5.136U/ml. the lowest extracellular activity was shown at pH 8 which was 0.697U/ml. The detailed values are mentioned in table 8 and are represented by the figure 9.

Discussion

For the research purpose, the pectinase enzyme was isolated from avocado peel which was good source of pectinase enzyme. On pectin agar plate, colonies of bacteria that degrade pectin, were produced after 24 hour of incubation.

The colonies were then purified by streaking method. Streaking was done in a contaminated free environment under laminar flow cabinet. After streaking, different morphological features were counted to determine the type of bacteria and it was suggested that there was *Bacillus species* on the plate. For more confirmation, we go for sequencing of our sample species and it was confirmed that *Bacillus halotolerans* was present in sample which was degrading pectin and was producing colonies. This species was then treated byhigh salt (NaCl) concentration and it produced yield of 7.0 Unit/ml.

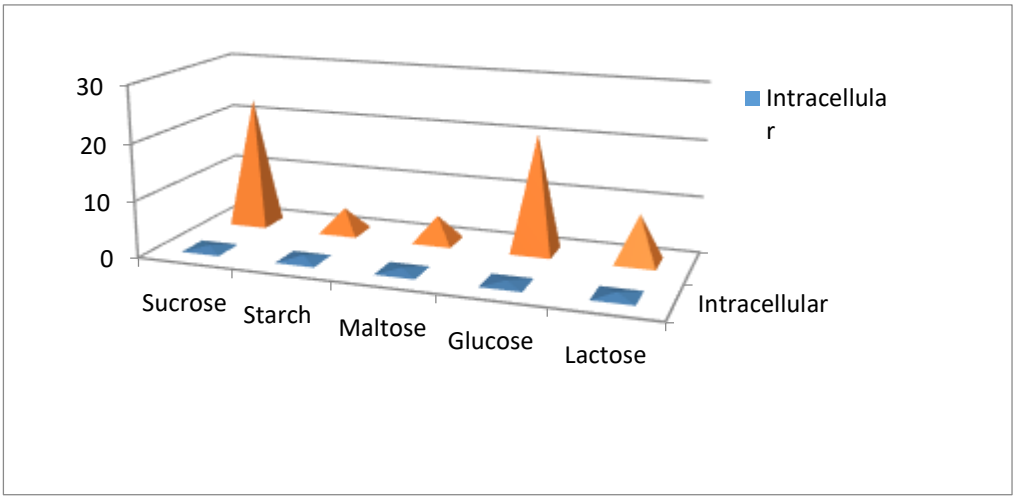

Figure 5: Graphical representation of different carbon sources. In these sources, bacteria at high sucrose concentration show high enzyme activity as compared to other sources.

Table 5
The pectinolytic bacteria grown on different Nitrogen sources showing its extracellular and intracellular activity

S.N.	Nitrogen sources	Extracellular O.D at 540nm	Extracellular enzyme activity (U/ml)	Intracellular O.D at 540nm	Intracellular enzyme activity (U/ml)
1	Casein	2.016	1.737	0.253	0.473
2	Yeast extract	3.072	5.886	0.337	0.378
3	Beef extract	3.672	5.683	0.381	0.742
4	Peptone	4.29	5.491	0.853	1.537
5	Ammonium oxalate	1.210	1.71	0.147	0.278
6	Ammonium sulphate	1.002	1.42	0.258	0.473

