Production of Polygalacturonase by *Aspergillus awamori sp. for* Clarification of Mosambi Juice

Project Report submitted in partial fulfilment of the requirement for the degree

of

Bachelor of Technology

In

Biotechnology

By

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Under the Supervision of

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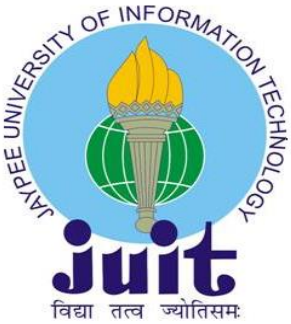

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DECLARATION

I hereby declare that the work reported in the B-TECH thesis entitled"Production of Polygalacturonase by Aspergillus awamori sp. For Clarification of Mosambi Juice"submitted at Jaypee University of Information Technology, Waknaghat , India , is an authentic record of my work carried out under the supervision of Dr.Garlapati Vijay Kumar. I have not submitted this work elsewhere for any other degree or diploma.

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CERTIFICATE

This is to certify that **Ms. C Pavithra (123815)**, of B. Tech Biotechnology, Jaypee University of Information Technology, Solan, HP-173234, has carried out her project titled "**Production of Polygalacturonase by *Aspergillus awamori sp. for* Clarification of Mosambi Juices**" as part of her project under my supervision. The work submitted is original to the best of my knowledge. I wish her all the success in her future endeavours.

(Dr. GARLAPATI VIJAY KUMAR)

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Date: (C.Pavithra)

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ABSTRACT

Polygalacturonase (PG) is necessary during fruit juice clarification process for the degradation of insoluble viscous pectinacious compounds. In the present study, PG (write full form) from *A. awamori* MTCC 9644 and *A.awamori brasiliensis* MTCC 1344 produced by solid-state fermentation of apple (*malus domestica*) and maosambi*(Citrus limetta)* which was procured from wine making industry and was dried and used it to produce the enzyme which was then purified using ammonium sulphate(60%) and activiated charcoal. Later the purified PG was used in clarification of fresh extracted mausambi juice and its increase in transmittance and reduction in viscosity was estimated.

Keywords: Polygalacturonase, pectinase, pectin, activated charcoal, decolorization, apple juice.

Chapter 1

Introduction & Literature Review

1. Pectic substances as substrates

Apple pomace is the processing waste generated after apple juice manufacturing and represents up to 30% of the original fruit. This solid residue consists of a complex mixture of peel, core, seed, calyx, stem, and soft tissue. This residual material is a poor animal feed supplement because of its extremely low protein content and high amount of sugar. The application of agro industrial by-products in bioprocesses offers a wide range of alternative substrates, thus helping solve pollution problems related to their disposal. Attempts have been made to use apple pomace to generate several value-added products, such as enzymes, single cell protein, aroma compounds, ethanol, organic acids, polysaccharides, and mushrooms. (Niharika Sood et al., 2014)

Pectin is the complex polysaccharide found in fruits and vegetables and helps in ripening. The highest concentration of pectin is found in the middle lamella of cell wall, with a gradual decrease as one passes through the primary wall toward the plasma membrane. Apple is particularly rich in pectin, the name applied to any one of a group of white, amorphous complex carbohydrates with a high molecular weight. Researchers have found that raw apples are the richest fruit sources in pectin. The characteristics structure of pectin is a linear chain of a (β-1-4)-linked D-galacturonic acid that forms the protein-backbone, a homogalacturonan. The highest concentrations of pectin are found in the middle lamella of cell wall, with a gradual decrease as one passes through the primary wall toward the plasma membrane. Pectic substanceslike pectin, protopectin and pectic acids, present in cell wall and middle lamella, contribute firmness and structure to plant tissues. (Niharika Sood et al., 2014)

In pectic substances, D-galacturonicacid units are linked together by a-1, 4-glycosidic linkages and the carbonyl side groups are 60_ 90% esterified with methanol. The rhamnose

units can be inserted into the main uronide chain and often sidechains of arabinan, galactan or arabinogalactan are linked to rhamnose. This indicates that various forms of pectic substances are present in plant cells and for this reason pectinases exist in various forms. Pectinasesdegrade pectic substances for nutritional purposes and they are responsible for plant pathogenesis. Pecticsubstances are widely distributed in fruits and vegetables (10_ 30% in turnips, peels of orange and in pulps of tomato, pineapple and lemon), hence they form important natural substrates for pectinases. In general pectinases are classified into de-esterification and depolymerizing enzymes based on the degradation mechanism.

Since the 1940s, pectinases have been exploited for many industrial applications. Pectinases are mainly used for increasing filtration efficiency and clarification of fruit juices, in wood preservation and used inmaceration, liquefaction and extraction of vegetable tissues. Various literature reports and reviews are available on the production and applications of pectinases. Recently pectinases have been used in the paper and pulp industry in addition to cellulases, plant pathologyand in protoplast fusion technology. Few recent reviews have highlighted thebiological and technological importance of pectinases. Nearly 75% of industrial enzymes in l995 has been contributed bypectinases. Novozymes (Denmark), Novartis (Switzerland), Roche (Germany) and Biocon (India) are some important commercial producers of pectinases.

