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EXTRACTION OF PECTIN METHYL ESTERASE FROM SWEET LIME PEEL

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***Abstract*-Pectin Methyl Esterase (PME) (3.1.1.11) is the pectin degrading enzyme which catalyses the hydrolysis of pectin methylester group, resulting in de-esterification. PME is widely distributed in plants, fungi, yeast and bacteria .Pectin Methyl Esterase (PME, E.C. 3.1.1.11) has wide application in different type of industries specifically in fruit juice clarifying industry. PME was extracted from sweet lime peel by various methods. The presence of enzyme was studied spectrophotometrically by bromothymol blue assay. Hydrolysis of ester was analysed qualitatively by TLC. It was found that maximum PME activity of 42.5 IU/ml was observed using PVPP extraction method. At pH 3.5, temperature 35℃ ,1 mM $CaCl_2$ and 0.2 % Pectin, the maximum concentration of galacturonic acid was produced.**

*Keywords: **PME, TLC, PVPP, pH***

I. INTRODUCTION

1.1 Pectin

Pectin is a complex high molecular mass glycosidic macromolecules and it is a principal polysaccharide composed of α- 1, 4- linked galacturonic acid and galacturonic acid methyl esters. It exists in higher plants. Pectin is rich in the albedo portion of citrus peels. This Pectin extracted from various fruit and vegetable waste materials by soaking it in acidic solution at different pH(Jain *et al*. 1984).For example, 15.92% of Pectin was extracted from Mosambi peels at pH 2.5.It is extensively used in the food industry in the conversion of low grade fruits into quality products like Jam, Jelly, Marmalade and Candies.

1.2 Pectin Methyl Esterase

Pectin Methyl Esterase (PME) is particularly abundant in citrus fruits.PME is classified under hydrolases. It is specifically categorised under pectinases. Pectin is responsible for the cloudiness in fruit juices. To overcome this cloudiness of the juices, PME is used alone or works in combination with other pectinases to modify Pectin .Pectin is hydrolysed by PME.Based on the action on Pectin,PME is categorised under de-esterifying enzymes.The galacturonic acid methyl esters are hydrolyzed by PME as follows:

$$\text{Pectin-COOCH}_3 + H_2O \longrightarrow \text{Pectin-COO}^- + H^+ + CH_3OH$$

Reaction 1.3 De-esterification o pectin by PME (Micheli, 2001)

1.3 Extraction of PME

PME is a cell wall bound enzyme (Cameron *et al.*, 1998, Corredig*et al.*, 2000) .It can be extracted by high salt concentration. Like early study for different sources, PME extraction procedure from sweet lime peel involves 5 different extraction procedures.

1.4 Characterisation and Purification of PME

For the purification of PME, Three Phase Partitioning (TPP) technique (Dennison & Lovrein 1997) is applied using varying volume of t-butanol and concentration of ammonium sulphate. By using TPP-t-butanol, about 25 enzymes and proteins are isolated. Normally t-butanol is immiscible with water which can be prevented by the addition of ammonium sulphate salt and produces two phases: a lower aqueous phase and an upper t-butanol phase. When protein is present, it shows the third phase which is an intermediate between the upper and lower phase (Dennison & Lovrein 1997).

1.5 Applications of PME

In order to obtain superior gelling agents and emulsion stabilizing in food industries, Pectin is modified by PME.PME is highly beneficial to the citrus industry since it acts as a causative agent for juice clarification (Rombouts & Pilnik 1986).It is also used in industrial process like pre-treatment of waste water, liquefaction, extraction and filtration. PME has a wide application in textile industry, paper making, and coffee and tea fermentation process.

II. MATERIALS AND METHODS

2.1 Materials

Sweet lime peels were purchased from a local market in Virudhunagar and Sivakasi. These peels were frozen and kept at -18℃until used in the experiments. Galacturonic acid was purchased from Sigma Chemicals. Pectin and P-Nitrophenylacetate was purchased from Hi-Media Chemicals.The other chemicals used is reagent grade.

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2.2 Extraction of PME from sweet lime peels

To extract PME from sweet lime peels, there are five different extraction procedures were used.

2.2.1 Procedure-1: Extraction of PME using cold distilled water

In the first procedure, 30g of sweet lime peels were homogenized using 100ml of cold distilled water. Then it was filtered using sieve and the pellet was discarded. The filtrate was then centrifuged at 3500 g for 15 min at 4℃.After centrifugation the supernatant was collected and this was used as an enzyme extract.

2.2.2 Procedure-2: Extraction of PME using NaCl solution

In the second extraction procedure, 30g of sweet lime peels were homogenized using 100 ml of 1M NaCl solution. The enzyme extract was obtained by filtration of the homogenized peels and followed by centrifugation at 3500g for 15 min at 4℃.

2.2.3 Procedure-3: Extraction of PME using NaCl

In third extraction procedure, 30g of peel was homogenized using 100ml of cold distilled water. Then it was filtered using sieve in which the water extract was removed and the pellet was collected. Again this pellet was homogenized with same volume of cold distilled water and the procedure was repeated. Now the pellet was mixed with 6-10g of NaCl (Wicker *et al.,* 2002; Laratta *et al.,* 2008) and it was make(made) up to 100g using distilled water. This mixture was then mixed for 30 or 40 minat 4℃in order to extract enzyme. After mixing it was filtered using sieve in which pellet was removed and the filtrate was collected. Thefiltrate was centrifuged at 3500g for 15 min at 4℃. After centrifugation the supernatant was collected and it was used as enzyme extract.