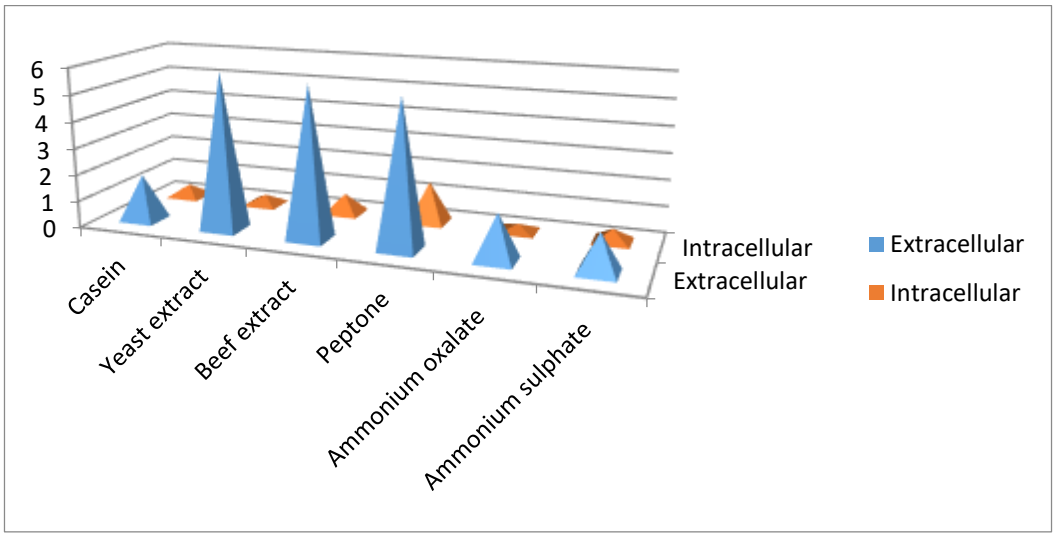

Figure 6: Graphical representation of different nitrogen sources. In these sources, bacteria high concentration of yeast extract show high enzyme activity as compared to other sources.

Table 6
The pectinolytic bacteria grown on different metal ion sources showing its extracellular and intracellular activity.

S.N.	Metal ions	Extracellular O.D at 540nm	Extracellular enzyme activity (U/ml)	Intracellular O.D at 540nm	Intracellular enzyme activity (U/ml)
1	Manganese sulphate	0.897	5.208	0.257	0.357
2	Magnesium chloride	2.157	3.738	0.617	0.647
3	Calcium chloride	2.133	4.482	0.411	0.675
4	Sodium chloride	5.431	6.928	0.595	0.537
5	Iron sulphate	1.71	5.844	0.572	0.642
6	Magnesium sulphate	2.166	2.526	0.722	0.525

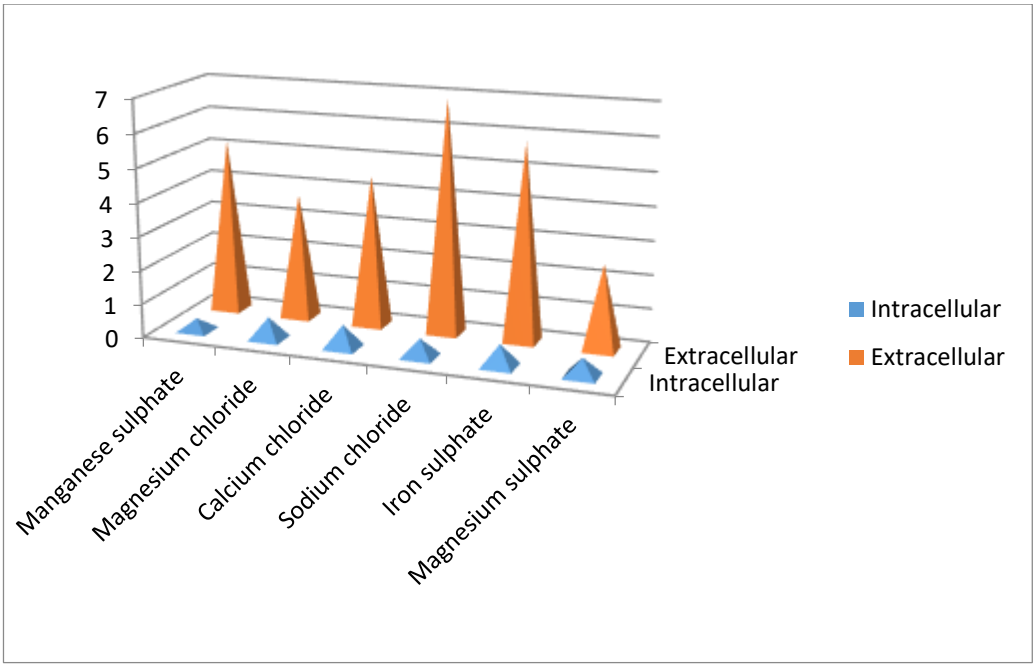

Figure 7: Graphical representation of different metal ion. In these metals, bacteria at high salt concentration of NaCl showed high enzyme activity as compared to other sources.

Table 7
The pectinolytic bacteria being treated with different substrate concentration showing its extracellular and intracellular activity.