1.1 Pectinases

Pectinases, the most important enzymes in the fruit juice industry, form a consortium of enzymes essential for the hydrolysis of pectin of plant tissues. It comprises about 25% of the total food enzymes sale (Jayani et al., 2005). Based on the pectin degradation mechanism they are classified mainly into pectinesterase, pectinlyase and polygalacturonase (PG). Among them, PG hydrolyses α-1, 4 glycosidic linkages of polygalacturonic acid chain and releases galacturonic acid residues (Kant, Vohra & Gupta, 2013). Although it is found in

higher plants and few parasitic nematodes, however, it is widely distributed in the microbial kingdoms including fungi, bacteria and yeast. Mainly microbial sources are used for the industrial production and application in several processes including fruit juice clarification, degumming and retting of plant fibers, treatment of pectic waste water, coffee and tea fermentation, paper and pulp industry, oil extraction etc. (sood et al., 2014).

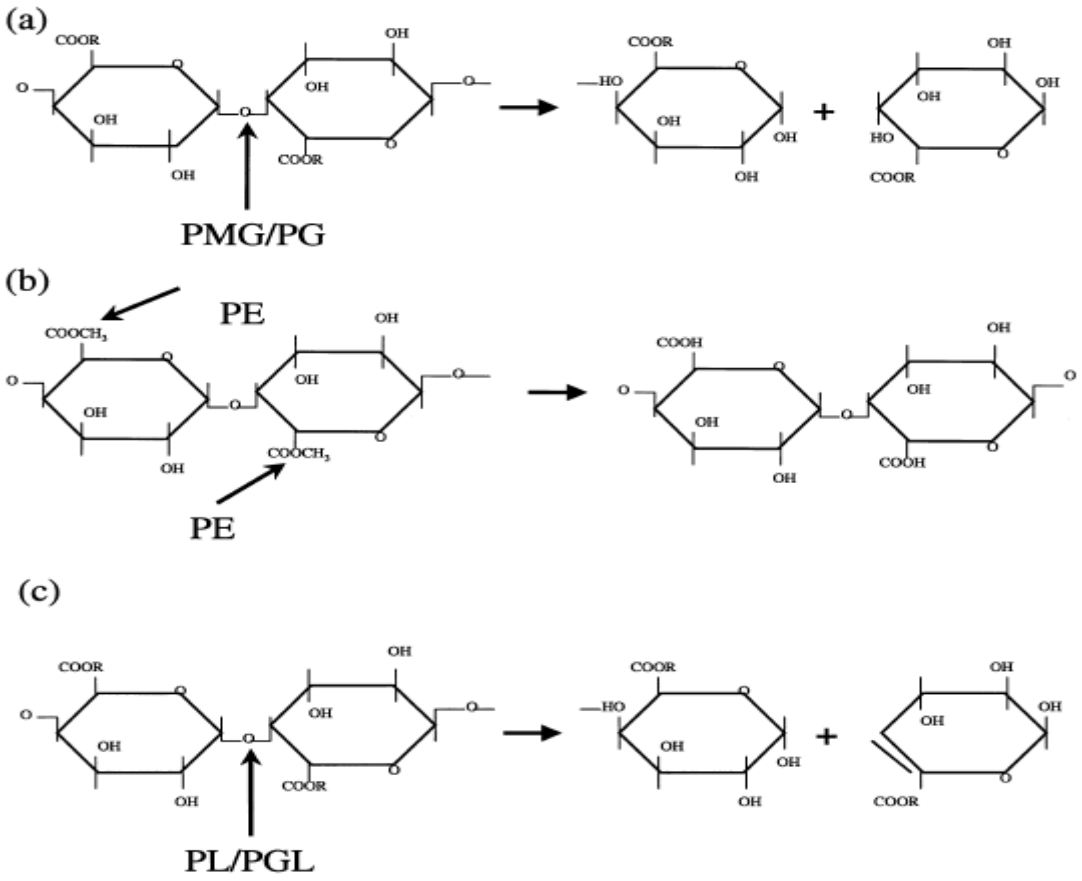

Figure 1: Different types of pectnases and their mode of action on pectic substrates (a)R=H for PG and CH3 for PG and CH3 for PMG (b) PEand (c) R=H for PGL and CH3 for PL. (K. Anuradha et al.,2010)

1.2 History of pectinase research

The history of pectinases began with an understanding the structure of pectic substances and the mechanism by which pectolytic enzymes degrade pectic substances later the microbial production of pectinases became prominent for many decades. Many microorganisms, viz.,

bacteria, yeast and fungi could produce pectinases. Evidence showed that pectinases are inducible and they can be produced from different carbon sources. In the course of time, numerous reports have appeared on the optimization of fermentation and microbiological parameters and different fermentation strategies for the production of pectinases. With the advent of molecular biology, vigorous research has been carried out on cloning and expression of pectinase genes in various hosts. The expression of cloned fungal genes including the pectinase genes from Aspergillus aculeatus in yeast was reviewed by DalbØge. In order to use pectinases as a suitable industrial biocatalyst and to apply these in various research fields, the purification and improved knowledge of enzyme properties is essential. Aspergillus niger pectinases are most widely used in industries because this strain posses GRAS (Generally Regarded As Safe) status so that metabolites produced by this strain can be safely used.

