DOI: https://doi.org./10.24163/ijart/2017/19 Available online at http://www.ijart.info/

INTERNATIONAL JOURNAL OF APPLIED RESEARCH AND TECHNOLOGY
ISSN 2519-5115

DAYANANDA SAGAR UNIVERSITY

School of Basic and Applied Sciences

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF PECTINASE

A dissertation submitted in partial fulfillment of the requirements of
Masters in Science

In
(Specialization **BIOTECHNOLOGY**)

Submitted by

(AQUEEF SHARIFF AND MARUDWATI J)
(BAS17MBT001 AND BAS17MBT005)

Guided by
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CHAPTER 1 - INTRODUCTION

Using microbes as a source for the enzyme involves no ethical issues when compared to the sources of enzymes from animals and plant sources and allows economical technology with low resource consumption (**Jayani et al., 2005**). These enzymes can be isolated by undergoing screening microorganisms, which are acquired from different environments and are modified by altering the already present enzymes using different methods such as molecular evolutions or protein engineering. Using these methods several different food-processing enzymes have been modified according to the need such as lipases and amylases (**Solbak et al., 2005**). Enzymes with desired biochemical and physico- chemical characters are commercially important and are the new focus for research.

Apart from fruit and vegetable industry these microbial enzymes are employed in various fields (environment friendly) and economic sectors. One such evolving enzyme in food industry is pectinases.

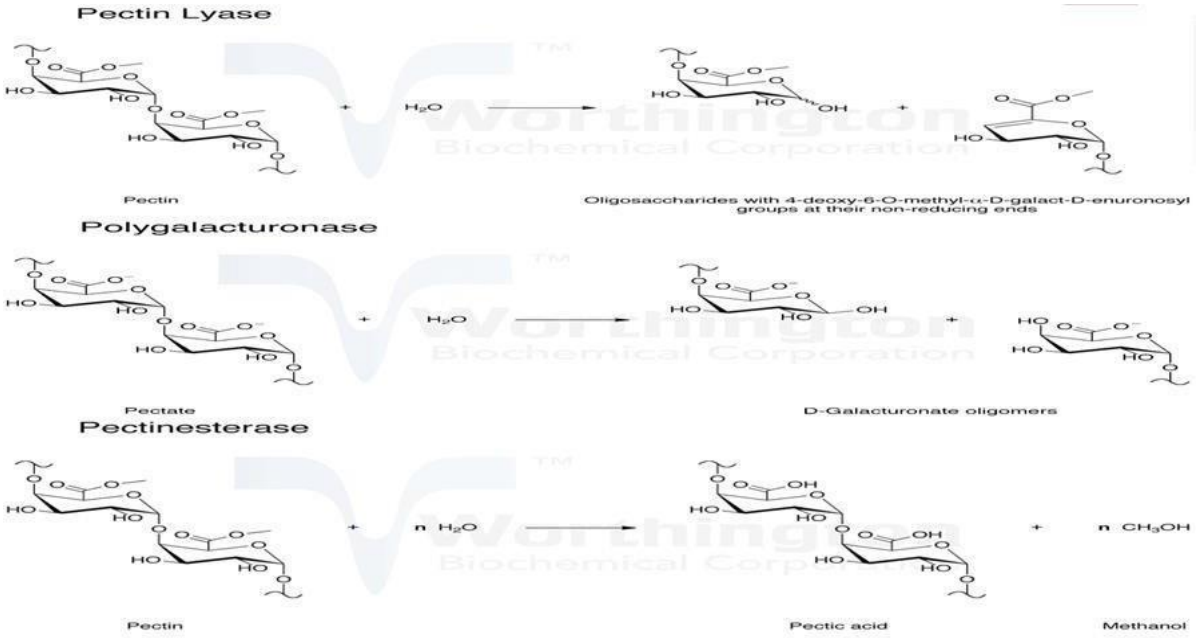


Figure 1: Mode of action: Pectinases

The enzyme which degrades the pectin are commonly referred to as pectinolytic enzymes or simply pectinases, they are made up of heterogeneous group of enzyme, these enzymes are grouped into three , based on their mode of action on pectin containing substrate. The pectate lyase (PLase) and polygalacturonase (PGase) split the molecular chains of the respective polymers. Methanol is liberated and methyl ester group of galacturonate unit is hydrolyzed by pectin methyl esterase.

Pectinase enzymes have great industrial value, they are commonly used in extraction, purification, clarification process in namely fruit and vegetable industries(**Naidu et al., 1998**). Pectinases reduce filtration rate upto 50%. These enzymes are used mainly in olive oil extraction (**Verhoef et al., 2009).**

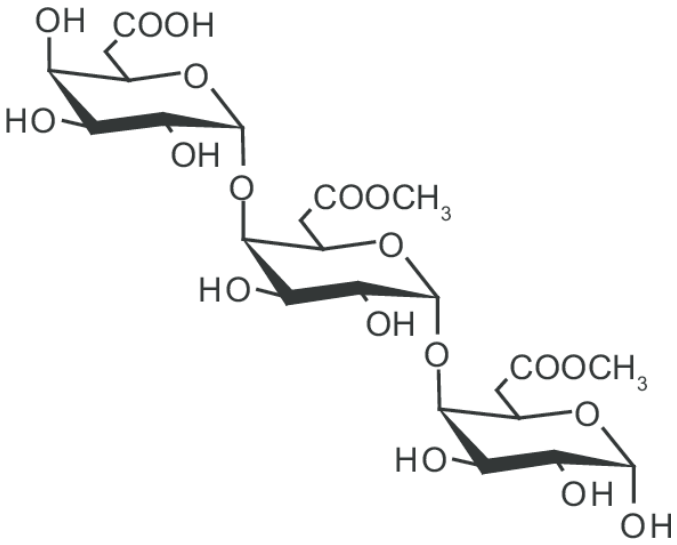


Figure 2 :- Structure of pectin

Pectin substanc are high molecular mass glycoside macromolecule found in higher plants, which are present in the primary cell wall. These are the major components of the middle lamllae, a thin extracellular layer formed between the walls of young cells.

CHAPTER 2 - REVIEW OF LITERATURE

PECTIN AND PECTINOLYTIC ENZYME

Pectinase enzymes are a group of enzymes that hydrolyze the pectic substances, present in plants. This group of enzymes contains pectin lyase (PL), pectin methylesterase(PME) , pectin polygalactouranase(PG) . Bacteria, yeast and fungi produce pectinases. Pectinases are produced by using different carbon sources. Pectinases have been produced by different fungal and bacterial strains using fruit waste and fermentation strategies as reported by researchers.