S.N.	Substrate concentration	Extracellular O.D at 540nm	Extracellular enzyme activity (U/ml)	Intracellular O.D at 540nm	Intracellular enzyme activity (U/ml)
1	0.5%	3.426	3.753	0.247	0.246
2	1%	6.838	7.863	0.537	0.545
3	1.5%	9.892	9.753	0.535	0.583
4	2%	8.531	8.374	0.528	0.547
5	2.5%	6.538	6.834	0.531	0.543

Table 8
The pectinolytic bacteria at different pH showing its extracellular and intracellular activity

S.N.	pH	Extracellular O.D at 540nm	Extracellular enzyme activity (U/ml)	Intracellular O.D at 540nm	Intracellular enzyme activity (U/ml)
1	pH 5	2.46	1.749	0.377	0.375
2	pH 6	3.066	1.833	0.532	0.596
3	pH 7	3.198	3.198	0.586	0.632
4	pH 8	5.135	5.136	0.633	0.697
5	pH 9	2.562	2.562	0.563	0.529

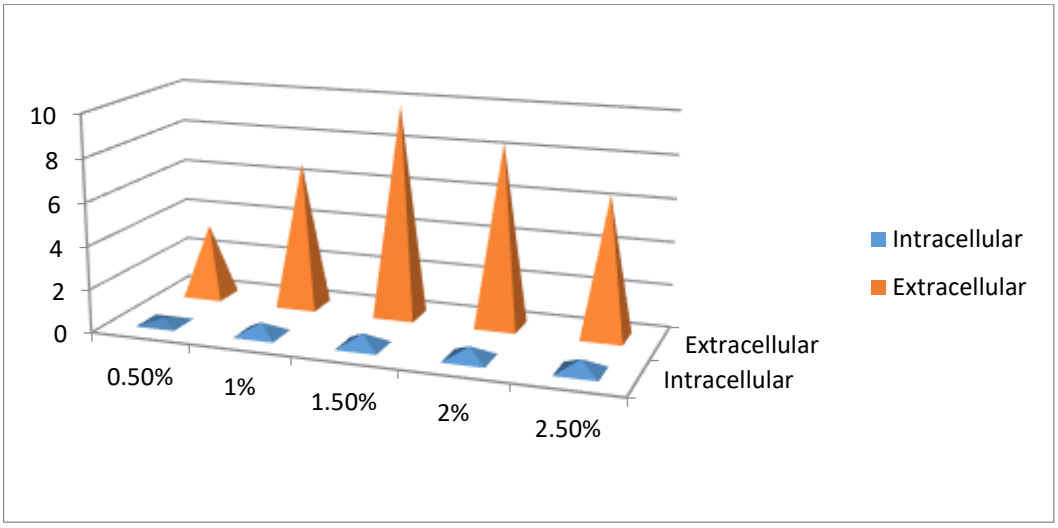

Figure 8: Graphical representation of different substrate concentration. In these pectin concentration, bacteria at 1.5% concentration showed high enzyme activity as compare to other concentrations.