This fungal strain produces various pectinases including polymethylgalacturonase (PMG), polygalacturonase (PG) and pectinesterase (PE). However, particular pectinases are used for specific purposes, for example only PG is used in baby food products. An effective approach is recombinant genetic techniques by which specific pectic enzymes can be expressed in different hosts. In order to achieve this, the original protein may be purified and characterized to determine the sequence. For this purpose, various researchers have focused on purification, stability and catalytic performance of pectinases from various sources. Fig 1. Explains how mode of action of various pectinase enzymes on the pectic substances, (a)In case of PG , it attacks R= H and CH3 is the site of action for PMG *(*polymethylgalacturonase) (b) PE (pectinesterase) and (c) and PGL will act on R=H and CH3 for PL. The arrow indicates the place where the pectinases reacts with pectic substances.

PMG: polymethylgalacturonase, PG: polygalacturonase (EC 3.2.1 15). PE: pectinesterase (EC 3.1.1 11), PL: pectinlyase (4.2.2 10). Apart from these other pectinases, viz.,

protopectinase (degrading protopectin), Oligogalacturonases (degrading D-galactosiduronates) exists. The pectinases are further subclassified based on the nature of reaction. Endopectinases cleaves the substrate in random fashion while exopectinases cleaves in end-wise fashion. (Sathyanarayana N. Gummadi et al., 2014)

Mosambi juice is one of the most popular juices consumed all over the world. Clarification of mosambi juice is essential before its commercialization because raw mosambi juice obtained after the mechanical pressing of mosambi is very viscous, turbid of cloudy, cream colored as well as it has a tendency to settle down during storage. Pectin is the key substance responsible for the above-mentioned problems present in the raw juice. Hence, degradation of pectic substances by pectinases is the main step in conventional clarification process in order to increase juice yield, clarity, sweetness and self-life with less viscosity. The pH and thermal stability of pectinases Enzyme deactivation and stability are considered to be the major constraints in the rapid development of biotechnological processes. Stability studies also provide valuable information about structure and function of enzymes. Enhancing the stability and maintaining the desired level of activity over a long period are two important points considered for the selection and design of pectinases. The stability of pectinases is affected by both physical parameters (pH and temperature) and chemical parameters (inhibitors or activators). The optimal pH for *Rhizopus stoloniferendo*-PG was stable inthe pH range 3.0-5.0 and this enzyme is highly specific to non-methoxylated PGA. They also isolated two PGs and PLs from *Aureobasidium pullulans* . The two PGs were stable at pH 5.0 and 7.5 and at a temperature of 50.8C whereas two PLs exhibited maximum stability at 5.0 and 7.5 and at a temperature of 40.8C. It has also been reported that PL from *Aspergillus fonsecaeus* was stable at 5.2. This PL does not react with PGA but it does with PGA pre-treated with yeast PG. The optimal pH for *A. niger* PMG was around 4.0. Most of the reports in the literature studied the pH and thermal stability by conventional optimization methods

(i.e. the effect of temperature on pectinase stability was studied at constant pH and vice versa).The interaction effect between pH and temperature is another interesting aspect, which alters the stability differently. The combined effect of pH and temperature on stability of three pectinases, viz., PMG, PG and PL from A. niger was studied in this laboratory using response surface methodology.

For this purpose, a central composite design was used and a quadratic model proposed to determine the optimal pH and temperature conditions at which pectinases exhibit maximum stability. The optimum pH and temperature were 2.23 and 23 8C, respectively, for PMG, 4.8 and 28.8c, respectively, for PG and 3.9 and 29.8c, respectively, for PL. PL was more stable than PMG and PG. The results showed that assay conditions of pH and temperature are not optimal conditions for stability, which is in agreement with the fact that catalytic performance (activity) and stability of pectinases are quite different aspects. This led further investigations on the catalytic performance of these enzymes. Deactivation kinetics has been conducted for both partially purified PMG and PG and crude enzymes. Thermodynamic parameters were estimated by considering the deactivation as first order process. The entropy of deactivation is negative for both partially purified and crude enzymes. The negative entropy of deactivation is contributed due to structural effects (unfolding of enzyme) or due to solvent effect (presence of charged molecules around the enzyme). Other thermodynamic parameters like, enthalpy, free energy and activation energy of deactivation was also determined. Similarly, the stability of pectic enzyme in sweet cherry brines has been studied by deactivation kinetics. Authors estimated thermodynamic parameters like enthalpy and entropy of deactivation based on first order deactivation rate constants for crude enzyme preparations. In addition, the interaction effect between the partially purified enzyme fractions was also studied. The stability of partially purified PMG increased in the presence

of partially purified PG and vice versa. In addition, the stability of pectinases was slightly enhanced by the presence of potassium ions.

Solid-state fermentation (SSF) has been carried out for the production 75 of industrially useful PGs by different microorganisms like *Penicillium* sp. (Silva, Martins, Da Silva, & Gomes, 2001; Silva, Martins, Leite, Silva, Ferreira, & Gomes, 2007), *Aspergillus*sp. (Nakkeeran, Subramanian, &Umesh-Kumar, 2010; Heerd, Yegin, Tari, & Fernandez Lahore, 2012), *Thermoascusaurantiacus*(Martins, Silva, Da Silva, & Gomes, 2002),*Trichoderma*sp. (Olsson, Christensen, Hansen, &Palmovist, 2003; Mohamed, Farid, Hossiny, &Bassuiny, 2006), *Lentinusedodes*(Zheng & Shetty, 2000), *Rhizopus*sp.2 (Saito, Takakuwa, &Oda, 2004), *Bacillus* sp. (Gupta, Kapoor, Sharma, Nair, &Kuhad, 2008; Rehman, Qader, &Aman, 2012). Among them, acid stable PGs from *Aspergillus* sp. are mainly used in fruit juice clarification process.