Pectinolytic enzymes have huge application in biotechnological industry. These applications are fruit juice clarification, coffee fermentation, extraction of oil and treatment of pectic wastewater **(kashyap et al., 2001; panda et al., 2003).**

Many organisms produce pettinesdfds : bacteria, fungi, yeast (**Visser, 1997; Rodriguez *et al*.,1991;; Zheng *et al*., 1992; Palanivalu et al., 1999 and Wubben *et al*.,2000).**

Pectinases from different microbial sources significantly differ from each other in their physicological properties and in the mode of action. pH range of 3.5–5.5 has been reported the optimal pH range and range of 30–50oCto be the optimal temperature (**Jayani*et al*., 2005**). 25 kDa to 85 kDa are the molecular masses range (**Baldwin and Pressey, 2005 and Kester and Visser, 2006**).

The major studies are on *Erwnia and Bacillus* amongst the bacteria and *Aspergillus* in the fungi family. Due to the dynamic advancements in the molecular biological techniques, cloning and expression of pectinases in various hosts are successful and are recombinant expressed (**Wang *et al*., 2011; Damak *et al*., 2013**).

From the literature, it is evident that pectinases are inducible and that they can be produced from varying carbon sources (**Aguilar *et al*., 1997; Maldonado Soriano *et al*., 2000*et al*., 1999; Friedrich *et al*., 1998; Nair *et al*., 1995; Nair and Panda, 1997**).

Several researchers (**Naidu and Panda 1998; Friedrich et al., 1989; Panda and Naidu 2000; Panda et al., 1999**) studied optimization microbiological parameters and different fermentation strategies for the production of pectinases.

These enzymes are utilized as biocatalysts in various research fields and Specific purpose is given for each type of pectinases. Bacterial pectinases have gained attention over fungal pectinases in the past few years.

Bacillus subtilis isolated by (**Martos*et al.* 2013**) from citrus peels produced pectianses in liquid medium with citrus pectin. Stability was shown between 3.0 and 6.0 pH range, temperature at 50oC at optimum pH. few studies are also conducted on bacterial pectinases even though fungal is currently (**Longo *et al*., 1992; Blanco *et.al.,2016)***

***(*Gainvors*et al.* (2000**) reported pectianses production from *Bacillus halodurans* s, which had a maximum activity of 2533.8U mg-1 by ultrafiltration with 219.4fold purification. In another study **(Lim *et al*. 1980)**, three forms of polygalacturanse from *Ralstoniasolanacearum* were separated by adsorption using Sephadex C-50 column chromatography. Purified PG had four proteins with molecular weights 47, 41, 35, and 33 kDa .

PECTIC SUBSTRATES

Among substrates like malt sprout, wheat bran, rice bran, pomegranate, lemon and orange, pectinase activity was found to be maximum at of 589.0±0.36. (**Reda et al. 2008)** saw the maximum productivity of pectinase from Solanumtuberosum (ST) peels when compared to agro industrial wastes (citrus peel) by *Bacillus firmus*-1-10104 at 37°C for 92 hrs. (**Venkatesh et al. 2009**) made use of cashew, banana, pineapple and grape fruit waste for production of pectinase. (**Baladhandayutham et al., 2010)** used sugarcane bagasse as substrate and observed maximum pectinase activity of 164.15 U/ml.

ISOLATION OF THE PECTIN DEGRADING BACTERIA

(Soares et al., 1999) studied the isolation of bacterial strains from vegetable decomposition sites and reported 168 bacterial strains as pectinase producers with citrus pectin as carbon source. . (**Ajit Kumar et al., 2012**) used enriched media and isolated bacterial strains producing pectinase outof rotten fruit waste. These were screened for production and morphological characterization. (**Kashyap et al., 2002)** obtained *Bacillus sp*. DT7 pectinase with growth conditions: 37C, 200 rev/min, and 12 h.

SCREENING

(Farooqui et al., 2012) screened bacterial strains, which were isolated from soil samples for enzyme production, with wheat bran as substrate. He observed the maximum diameter of zone of clearance and pectinase production as *Bacillus subtilis*. (**Patil et al., 2012)** obtained pectinolytic bacteria depending on the zone of translucent clearance around the colonies. The colony showed 2.9 cm as maximum zone and 39.44 U/ml pectinase activity. (**Oyeleke et al., 2012)** reported isolates of bacteria from corn with clear zone production for pectinase. **(Garg et al., 2014)** used orchid soil for isolation of pectinase from kurukshetra Haryana (India). Areca nut husk was used by (**Naveenkumar et al., 2014)** obtained 24 isolates, which showed maximum zone of clearance.

EFFECT OF pH AND TEMPERATURE

(Panda et al., 2003) studied the biochemical characters of extracted bacterial pectinase, which showed stability at 30°C and at pH of seven. (**Ranveer et al., 2005)** (**Phutela et al., 2005)** obtained pectinases with maximum activity at 50 and 60ºC (925 Ug-1). **(Divakar et al., 2013)** used different pH ranges of 5,6,7,8,9 and 10 for pectinase production at 37°C and temperatures of (10, 20 30 40 50 60 70 and 80ºC) to check the maximum production and activity. Maximum activity of 647.0 (Islam et al., 2013) observed U/g at 40oC, and others with 583 U/g, 516 U/g, 515 U/g and 392 U/g respectively. **(Madu et al., 2014)** observed physico-chemical properties influencing production of pectinases by *Bacillus licheniformis*, the maximum enzyme activity was seen at pH of 9.0 with bagasse as carbon source. **(Garg et al., 2014)** investigated the optimization of enzyme by *Bacillus sp*. At pH (3.0-12.0) and temperature (25 ° C- 50 ° C), they observed maximum production of pectinase (168.83 IU/ml). (**Kaur et al., 2014)** studied the fruit spoilage soil cultural conditions for the growth of pectinase producing bacteria at different pH ranges and different temperature ranges.

THE APPLICATION OF THE ENZYME PECTINASE

FOOD SECTOR

The use and application of pectinases enzyme in industries like food processing has prolonged remarkably in the past years mainly in the process like extraction, clarification and stabilization (**Suneetha et al.,2011**).