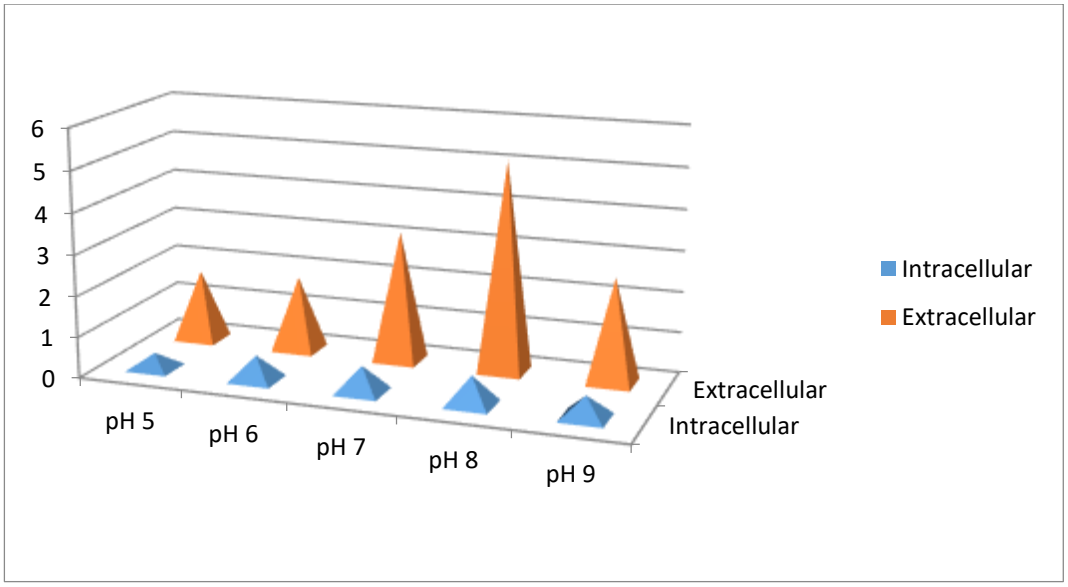

Figure 9: Graphical representation of different pH ranges.
In these range, bacteria at pH 8 showed high enzyme activity as compare to other ranges.

Table 9
Representation of total protein content and specific activity of enzyme

Purification	Total volume (ml)	Total activity (U/ml)	Total protein (mg)	Specific actitvity (U/mg)
Crude pectinase	100	197	30	6.56
Ammonium Sulphate (70%)	50	98.5	15	6.56
Dialysis	40	78.8	12	6.56

The best pectinolytic isolates were found utilizing the 16S rRNA gene in a molecular analysis. About 70% of the molecularly identified isolates belonged to the genus Bacillus and our results agreed well with those of Namasivayam et al[9] who claimed that *Bacillus sp.* can produce high amounts of extracellular pectinase enzyme. According to Priest[12], the pectinolytic activity of the *Bacillus* genus is widely distributed. Numerous strains of this species had also been used in other studies to generate pectinase. Additionally, during screening, the isolates that secretedthe most pectinase belonged to the genus *Bacillus.* Murugan et al[8] also reported few other bacteria like *Erwinia specie*, *Pseudomonas specie* and *Bacillus specie* were able to produce the pectinases.

Mohandas et al[7] reported that higher pectinase activity was done at pH 8.0. The same was reported by Sohail and Latif[18]. They reported optimal poly galacturonase production of

Bacillus mojavensis at the pH of 8.0. Here *Bacillus halotolerence* also showed its best activity at pH 8 because the alkaline pectinase enzyme showed its best activity at pH 8 and 9. According to Kashyap et al[6], the activity lose was observed at acidic pH less than 6.0 and was unable to performed its activity at highly basic pH of 9.0. Our results for *Bacillus halotolerans* showed similarity with *Bacillus pumilus* reported by Kashyap et al[6] who also displayed the excellent growth at pH 8.0 and lower growth at pH less then 6.0.

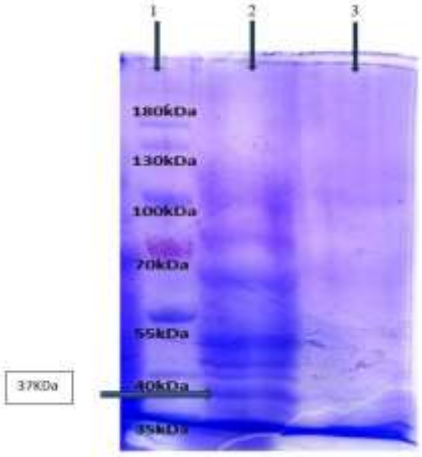

Figure 10: Image of 12% SDS gel showed the bands where protein size of approximately 37KDathat was isolated from pectinolytic bacteria from Avocado peel. 1 is ladder, 2 is extracellular and is intracellular.

PCR Primer name and Sequence
27F 5' (AGA GTT TGA TCM TGG CTC AG) 3'
1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' B
Sequencing Primer Name/Sequence
785F 5' (GGA TTA GAT ACC CTG GTA) 3'
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' A

Figure 11: (A) Sequencing primer along with its sequence is shown.
(B) PCR primers along with their sequences is shown.