The crude enzyme extract obtained after SSF process contains a mixture of metabolites including a significant amount of melanine like color compounds (Aikat, Maiti, & Bhattacharyya, 2001) that can interfere in the juice clarification process. Hence, downstream processing is essential for the purification of the targeted enzyme prior to specific commercial application. Selection of minimum number of separation steps with high specific activity or purity and high recovery is recommended for the cost effective application of purified enzyme.(Tapati Bhanja Dey et al ., 2014).

Pectinases are a complex group of enzymes that degrade various pectic substances present in plant tissues. Pectinases have Potential applications in fruit, paper and textile industries. Apart from these industrial applications, these enzymes possess biological importance in protoplast fusion technology and plant pathology. Since applications of pectinases in various

fields are widening, it is important to understand the nature and properties of these enzymes for efficient and effective usage.

Fungal isolates from natural pectic substrates for polygalacturonase and multienzyme production (K. Anuradha · et al') showed that the maximum out of eighty pectinolytic fungal isolates that were obtained by enrichment culturing and ruthenium red plateAssay the isolate of *Aspergillus awamori MTCC* 9166was with highest polygalacturonase activity which led to the selection of *A. awamori sp.* for production of PG.

In the paper-'Use of Pectin Rich Fruit Wastes for Polygalacturonase Production by *Aspergillus awamori* MTCC 9166 in Solid State Fermentation by (P.Naga Padma2 et al') the author studied the production of PG using different pectin-rich fruit wastes like apple peel, banana peel, citrus (orange) peel, jackfruit rind, mango peel, and pine apple peel and it was seen that jackfruit rind showed highest production. Also in research article by (Katsuichi Saito et al., 2004, purification of the extracellular pectinolytic enzyme from the fungus *Rhizopus oryzae* NBRC 4707), solid-state culture of *Rhizopus oryzae* NBRC 4707 was purified to homogeneity by column chromatography on CM-Toyopearl 650 M and hydroxylapatite. The molecular weight of the enzyme was estimated by SDS-polyacrylamide gel electrophoresis to be 31,000 and was reduced to 29,700 after treatment with endoglycosidase H. Maximal activity was observed near pH 4.5 at 451C.

The enzyme was shown to be endopolygalacturonase, as judged from the formation of oligogalacturonides as its reaction products. The addition of purified enzyme, as expected, enhanced the formation of lactic acid and ethanol in potato pulp grown with *R. oryzae*. In the paper – 'Purification of polygalacturonase from *A. awamori nakazawa* MTCC 6652 and its application in apple juice clarification by Tapati Bhanja Dey et al published in LWT Food and science technology (2014)' the author used *A awamori nakazawa* MTCC 6652 for the production of PG using mosambi pomace as a substrate for the Aspergillus to feed on.It was

found that using activated charcoal, PG was purified efficiently from solid-state culture of *A.awamori nakazawa* in a single step with very high purification fold and yield. The purification process is very simple, time saving and economic. The purified PG was partially characterized and applied successfully for the apple juice clarification.

Objective(s)

- Cultivation of the Apergillus sp. using PDA
- Production of the polygalacturonase enzyme through Solid State Fermentation using-Apple and Mosambi Pomace, and its extraction
- Selection of parameters for polygalacturonase production
- Concentration and purification of the polygalacturonase enzyme
- Study of effect of pH and temperature on the enzyme activity
- Application of the polygalacturonase enzyme in clarification of mosambi juice

Chapter 2

Materials and Methods

2.1 Materials

The following materials and chemicals (stated in Table 1) were used for carrying out various tests and experiments.

- **Substrate**

Apple Pomace and Mosambi Pomace were used as the substrates. The peels were dried in a hot air oven at 80oC for 24 hours and ground using a lab mixer grinder.

- **Fungus**

The production of Polygalacturonase (PG) by *Aspergillus awamori sp.* procured form the repository of Jaypee University of Information Technology –
MTCC-1344 and MTCC-9644.

- **Chemicals**

For the Revival of the strains namely *A. awamori* MTCC No. - 1344 and *A. awamori* MTCC No.-9644 - Potato Dextrose Broth cultivated and maintained on - potato dextrose agar (PDA)

For setting up of solid state fermentation Czapekdox medium was used. Table 2 describes the composition of the Czapekdox media used.

Table 1: Composition of the PDA medium used

PDA Medium (for maintenance of Microbes)	
Component	**Amount (g/100ml)**
Potato Dextrose Agar	3.9
Water	Up to 100 ml

Table 2: Composition of the Czapedox media used

Czapekdox Media(for Production)	
Component	**Amount (g/l)**
Yeast extract	5
Di-potassium	1
Sodium Nitrate	0.3
Potassium Chloride	.05
Magnesium Sulphate	.05
Ferrous Sulphate	.001
Zinc Sulphate	.001
Copper Sulphate	.0005
Sucrose	30

2.2 Methods

- **Reviving of Microbial Culture**

  The strains maintained in the repository were inoculated in potato dextrose broth and incubated for 3 days at 30 degree Celsius. Streaking of PDA slants. Spore suspension was prepared with the addition of water in heavily sporulated PDA slants. Streaking was done within 3 days in order to maintain the culture.

- **Solid State Fermentation**

  Procured the substrate for enzyme production – Apple Pomace, Mosambi Pomace. Substrate preparation for Solid state fermentation was done which included the following steps.