The acidic pectinase enzyme has a important role in the vegetable and the fruit industry, this enzyme is produced by fungi which is mainly Aspergillus spp. The enzyme pectinase can be used along with other enzymes like arabinases ,cellulases , or xylanases which increases the pressing fruit efficiency for the removal of the juice. When pectinase used with the enzyme cellulose it increases the juice yield up to 100% (**Sarioglu at al.,2001**)

The enzyme pectinase easily removes the fruit peel by the softening process. The juice yield in increased by the industry by reducing the viscosity and maceration. The use of this enzyme reduces the filtration time up to 50% and also increases the clarification. This enzyme not only increases the clarification but also enhances the taste of the juice in particular apple juice. Use of this enzyme makes it easier for the production (**Suneetha et al.,2011).**

EXTRACTION OF CLEAR JUICE

The undissolved cloudy matter in the juice suspension is removed by adding pectinase during the pressing stage of juice extraction. The mixture of enzymes that contain pectinase , cellulose , hemicellulase are used in the treatment of suspension (**Kashyap et al., 2009**).

The enzymes which catalyze the depolymerization of very highly esterified pectin are commonly used in apple juice clarification. The enzyme parts PG and PME in a mixture is found effective in juice clarification but the best is the pure form of pectin lyase at a pH OF 3-4 , even 91 – 92% of esterified pectin can be removed by this enzyme pure lyase (**Ishii et al., 1973**).

the juice extraction from apple is a two stepprocess,in the first step the apple mush is crushed and treated with pectinase to produce a good quality of juice. In the second step is liquefaction treatment in which cellulose and pectinase are added finally to the juice for the extraction(**Will et al., 2000**) . For the effective juice clarification 1000 – 2000 U of pectinase should be added to 1L of juice for 1 to 3 hour (**Ishii et al., 1973**).

During grape juice preparation the pectinase is added during grape crushing. The enzyme enhances the economic value of orange juice. The old process or the traditional processing of orange juice was done by heating which use to spoil the natural taste and also was expensive (**Braddock et al.,1981**)

COCOA, COFFEE AND TEA FERMENTATION

In various types of fermentation pectinase is used, to speed up the fermentation process. In the tea fermentation, the cell walls tea leaves is broken down by the pectineas enzyme of the fungal origin but if extra amounts of pectineas is added it may damage the tea leaves, therefore an appropriate concentration of the enzyme should be maintained during fermentation. The pectinease also acts as an anti-foam agent, that is by destroying the pectin in the instant tea powder (**will et al., 2000**)

.

The mucilage layer is removed by using pectineases during coffee fermentation, because of this the pectinease preparations are used as sprays and sprayed all over cocoa beans and fermentation is carried out. The enzymatic preparations of the enzyme are highly efficient as it reduces the time of fermentation process and increases or develops the flavor (**Braddock et al., 1981**).

During the fermentation of cocao many micro organisms including pectinolytic bacteria play important role.These different microorganisms are used for the degradation of the pulp of cocao and by realizing pectinease, giving the finest quality of cocoa beans (**serrat et al.,2002**) .

THE USE OF PECTINASE IN JAMS, JELLIES PREPARATION

The enzyme part which is pectin esterase, convertsthehighmethoxylatedpectins into a low methoxylated pectin by the process known as demethylation. This enzyme part has dependency on calcium for gelation property due to which gel is formed which results in reducing sugar requirement.the enzyme pectin esterase is usedfor making sauces, soups, jams, jellysetc (**Grassin et al., 1996**).

PECTINASE USED IN PICKILING

The vaccum pressure which is applied to the resin to drive it into a laminate is called vaccum infusion. In fruits and vegetable industry pectinease is used a lot for softening process because of its property of vaccum infusion. Due to this process pectinease used in picikling industry (**Iconomou et al., 2010**)

AGRICULTURAL INDUSTRY

PLANTVIRUSPURIFICATION

In the isolation of plant virus pectinases is used. The pectinase with a mix of cellulose is used in the extraction of plant virus from the phloem tissue. Due to this process the pure preparation of plant virus is used (**will et al,.2000**)

EXTRACTION OF OIL

In oil extraction the use of pectinase along with other enzymes is varycommon . The enzyme cellulases and the enzyme hemicellulose are used for preparation and extraction of olive oil. During the preparation of oil extract the enzyme are added at grinding stage (**Scott et al., 1978**). Organic solvents like hexane and by treatment of pectinase the extraction of sunflower, coconut, lemon, palm and canola oils is possible, more preferred is of alkaline nature because they help in the extraction of aqueous process. The stability and oil yield with enzyme treatment is improved. Enzyme concentration, pH and temperature affect the oil yield.

An enzyme preparation called the olivex which is produced by fungus, Aspergillus aculeate, the preparation commercially has pectinolytic, cellulolytic and hemicellulases activity which is helpful in oil yield and in stability when it comes to storage.the treatment of olivex there is high level of polyphenols and also vitamin E content (**Iconomou et al.,2010**)

DEGUMMING AND RETTING

In the degumming of fiber crops pectinase is used. From the the fiber of the crop the gum is obtained, the ramie fiber is one of the best natural textile and it contains 20-30% ramie gum which mostly has pectin and also hemicellulose and therefore it should be removed before the further treatment of the textile processing. The process of degumming can be done by two methods that

are enzymatic treatment or chemical treatment , in the treatment of chemical, 12-20% NaOH of the solution is used in the removal of the gum of the decorticated fiber (**Jayani et al.,2005**) .

The drawback is chemical method is not that efficient and degumming can be toxic, non – biodegradable and contaminating. The use of the enzyme reduces the energy consumption and chemicals. In all the pectinase the pectate lyase from the actinomycetes is the most useful for the separation of bast fiber by the removal of gum.

The fermentation process called retting in which the prokaryotes and the eukaryote microorganism do the fermentation where the pectin is degraded and a good quality of fiber is released. during the process of retting the most commonly used bactria is *clostridium* and *bacillus* and the common fungi is *Aspergillus* (**Chesson et al.,1978**)

SACCHARIFICATION OF BIOMASS AND LIQUEFICATION

In the fermentation process, the fermentation sugar is an important biomass and the raw material is wheat, potatoes, rice and sugarcane. By the action of the enzyme pectinase, hemicellulases and cellulases , for producing fermentable sugar. These enzymes degrade plant cell walls, these enzymes disrupt and degrade the cell wall matrix which result in the liquefying the material and release of intracellular carbohydrates (**Beldman et al,. 1984**)

AIM AND OBJECTIVE

Pectinases are increasing in commercial importance. The role of pectinases in fruit juice preparation is well established.

Application of pectinases is mainly in extraction, clarification and stabilization process in fruit and vegetable juice industry. Usage of pectin producing bacteria reduces filteration time by 50% during juice clarification. Used in Coffee, cocoa and tea fermentation to breakdown the cell wall of tea leaves.(used in specific concentrations).