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct. (%)
OQ306542.1	Bacillus halotolerans	1511	01	1511	100	2791	0.0	1511/1511	100

Kingdom	Family	Genus	Species
Bacteria	**Bacillaceae**	**Bacillus**	**halotolerans**

Figure 12: Showing the accession number and description of identified specie

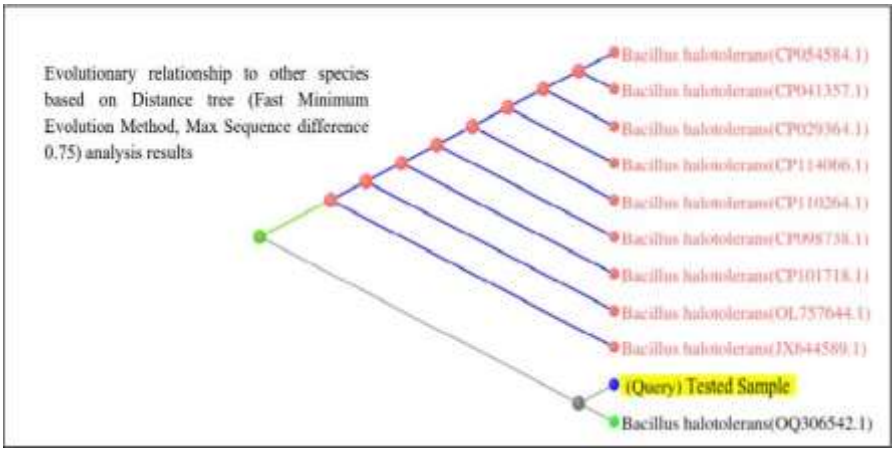

Figure 13: Showing the evolutionary relationship of our species with other species based on theDistance tree method.

For pectinase production by *Bacillus polymyxa* reported by Silley[17], *Bacillus stearothermophillus* at pH 9.0 accounted for good production of pectinase and also displayed excellent growth at 9.0 and pH 10.0 reported as best condition *for Bacillus strain RK9* reported by Karbassi and Vaughn[5]. The pectinolytic bacteria from avocado peel grew best at optimum temperature of 72 hours and showed the activity of 6.7unit per ml. Haile et al[4] also reported that after 72hours of incubation, *Serratia marcescence* showed the activity of 6.8unit/ml.

Temperature is one of the most basic parameters for effective production of pectinase enzyme. *Bacillus sp.* was observed to grow best at an optimum temperature of 40°C. The confirmation and configuration of pectinase enzyme remain intact at temperature of 37-50°C. According to Kashyap et al[6], temperature stability profile of pectinase activity revealed that the enzyme is maximally activated at moderately high temperatures ranging from 40° to 60°C with highest activity (63 U/ml) while Haile et al[4] showed that bacteria performs its best growth at 35°C temperature.

Darah et al[2] got higher polygalacturonase activity at the concentration of 1% of. the pectin for *Entero bacteraero*genes. In our research, the pectin substrate was used in the different concentrations for bacterial growth and it was observed that 1.5% of substrate concentration was the most appropriate. Few bacterial species such as *Entero bactertabaci NR14667*, can produce the higher pectinase enzyme activity at 0.3% of pectin concentration as reported by Shrestha et al[16]. Also higher pectinase enzyme activity at 2% of the citrus pectin concentration was also observed for *Chryseo Bacterium Indologenes* strain SD.

Through SDS analysis, it was observed that the size of protein was approximately 37KDa and pore size of polyacrylamide gel used was 12%. For nitrogen sources and carbon sources, *Bacillus* showed its best activity with yeast extract and sucrose respectively. The glucose is generally preferred source of carbon by Gram positive bacteria and here *Bacillus halotolerans* is also a Gram positive bacteria. Yeast extract is most widely used as organic nutrient sources in microbiologicalculture media for cultivation of variety of microbes. *Bacillus halotolerans* showed its maximum activity at high concentration of NaCl and lowest activity at magnesium sulphate.

Conclusion

Avocado peel was taken to produce pectinolytic bacteria using pectin agar media. Pectinolytic bacterium was isolated using repeated streaking method. Through 16S rRNA sequencing, it was confirmed that the bacteria that was producing pectinase, was *Bacillus halotolerans*. The future perspective of this research work is to use the application of pectinase enzyme in different industrial sectors.

Gene that is responsible for halotolerant behavior can be expressed in plants to make them halotolerantto different salt concentrations under stress condition. Halotolerant gene from *Bacillus halotolerants* can be introduced into those plants which are facing high salt stress and convert them into salt tolerating plants.

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