  - Drying- dried in a hot air oven at 80°C for 24hrs.
  - Grinding- used lab mixer grinder
  - The ground peels were then sieved using mesh size of 60 BSS(2.382-1.41mm)
  - The grounded peels were then autoclaved at 121°C

Czapek-dox medium with pH 4.0 was mixed with 5 g of ground peels in 1:2 ratio (w/v). It was taken in a 250 ml Erlenmeyer flask. Autoclaved at 103.42 KPa pressure and 121°C temperature for 20 min. Then it was cooled. SSF was carried out inoculating 0.5 ml spore suspension (1×10^6 spores/ml) of *A. awamori.* The flasks were then kept static for incubation at 30 °C for 72 hrs.

- **Set ups of solid state fermentations**

    1. **To check the best suited substrate for each strain**

Four SSF's were set in order to know which is the best suited pomace (Apple or Mosambi) for each *A. awamori*strain i.e. MTCC-1344 and MTCC-9644

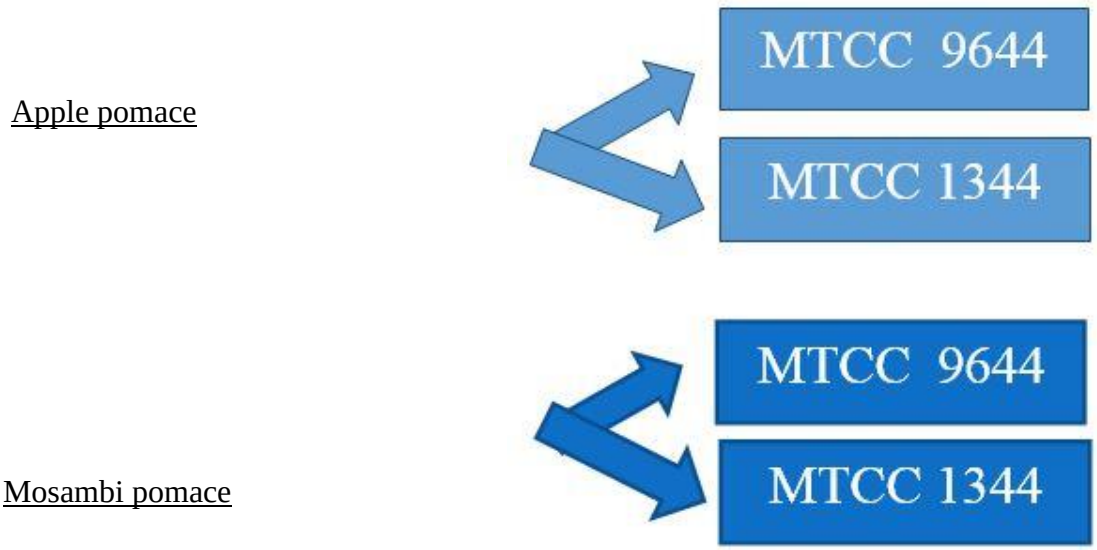

Figure 2: Depicts the pattern of fermentation set both apple

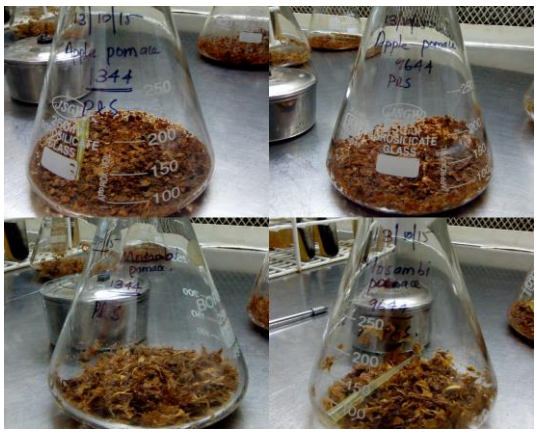

Figure 3: Showing solid state fermentation setups

- **Selection of production variables**

  Two parameters were tested that is time and temperature.

  - **Selection of incubation time for polygalacturonase production**

    The optimum time required for the production of PG was seen by placing 4 SSFs for each strain with different incubation time using mosmabi pomace as substrate. Time spans of 24hr, 48hr, 76hr and 96hr was used with a 24hrs difference of incubation time. Eight SSFs were set at 30 degree Celsius each for different incubation period in totality.

  - **Selection of temperature for polygalacturonase production**

    The best suited temperature required by *A. awamori* MTCC-9644 and MTCC-1344 for production of PG was tested by placing 4 SSFs at varied temperature. Incubation temperatures: 25 °C, 30 °C, 35 °C & 40 °C were used with a gap of 5°C in each incubation temperature.

  - **Solid liquid ratio**

    The solid to liquid ratio that is the amount of substrate and media used for SSF was at pH 4.0 the Czapek-dox medium [NaNO3 (2.5 g/L), KH2PO4 (1 g/L), KCl (0.5 g/L) and 109 MgSO4.2H2O (0.5 g/L)] was mixed with 5 g of ground peels in 1:2 ratio (w/v) taken in a 250 ml Erlenmeyer flask.

  - **Extraction of Enzyme**

For the extraction of the enzyme from the ssf after 72 hrs, these flasks were incubated at 30°C for 1 hr after adding 20 ml of distilled water. After incubating the enzyme was harvested, the fermented slurry was squeezed through muslin cloth and the extract was collected.Centrifugation was carried out at 2200g for 10 min. The resulting extract was used for assay of enzyme activites and lowry.