With bacterial pectinases having an advantage of easy to harvest pectinase than fungus as it is an extracellular product in bacterial culture, ease of fermentation process,implementation of strain improvement techniques , enzyme production is achieved in less time.

Therefore, the aim and objectives of the study are:

- Isolation and screening of pectinase producing bacteria.
- Identification of unknown pectinase producers.
- Partial purification of the enzyme.
- Physico chemical characteristics of the enzyme.

: Effect of pH on the enzyme activity

: Effect of temperature on the enzyme activity

CHAPTER 3 – METHODOLOGY

ISOLATION OF PECTINASEDEGARDING BACETRIA FROM FRUIT WASTE SOIL

Collection of samples

Soil samples from 30 different fruit markets were collected and screened for pectinase producing bacteria using PSA media.

PSA Medium contains:-

containing 3 gm KH_2PO_4, 3 gm Na_2HPO_4, 5 gm NaCl, 2 gm $MgSO_4$ (separately autoclaved), 5 gm pectin, 20 gm agar and a pinch of peptone at pH 7(**Kashyap*et al*., 2001; Gummadiand Panda, 2003)**

SCREENING:

PRIMARY SCREENING (PLATE ASSAY)

1g of soil was dissolved in autoclaved distilled water and 100 micro litre was over laid on PSA agar plate. The plate was incubated at 37°C for 48 hours. Colonies showing clear zone were sub cultured on fresh PSA agar plates and added with solution of iodine (1.0 g iodine, 5.0 g of potassium iodide and 330 ml water) for checking clear halo zones. Pure cultures were streaked on the slants and stored at 4°C for further studies**.(Oyeleke et al. (2012).**

SECONDARY SCREENING

Pure cultures were inoculated in nutrient broth media and were incubated for 48 hours at 37 degree. Cultures were centrifuged at 10000 rpm at 4 degrees and 100 microliters from the supernatant was added to the puncture made in the PSA agar medium, which were incubated for 48 hours. The plates were flooded with iodine solution to detect themaximumzone of clearance. (**Bhardwaj and Garg et al.,.2014, Naveenkumar et al. 2014**

PECTINASE PRODUCTIONASSAY

The obtained pure cultures were inoculated in the PS medium and incubated to check the optimum time periods of incubation. After every time interval of 24, 48,72 and 96 hours the amount of enzyme produced was assayed by DNS method. A reaction mixture containing the enzyme and the substrate was made upto 1 ml and was added with 3 ml DNS reagent, incubated for 10 minutes and absorbance was calculated.(**Miller, G.L. 1959**.)

BIOCHEMICAL CHARACTERIZATION AND IDENTIFICATION OF PECTIN DEGRADING BACTERIA

- **Gram staining-**

The 5 bacterial isolates where identified by gram staining. (**AnejaK.R., 2007)**

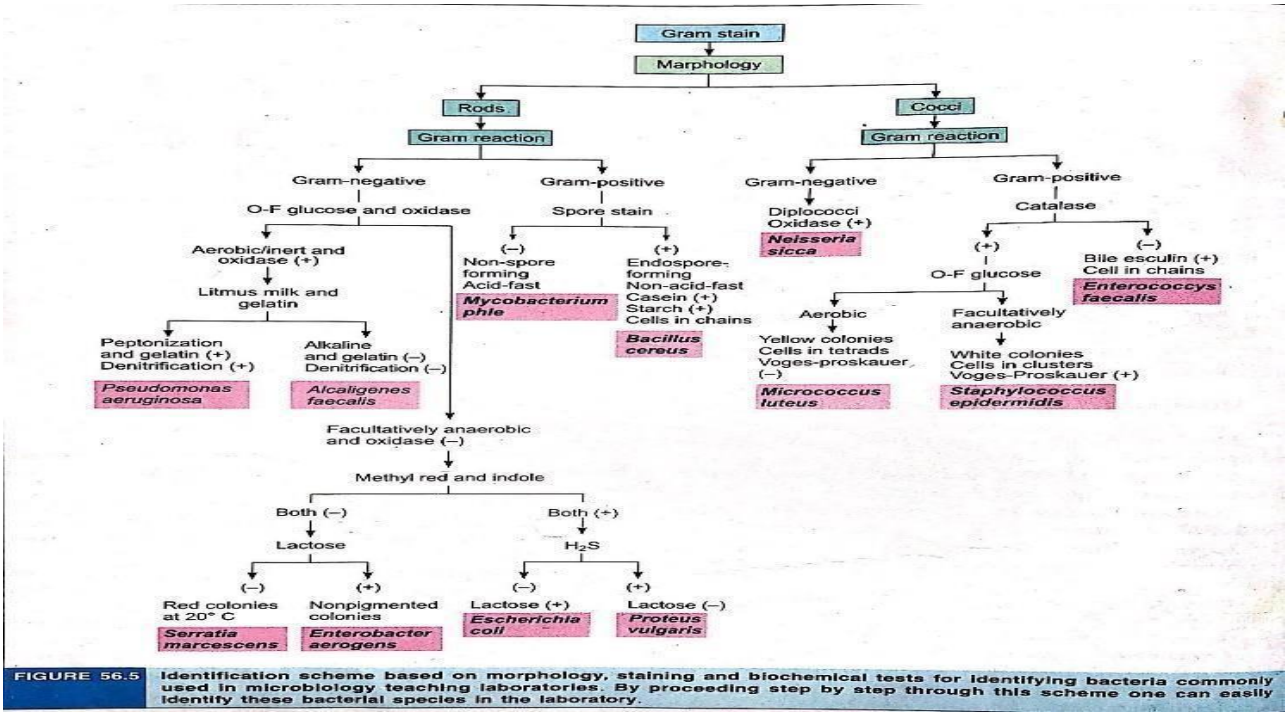


Figure 3: Flow chart of the series of biochemical test performed

- **OXIDASE TEST**: -

The isolates were grown on agar plates and 2-3 drops of oxidase test reagent(tetra methyl phenylene diamine) was added over the plate of the organism and change in colour was observed within 10 -30 seconds.**(AnejaK.R ., 2007)**

- **CATALASE TEST** : -

2-3 drops of3% of hydrogen peroxide was taken on a glass slide. One loop full of the culture was added to it. Slide was then observed for the of effervescence. (**AnejaK.R ., 2007)**

- **OXIDATION FERMENTATION (O-F) TEST**: -

The test was conducted using the O-F media containing sodium chloride 5g, Di- potassium phosphate 0.3g, peptone 2g , bromothymol blue 0.03g , agar 3g, glucose 10g, water 1000mL. duplicates of O-F medium were taken in test tubes and inoculated with pure cultures, with one of the two tubes was overlaid with 2-3ml of mineral oil and incubated for 24 hours at 37 degree. Test tubes were observed for colorchange.**(AnejaK.R ., 2007)**

- **VOGES-PROSKAUER TEST(V-P TEST)**

Cultures were inoculated in test tubes containing MRVP broth containing buffered peptone 7g, dextrose 5g, Di potassium phosphate 5g in 1000mL of water, incubated all the tubes at 37°C for 48 hours. 2.5ml of the broth was taken to another tube and 6 drops of 5 % α-naphthol and 2 drops of 40% KOH was added and was observed for color change.**(AnejaK.R ., 2007)**

PHYSIO CHEMICAL PROPERTIES:

PARTIAL PURIFICATION:

BY ACETONE:

The pure culture was inoculated in PSA medium, grown for 48 hours at 37 degrees. The culture was centrifuged at 10,000 rpm at 4 degree. The supernatant containing the crude enzyme was mixed along with three volume (3:1)of chilled acetone and was kept in -4 ℃ one hour without being disturbed. The sample after incubation was centrifuged at 10000rpm for 10 minutes and the pellet was allowed to air dried to remove the acetone, and dissolved in respectivebuffer. (**Sin et al., 2006)**

ESTIMATION OF PROTEIN CONTENT IN THE PARTIALLY PURIFIED ENZYMEUSING FOLIN LOWERYS METHOD.

The protein content in the sample was estimated by folinlowry`s method and was plotted on a standard BSA.0.5mL of partially purified enzyme was made upto 1mL using respective pH buffers. 4.5mL of Reagent 1 (2% Na_2CO_3 in NaOH , 1% NaK tartrate and 0.5 % $CuSO_4$)was added and incubated for 10mins0.5mL of Reagent 2 (Folin: phenol reagent 2:1)was added and incubated for 30mins. Absorbance was at 660nm.(**lowry O H et al ., 1951**)

EFFECT OF pH:

Optimum activity of the partially purified enzyme was checked at different pH buffers. Different buffers such as acetate buffer, phosphate buffer and tris buffer were used to obtain a pH range .(Phutela et al.2005) The enzyme activity was measured by performing DNS method.(Raju and Divakar et al., 2013)

BUFFER	**pH**
ACETATE BUFFER	4,5
PHOSPHATE BUFFER	6,7
TRIS BUFFER	8,9,10,11

Table 1: List of buffers used for the study

EFFECT OF TEMPERATURE

To measure the optimum temperature at which the enzyme was most active . pectinase activity using DNS method was checked at different temperature ranging from 20-80 ℃.(**Kaur and Kaur et al.,2014) (Prakash et al.,2014).**

APPLICATION

JUICE CLARIFICATION

Thepectinase enzyme was checked for its effectiveness in juice clarification study**(Vijayanand et al.,2010**) .Fresh citrus juice was autoclaved and different concentrations (0.2,0.5 and 1.0) of crude as well as partially purified enzyme was added and incubated for 72hours.The clarification of juice the turbidity of the juice was checked**(Robin et al.,2013).**

CHAPTER 4 - RESULT

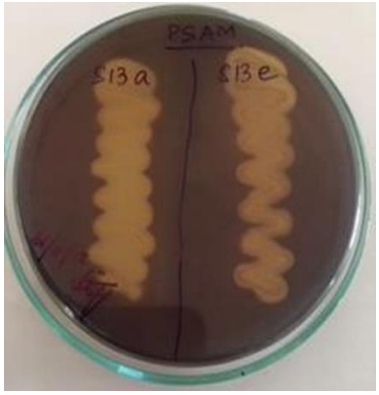
SCREENING

PRIMARY SCREENING

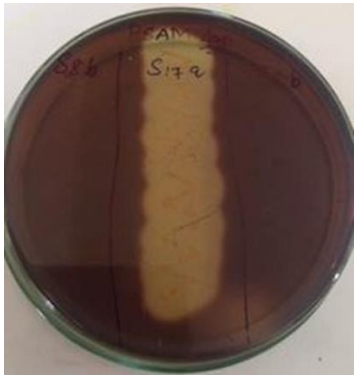
15 isolates were screened for good pectinase production

As a primary screening, the pure cultures were streaked on the PSA medium and were detected for clear zone with the addition of iodine solution. Out of which 5 isolates showed promising results .

P1



P2 **P3** **P4**

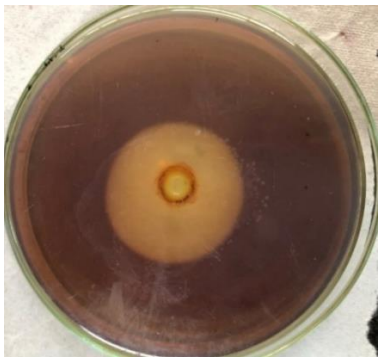


P5

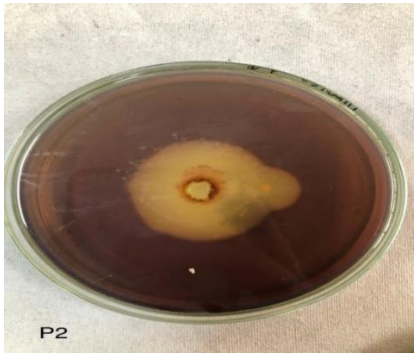
FIGURE 4: Primary screening of pectinase producing bacteria

SECONDARY SCREENING

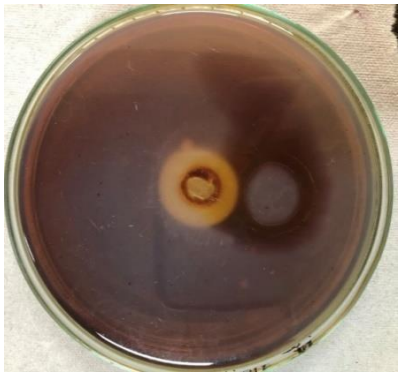
Further confirmation was carried out by secondary screening. P2 followed by P1 showed maximum zone of clearance.Hence, further studies were carried out with P2 isolate.