2.3 Polygalacturonase assay

The assay was performed with the crude enzyme extract from both the ssf setups of apple and mausambi which had been setup using strains mtcc 1344 no. and mtcc no. 9644.The assay was performed according to the method of Dong and Wong (2011) with few modifications. A reaction mixture (1ml) containing equal amounts of polygalacturonic acid as substrate (1%) prepared in acetate buffer (ph 4.5) and suitably diluted enzyme was incubated

In water bath for 50c for 10 min. After incubation 3ml DNS solution was added to stop this reaction and the tubes were kept for incubation in boiling water (100c) for 15 min. then 1 ml of 40% of Rochelle's salt solution was added and tubes were then left for cooling. After cooling the developed color was read spectrophotometrically at 575 nm. The amount of released reducing sugar was quantified using galacturonic acid as standard. The enzyme activity was calculated as the amount of enzyme required to release one micromole of galacturonic acid per min under assay condition.

2.4 Estimation of protein by lowry method

The protein concentration per mg/ml was estimated using BSA as standard following the method of lowry. For every enzyme sample of crude enzyme, ammonium sulphate precipitated enzyme (60%), activated charcoal purified enzyme the protein content was measured.

2.5 Purification of enzyme:

- **Ammonium sulphate precipitation (60%):**

  The crude enzyme extract (100ml) was subjected to ammonium sulphate precipitation (60%).the sulphate precipitation is a method that is used to purify proteins by altering their solubility. Ammonium sulphate has been commonly used as its solubility is so high that salt solutions with high ionic strength are

allowed. Later the enzyme activity for the enzyme sample from this purification was done.

- **Activated charcoal purification:**

  Activated charcoal at the concentration of 10 g/ 100 ml. It was kept at 30 degree Celsius for 15 min. Then the mixture was centrifuged at 2200 g for 10 min and filtered. Later the enzyme activity for the enzyme sample from this purification was done.

Figure 4: Extracting Enzymes

Figure 5: Crude Enzyme

2.6 Effect of pH and temperature on the enzyme activity

The effect of temperature and pH on the activity of the purified enzyme was studied .Optimum pH and temperature of purified PG activity was analyzed by carrying out assays at different pH ranging from 2 to 8.0 using different buffers (acetate and phosphate) of 0.1 mols/L concentration at 50°C and at various temperatures (30°C-80°C) at optimum pH. .

2.7 Application of purified polygalacturanase in clarification of mosambi juice

Fresh Mosambi juice was purchased from local market and stored at 4oC until used. The purified enzyme (7.21 U/ml) was mixed with the juice (1%; v/v) and incubated at 50oC for 2 h. The reaction was terminated by increasing the temperature of the mixture to 90oC for 1 min. The PG treated clarified juice was centrifuged at 2200g for 10 min and then it was analysed for juice yield, clarity (%T660 nm), viscosity reduction and pH. Viscosity was measured using Oswald viscometer.

CHAPTER 3

Results and discussion

3.1 selection of polygalacturonase production variables

- Fermentation conditions (temperature, time period, humidity, pressure) are few examples of variables that can influence the amount and the enzyme reduced.
- In study up till now I have tried to select temperature and time period. The optimum temperature for the production of the pectinase enzyme is 30°c and pH 4.5
- The pomace (apple,mausambi)they were sieved using mesh size of 60 BSS

Table 3: Time for incubation

S.no	Time period for incubation
1)	24hrs
2)	46hrs
3)	72hrs
4)	96hrs

Table 4: Temperature of incubation

S.no	Temperature of incubation
1)	25°C
2)	30°C
3)	35°C
4)	40°C

3.2 Solid state fermentation setup

- *Aspergillus awamori*mtcc 9644 and *Aspergillus awamori* mtcc 1344 were grown on apple pomace and mausambi pomace, using czapek dox media-they grew successfully at 30°C incubated for 72 hrs.

3.3 Extraction of crude enzyme

- The enzyme was successfully extracted and it was seen that more amount of crude enzyme was extracted from fermentation of mausambi pomace using muslin cloth and it was centrifuged at 2200g for 10minutes.

3.4 Polygalacturonase assay

When the assay was performed on the crude enzyme that was extracted from mausambi SSF, it was seen that crude enzyme that was extracted from mausambi SSF inoculated with A.awamori (mtcc 1344) had more activity than the crude enzyme extracted from the SSF inoculated with A.awamori (mtccc 9644)

Table 5: enzyme activity estimated for crude enzyme through PG assay

Substrate(a.awamori strain)	Polygalacturonase enzyme activity(U/ml)
SSF(mosambi ,MTCC 1344)	7.21
SSF(mosambi,MTCC 9644)	4.33

As observed,since enzyme produced by MTCC 1344 had more enzyme activity,I have used this enzyme further for purification and clarification studies.

3.5 Purification of the crude PG enzyme

- The enzyme was purified using ammonium sulphate precipitation (60%) method and activated charcoal and the enzyme activity, protein content, specific activity was measured.