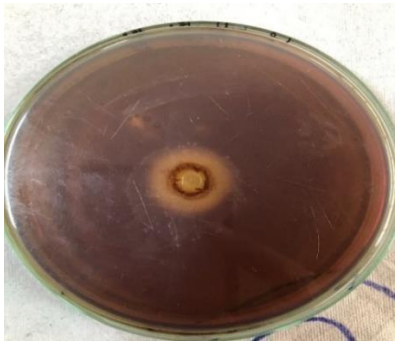
P1



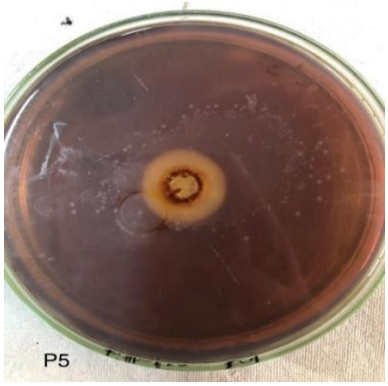
P2



P3



P4



P5

FIGURE 5: Secondary screening: halo zone detection

ISOALTE	ZONE OF CLEARANCE DIAMETER (cm)
P1	3.5
P2	3.8
P3	2.0
P4	2.0
P5	2.0

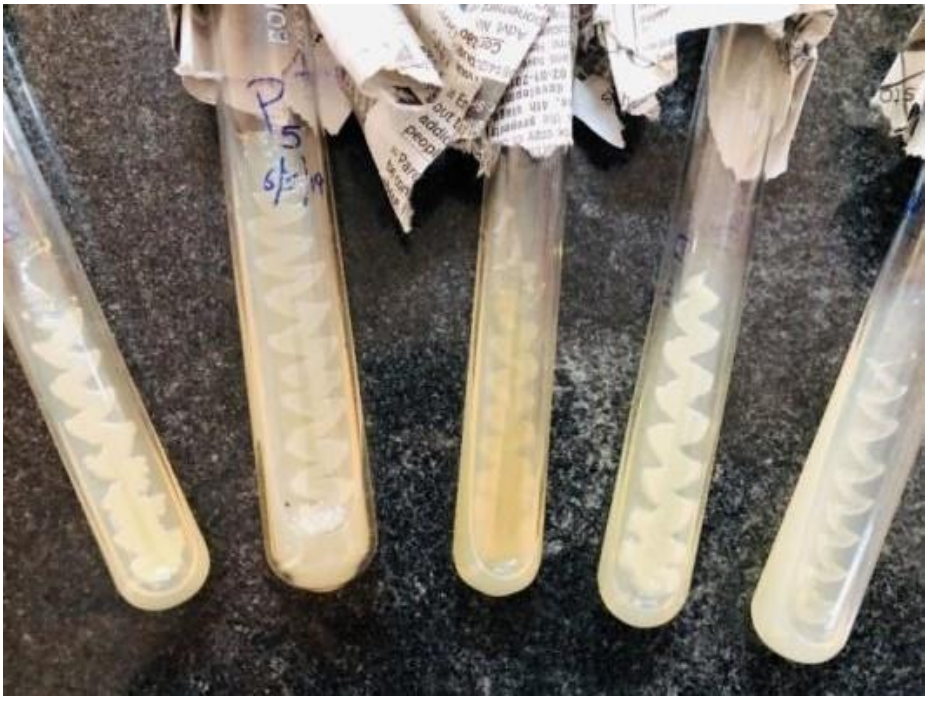
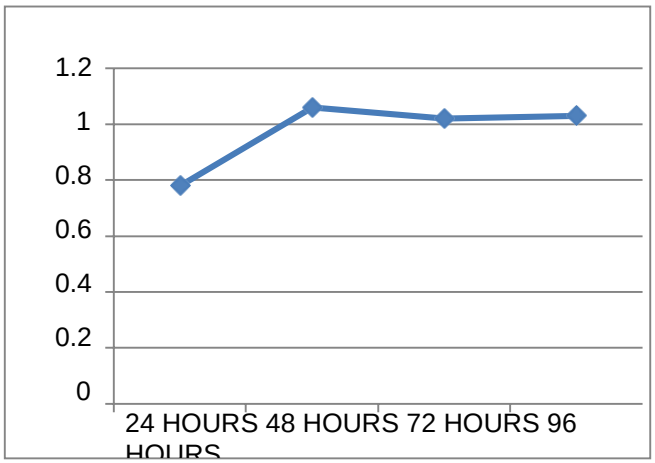


FIGURE 6: Pure cultures of the pectinase producing bacteria

PECTINASE PRODUCTION ASSAY

The optimum incubation time required for the production of enzyme was maximum at 48 hours of incubation at 37℃. Therefore, for further studies the enzyme used was incubated for 48 hours

INCUBATION TIME	ABSORBANCE AT 540 nm
24 HOURS	0.78
48 HOURS	1.06
72 HOURS	1.02
96 HOURS	1.03



IDENTIFICATION OF PECTINASE PRODUCING BACTERIA

The bacterial isolates were subjected to series of biochemical test to identify tentative bacterial species. Table 3, summarizes the entire test, Gram staining showed that P2, P3, p4 was gram positive and P1 and P5 was gram negative. P1 and P5 were positive for oxidase where as P2 P3 P4 were positive for catalase. P2 P3 P4 are aerobic and P1 P5 are anerobic. P2 P3 P4 were found to be negative for VP test. P1 and P5 were *nesierria* species and P2, P3, P5 were *micrococcus* species.

ISOLATES	GRAM`S NATURE	OXIDASE	CATALASE	O-F TEST	V-P TEST
P1	NEGATIVE	POSITIVE	-	-	-
P2	POSITIVE	-	POSITIVE	OXIDATIVE	NEGATIVE
P3	POSITIVE	-	POSITIVE	OXIDATIVE	NEGATIVE
P4	POSITIVE	-	POSITIVE	OXIDATIVE	NEGATIVE
P5	NEGATIVE	POSITIVE	-	-	-

TABLE 2: The overall view of the identification of pectinase producing bacteria

PHYSICO CHEMICAL PROPERTIES

PARTIAL PURIFICATION OF PECTINASE

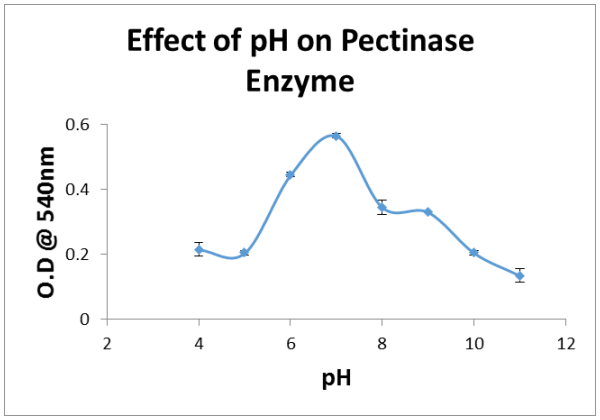
The crude enzyme, which was secreted out was collected by centrifugation and partially purified by acetone precipitation. The protein obtained was estimated using lowerys method, the table below shows the amount of protein per ml at different pH range.