Table 6: enzyme activity, protein content, specific activity measured at various level of purification

Sample ,1344 MTCC mausambi SSF	Enzyme activity(U)	Protein content(mg)	Specific activity(U/ml)	Purification fold(%)
Crude enzyme	180.25	11.5	15.67	1
Ammonium sulphate(60%)	186.75	9.75	19.15	1.23
Activated charcoal	195.75	6.75	29	1.85

- The results have been summarised in the table. Utilizing ammonium sulphate precipitation method the enzyme was partially purified, there was increase in the enzyme activity and reduction in the protein content. Then when further purified with activated charcoal the enzyme activity increased and protein content was reduced.

3.6 Effect of pH and temperature on the enztme activity of polygalacturonase

Further study of the effect of pH and temperature had been carried out on the purified enzyme extracted from ssf inoculated with Aspergillus awamori(mtcc no.1344). It was seen that the enzyme showed maximum activity at pH 4.5 and at temperature 50°C similar to the results obtained by Tapati Banja Dey and Sunita(2014).

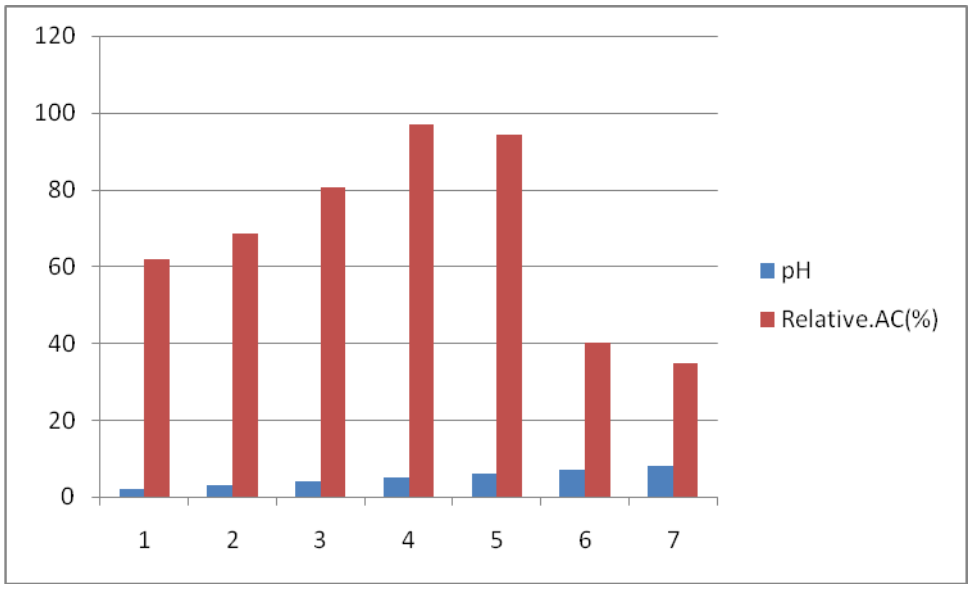

Figure 6. Efect of pH on the activity of purified PG

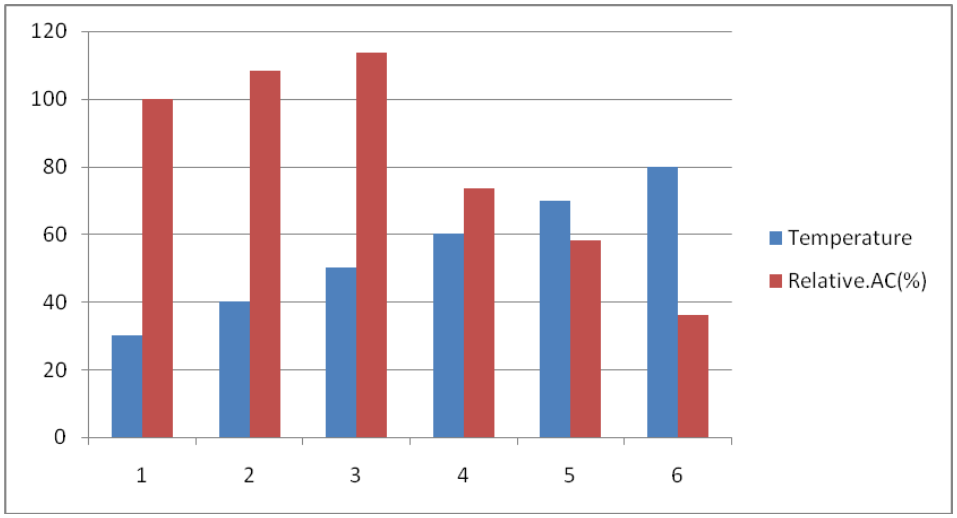

Figure 7: Effect of Temperature on the activity of purified PG

3.7 Clarification of mosambi juice using purified PG

- When the fresh mosambi juice brought from local market was analysed upon purified PG treatment, the % T660 were increased with the reduction of viscosity, pH. The % T660 was increased in clarified juic as PG degrades insoluble pectin molecules into soluble galacturonic acid. During this process, the formation of pectin-protein flocs facilitated production of a clear supernatant with the removal of colloidal part of the

juice. In the present study, the purified PG from *A. awamori* with only 7.21 U/ml activity and 2 h of incubation at 50oC, clarified the juice to some extent with %T660 increasing from 20.6 to 22.3. There was reduction in the viscosity of the fruit juice, initially it was 1.28cps and after enzymatic treatment reduced to 1.08cps.

Table 3: Application of purified PG in clarification of mausambi juice

• Factors	Raw extract of juice	After incubation with purified enzyme
• Viscosity(CPS)	1.28	1.08
• Transmittance(%T660)	20.6	22.3

CHAPTER 4

Conclusion

- Mosambi pomace support the growth of *A. awamori* for the production of PG.
- SSF provides the fungus natural conditions for its growth due to which high growth rate is seen.
- Parameters including pH and temperature that influenced the growth rates were optmised.
- Using ammonim sulphate (60%) and activated charcoal, PG was purified efficiently from solid-state culture of *A. awamori* in a single step with a purification fold 0f 1.85%.
- The purification process is very simple, time saving and economic.
- From the studies conducted on the effect of pH and temperature on the enzyme activity, the PG enzyme exhibited maximum activity of 7.8(U/ml) at temperature 40°c and pH 5.0 The optimum temperature being 30°C and pH of 4.5 had been estimated by studying the effect of temperature (30-80°C) and pH (2-8) with an activity of 7.21(U/ml) of purified enzyme.
- The application of the purified enzyme PG for clarification of fresh mosambi juice had been studied and seen that the transmittance increased to some extent and the pH reduced.

REFERENCES

[1] Dey.TB, Adak.S , Bhattacharya.P , Banerjee.R, Purification of polygalacturonase from *A. awamori* Nakazawa MTCC 6652 and its application in apple juice clarification, *LWT - Food Science and Technology*(2014)

[2] K. Anuradha · P. Naga Padma · S. Venkateshwar · Gopal Reddy, Fungal isolates from natural pectic substrates for polygalacturonase and multienzyme production**,** Indian J Microbiol (2010)

[3] P.Naga Padma2et al Use of Pectin Rich Fruit Wastes for Polygalacturonase Production by Aspergillus awamori MTCC 9166 in Solid State fermentation, J Bioproces Biotechniq (2012)

[4] Sathyanarayana N. Gummadi , T. Panda, Purification and biochemical properties of microbial pectinases a review, Process Biochemistry 38 (2003) 987_ 996, (2002)

[5] Heerda.D, Sirma Yegina,b, Canan Tari c, Marcelo Fernandez-Lahorea, Pectinase enzyme-complex production by Aspergillus spp. In solid-state fermentation: A comparative study, Food and Bioproducts Processing, (2012)

[6] Jayani.RS , Saxena.S , Gupta.R, Microbial pectinolytic enzymes: A review, Process Biochemistry 40 (2005)

[7] D.R. Kashyap, Applications of pectinases in the commercial sector: a review, Bioresource Technology 77 (2001)

[8] Ivana Greice Sandri, Clarification of fruit juices by fungal pectinases, LWT - Food Science and Technology,(2011**)**

[9] Sibel Uzuner Deniz Cekmecelioglu, Enhanced pectinase production by optimizing fermentation conditions of *Bacillus subtilis* growing on hazelnut shell hydrolyzate, *Journal of Molecular Catalysis B: Enzymatic* ,(2015)

[10] Niharika Sood and Abhishek Mathur, Evaluation of pharmacological activities of pectin extracted from apple citrus pomace , Biolife, 2(4):1203-1217

[11] Tsen, H. Y.: Debittering of grapefruit juice with immobilized naringinase. Nat. Sci. Council Monthly. R.O.C., 9, 871-881 (1981).

[12] Tsen, H. Y.: Factors affecting the inactivation of naringinase in chitin during debittering of fruit juice. J. Ferment. Technol., 62, 263-267 (1984)

[13] Carla M. Stinco a , Rocío Fernández-Vázquez a , Dolores Hernanz b , Francisco J. Heredia a , Antonio J. Meléndez-Martínez a , Isabel M. Vicario a, Industrial orange juice debittering: Impact on bioactive compounds and nutritional value(2012)

[14] Maria Teresa Pretel, a* Pedro Lozano, b Fernando Riquelme c and F61ix Romojaro c, Pectic enzymes in fresh fruit processing: optimization of enzymic peeling of oranges

[15] Tapati Bhanja Dey, Rintu Banerjee, Application of decolourized and partially purified polygalacturonase and -amylase in apple juice clarification(2013)

[16] Nagai, M., Katsuragi, T., Terashita, T., Yoshikawa , K., & Sakai, T. (2000). 274 Purification and characterization of an endo-polygalacturonase from Aspergillus 275 awamori. Bioscience, Biotechnology, and Biochemistry , 64 ,1729-1732.

[17] Nakkeeran, E., Subramanian, R., & Umesh-Kumar, S. (2010). Purification of 277 polygalactouronase from solid-state cultures of Aspergillus carbonarius. Journal 278 of Bioscience and Bioengineering,109, 101–106.

[18] Ngo, L.T.A., Pham, T.L., & Le, V.V.M. (2008). Purification of 280 endopolygalacturonase from Submerged Culture of Aspergillus awamori L1 using 281 a Two-step Procedure: Enzyme Precipitation and Gel Filtration. International Food Research Journal ,15,135-140.

[19] Olsson, L., Christensen, TMIE., Hansen, KP., & Palmovist, EA. (2003). 284 Influence of the carbon source on production of cellulases, hemicellulases and 285 pectinases by Trichoderma reeseiRut C-30. Enzyme Microbiol Technol, 33, 612– 286 619.

[20] Oszmianski, J., Wojdylo, A., & Kolniak, J. (2009).Effect of enzymatic mash 288 treatment and storage on phenolic composition, antioxidant activity, and turbidity 289 of cloudy apple juice. Journal of Agricultural and Food Chemistry, 57, 7078– 290 7085.