PROTIEN IN DIFFERENT pH	Amount of protein(per mL)
pH4	32 µg
pH 5	19.6 µg
pH 6	32 µg
pH 7	48 µg
pH 8	128 µg
pH 9	144 µg
pH 10	128 µg
pH 11	144 µg

Table 3: amount of protein

EFFECT OF pH

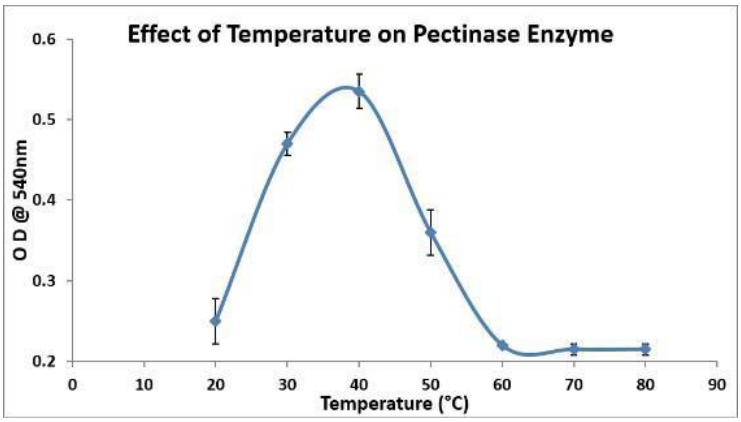
The optimum pH at which the enzyme was most active was found to be at pH 7. For further studies, the enzyme was used at the same pH.



pH	OD at 540nm
4	0.23
5	0.20
6	0.45
7	0.56
8	0.33
9	0.33
10	0.21
11	0.15

EFFECT OF TEMPERATURE

The maximum activity was found to be at 40 degree.



TEMPERATURE	ABSORBANCE AT 540 nm
20	0.23
30	0.48
40	0.55
50	0.38
60	0.22
70	0.21
80	0.21

JUICE CLARIFICATION

The partially purified enzyme with the optimum pH and temperature was estimated for its effectiveness in juice clarification, which showed maximum clarification with certain amount of enzyme.

FIGURE 7: Juice clarification

CHAPTER 5 - CONCLUSION

The study was concerned with isolating novel bacteria from different fruit waste market locations that are able to produce economically valuable pectinase enzyme and are potentially used in different industries. The isolation of the 5 pure strains (P1 , P2 , P3, P4 , P5) of the pectinase producing bacteria was done through primary and secondary screening.

The pure cultures once obtained were identified and characterized through a series of biochemical testes which included oxidase test, catalase test , oxidative fermentation test , vogues proskeur test. This biochemical tests helped in tentatively identifying the bacteria as *Nesierria* species and *Micrococcus* species. In the further study physio chemical properties were carried out, this was done by first partially purifying the enzyme produced through acetone precipitation, the partially purified enzyme which was obtained was taken for studying the effect of temperature and effect of pH, with the results showing enzyme activity maximum at pH 7 and at temperature 40 degree. Lastly an application of the enzyme was done by juice clarification and the enzyme showed clear results by clarifying the juice.

REFERNCES

1. Aguilar G, Huitrón C. Constitutive exo-pectinase produced byAspergillus sp. CH-Y-1043 on different carbon source. Biotechnology Letters. 1990 Sep 1;12(9):655-60.
2. Abhishek M, Akhilesh K, Singh AK, Ankur K. Isolation, purification and characterization of alkaline pectinase from Bacillus subtilis isolated from soil. Der Pharmacia Sinica. 2014;5(6):1-6.
3. Abdullah AL, Sulaiman NM, Aroua MK, Noor MM. Response surface optimization of conditions for clarification of carambola fruit juice using a commercial enzyme. Journal of Food Engineering. 2007 Jul 1;81(1):65-71.
4. Aneja K. R . Experiments in microbiology,plant pathology and biotechnology . New age international ; 2007 .
5. Blanco P, Sieiro C, Villa TG. Production of pectic enzymes in yeasts. FEMS Microbiology Letters. 1999 Jun 1;175(1):1-9.
6. Baladhandayutham S, Thangavelu V. Optimization and kinetics of solid-state fermentative production of pectinase. International Journal of ChemTech Research. 2011 Oct;3(4):1758-64.
7. Bhardwaj V, Garg N. Exploitation of micro-organisms for isolation and screening of pectinase from environment. InGlobelics 2010 8th International Conference, Universityof Malaya, Kuala Lumpur, Malaysia 2010 Nov (Vol. 13).
8. Brown RL, Cleveland TE, Cotty PJ, Mellon JE. Spread of Aspergillus flavus in cotton bolls, decay of intercarpellary membranes, and production of fungal pectinases. Phytopathology. 1992 Apr 1;82(4):462-7.
9. Damak N, Hadj-Taieb N, Bonnin E, Bacha AB, Gargouri A. Purification and biochemical characterization of a novel thermoactive fungal pectate lyase from Penicilliumoccitanis. Process Biochemistry. 2011 Apr 1;46(4):888-93.
10. Evangelista DE. Recombinant production, enzymatic characterization and studies on the occurrence of pectinases in sugarcane boll weevil (Sphenophoruslevis, Curculionidae).
11. Farooqui MJ. Cost-effective production and process optimization of Pectinaseunder submerged fermentation. Asiatic. J Biotechno. Resour. 2012;3:1419-23.

12. Gummadi SN, Panda T. Purification and biochemical properties of microbial pectinases—a review. Process biochemistry. 2003 Feb 28;38(7):987-96.
13. Grainger JN, Shillitoe AJ. Histochemical observations on galactogen. Stain technology. 1952 Jan 1;27(2):81-5.
14. Gailing MF, Guibert A, Combes D. Fractional factorial designs applied to enzymatic sugar beet pulps pressing improvement. Bioprocess Engineering. 2000 Jan 1;22(1):69-74.
15. Horikoshi K. Production of Alkaline Enzymes by Alkalophilic Microorganisms: Part III. Alkaline Pectinase of Bacillus No. P-4-N. Agricultural and Biological Chemistry. 1972 Feb 1;36(2):285-93.
16. Hoondal G, Tiwari R, Tewari R, Dahiya NB, Beg Q. Microbial alkaline pectinases and their industrial applications: a review. Applied Microbiology and Biotechnology. 2002 Jan 1;59(4-5):409-18.
17. Ibrahim D, Weloosamy H, Lim SH. Effect of agitation speed on the morphology of Aspergillus niger HFD5A-1 hyphae and its pectinase production in submerged fermentation. World journal of biological chemistry. 2015 Aug 26;6(3):265.
18. Islam S, Feroza B, Alam AK, Begum S. Pectinase production by Aspergillus niger isolated from decomposed apple skin. Bangladesh Journal of Scientific and Industrial Research. 2013 Jun 22;48(1):25-32.
19. Jayani RS, Saxena S, Gupta R. Microbial pectinolytic enzymes: a review. Process Biochemistry. 2005 Sep 1;40(9):2931-44.
20. Kashyap DR, Soni SK, Tewari R. Enhanced production of pectinase by Bacillus sp. DT7 using solid state fermentation. Bioresource Technology. 2003 Jul 1;88(3):251-4.
21. Kaur A, Mahajan R, Singh A, Garg G, Sharma J. Application of cellulase-free xylano-pectinolytic enzymes from the same bacterial isolate in biobleaching of kraft pulp. Bioresource Technology. 2010 Dec 1;101(23):91
22. Kuhad RC, Kapoor M, Rustagi R. Enhanced production of an alkaline pectinase from Streptomyces sp. RCK-SC by whole-cell immobilization and solid-state cultivation. World Journal of Microbiology and Biotechnology. 2004 Apr 1;20(3):257-63.
23. Kumar A, Sharma R. Production of alkaline pectinase by bacteria (Cocci sps.) isolated from decomposing fruit materials. Journal of Phytology. 2012 Mar 13.

24. Liang S, Wang H, Yang M, Wu H. Sequential actions of pectinases and cellulases during secretory cavity formation in Citrus fruits. Trees. 2009 Feb 1;23(1):19.
25. Lubrano C, Flavet L, Saintigny G, Robin JR, inventors; Laboratories de BiologieVegetale Yves Rocher, assignee. Methods of treating aging of skin with oligosaccharides in cosmetic or dermatological compositions that stimulate adhesion of keratinocytes to major proteins of the dermoepidermal junction and restore epidermal cohesion. United States patent application US 11/894,945. 2007 Dec 20.
26. Martos MA, Martinez Vazquez F, Benassi FO, Hours RA. Production of pectinases by A. niger: influence of fermentation conditions. Brazilian Archives of Biology and Technology. 2009 Jun;52(3):567-72.
27. Maldonado MC, Navarro A, Callieri DA. Production of pectinases byAspergillusspusing differently pretreated lemon peel as the carbon source. Biotechnology letters. 1986 Jul 1;8(7):501-4
28. Nair SR, Panda T. Statistical optimization of medium components for improved synthesisof pectinase by Aspergillus niger. Bioprocess Engineering. 1997 Feb 1;16(3):169-73.
29. Oyeleke SB, Oyewole OA, Egwim EC, Dauda BE, Ibeh EN. Cellulase and pectinase production potentials of Aspergillus niger isolated from corn cob. Bayero Journal of Pure and Applied Sciences. 2012;5(1):78-83.
30. Oumer OJ, Abate D. Characterization of pectinase from Bacillus subtilis strain Btk 27 and its potential application in removal of mucilage from coffee beans. Enzyme research. 2017;2017.
31. Panda T. Purification and biochemical properties of microbial pectinases—a review. Process biochemistry. 2003 Feb 28;38(7):987-9
32. Phutela U, Dhuna V, Sandhu S, Chadha BS. Pectinase and polygalacturonase production by a thermophilic Aspergillus fumigatus isolated from decomposting orange peels. Brazilian Journal of Microbiology. 2005 Mar;36(1):63-9.
33. Patil SR, Dayanand A. Exploration of Regional Agrowastes for the Production of Pectinase in Food Technology & Biotechnology. 2006 Apr 1;44(2).
34. Palaniyappan M, Vijayagopal V, Viswanathan R, Viruthagiri T. Screening of natural substrates and optimization of operating variables on the production of pectinaseby

submerged fermentation using Aspergillus niger MTCC 281. African journal of Biotechnology. 2009;8(4).

35. Pavan ME, Abbott SL, Zorzopulos J, Janda JM. Aeromonassalmonicida subsp. pectinolytica subsp. nov., a new pectinase-positive subspecies isolated from a heavily polluted river. International journal of systematic and evolutionary microbiology. 2000 May 1;50(3):1119-24.
36. Rodriguez MS, Zalba MS, Debbaudt AL, Agullo E. New chitosan–calcium pectinate pellets and their adsorption capacity. Colloid and Polymer Science. 2006 Oct 1;285(1):119-24.
37. Raju EV, Divakar G. Production of pectinase by using Bacillus circulans isolated from dump yards of vegetable wastes. International Journal of Pharmaceutical Sciences and Research. 2013 Jul 1;4(7):2615.
38. Soriano M, Diaz P, Pastor FJ. Pectinolytic systems of two aerobic sporogenous bacterial strains with high activity on pectin. Current microbiology. 2005 Feb 1;50(2):114-8.
39. Shobha MS, Kumar AV, Tharanathan RN, Koka R, Gaonkar AK. Modification of guar galactomannan with the aid of Aspergillus niger pectinase. Carbohydrate Polymers. 2005 Dec 1;62(3):267-73.
40. Soares MM, Silva RD, Gomes E. Screening of bacterial strains for pectinolytic activity: characterization of the polygalacturonase produced by Bacillus sp. Revista de Microbiologia. 1999 Dec;30(4):299-303.
41. Tariq A, Latif Z. Isolation and biochemical characterization of bacterial isolates producing different levels of polygalacturonases from various sources. African Journal of Microbiology Research. 2012 Nov 27;6(45):7259-64.
42. Venkatesh M, Pushpalatha PB, Sheela KB, Girija D. Microbial pectinase from tropical fruit wastes. Journal of Tropical Agriculture. 2009 Dec 18;47(1):67-9.
43. Vijayanand P, Kulkarni SG, Prathibha GV. Effect of pectinase treatment and concentration of litchi juice on quality characteristics of litchi juice. Journal of food science and technology. 2010 Mar 1;47(2):235-9.
44. Voragen F, Schols H, Visser RG, editors. Advances in pectin and pectinase research. Springer; 2013 Mar 9.

45. Wubben JP, Mulder W, Ten Have A, Van Kan JA, Visser J. Cloning and partial characterization of endopolygalacturonase genes from Botrytis cinerea. Appl. Environ. Microbiol.. 1999 Apr 1;65(4):1596-602.