2.2.4 Procedure-4: Extraction of PME using Acetone

In the fourth procedure, the sweet lime peel (30g) was homogenized using 100 ml of cold acetone at -18℃ to remove the phenolic compounds. Then the homogenate was filtered using sieve in which the acetone extract was discarded. The collected pellet was homogenized with cold distilled water and again it was filtered using sieve. In this the filtrate(water extract was discarded) and the pellet was mixed with 6g of NaCl and made up to 100g using distilled water. This mixture was mixed for 45 min at 4℃in order to extract the enzyme. It was centrifuged at 3500g for 15 min at 4℃.After centrifugation the supernatant was collected and it was used as enzyme extract.

2.2.5 Procedure-5: Extraction of PME using PVPP

In the fifth extraction procedure, the third procedure was repeated. In addition to 6g of NaCl, 2g of PVPP (phenolic scavenger) was used and made up to 100g using distilled water. This mixture was stirred for 30-40 min. After stirring it was filtered and followed by centrifugation to obtain the enzyme extract.

III. BROMOTHYMOL BLUE ASSAY

The activity of enzyme was measured quantitatively by the acid produced from Pectin. The assay was carried out in triplicates for each extraction procedure. The reaction (extraction) mixture was formed by mixing 2ml of 0.5%pectin prepared in 0.1M NaCl 0.5 ml of 0.01% (w/v) bromothymol blue in 0.003 M sodium phosphate buffer at pH 7.5and 0.1ml of crude enzyme extract and it was incubate for different time intervals. The absorbance was measured using Hitachi U-2800 UV-Vis Spectrophotometer at 620nm (Hagerman and Austin, 1986).

3.1. Determination of Enzyme Activity using Acid Base Titration

In order to determine PME activity, the carboxyl can be titrated during the enzymatic hydrolysis with sodium hydroxide solution at constant temperature and pH. Activity of the enzyme was measured quantitatively by polygalacturonic acid produced from Pectin. Each assay was carried out in triplicates, by titrating 20 mM NaOH with 20 ml of 1% (w/v) Pectin solution prepared in 0.1 M NaCl of pH 7.5 at 30℃. By the addition of small volumes of 20 mM NaOH, the pH was maintained at 7.5. Then 0.2 ml of enzyme extract was added and the reaction (extraction) mixture was kept for 1-5 min. The same procedure was done without enzyme. With that 0.2 ml of enzyme diluents (1.7 M NaCl) was added (Blank). 20 mM NaOH was added to maintain the pH at 7.5. The time and volume of NaOH required to maintain the pH at 7.5 was noted (Kertesz, 1955). The amount of polygalacturonic acid produced was measured titrimetrically using pH meter. One unit of enzyme (PME) activity was defined as the amount of enzyme required to release 1µmol of acid from Pectin per min at pH 7.5 at 30℃.

3.2Analysis of PME

PME was qualitatively analysed by means of TLC by using a silica gel plate. After the P-Nitro phenol was developed with a solvent system of Petroleum ether: Ethyl acetate (7:3 w/v), the P-Nitro phenol spot was visualized by spraying 10 mg/ml of potassium permanganate solution.

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3.3. Effect of pH on Galacturonic acid production

To find the optimum pH the Galacturonic acid production was measured in acetate buffer of pH 3-5 by the method of assay of PME with 0.5% Pectin as a substrate.

3.4. Effect of temperature on Galacturonic acid production

To find the optimum temperature the reaction mixture containing 0.1 ml enzyme, 2 ml substrate and 0.5 ml bromothymol blue was incubated at 30℃, 35℃, 40℃, 45℃, 50℃, 55℃ and 60℃ and the Galacturonicacid production was measured by the method of bromothymol blue.

3.5. Effect of $CaCl_2$on Galacturonic acid production

To find the effect of $CaCl_2$ on acid production, Galacturonic acid production was determined in the presence of 1-50 mM $CaCl_2$.Galacturonic acid production was determined.
Spectrophotometrically using bromothymol blue by adding varying concentrations of 0.1 mL of $CaCl_2$ to the reaction mixture.

3.6. Effect of Substrate Concentration on Galacturonic acid production

To find the effect of substrate concentration on the production of Galacturonic acid, the enzymatic assay was performed in the presence of different concentration of pectin: 0.2%, 0.4%, 0.6%, 0.8% and 1%.Galacturonic acid production was determined spectrophotometrically using bromothymol blue by adding varying concentration of 2 ml of pectin to the reaction mixture.

IV. RESULTS AND DISCUSSION

4.1 Source of PME

Sweet lime peels purchased from the local market were stored at -18℃and maintained for further usage.

4.2 Extraction of PME

4.2.1Extraction of PME using cold distilled water

The sweet lime peels were first homogenized by mortar and pestle using cold distilled water. It was filtered and centrifuged at 3500 g for 15 min at 4℃.The supernatant was used as an enzyme extract and it was shown in (fig: 4.2.1.1).

Fig: 4.2.1.1 Enzyme extract obtained using cold distilled water

4.2.2Extraction of PME using NaCl solution

The sweet lime peels were homogenized using 1M NaCl solution. It was filtered and again homogenized with distilled water. The filtrate was centrifuged at 3500g for 15 min at 4℃.The supernatant was used as an enzyme extract and it was shown in(fig:4.2.2.1).

Fig: 4.2.2.1 Enzyme extract obtained using 1M NaCl solution

4.2.3 Extraction of PME using NaCl

The sweet lime peels were homogenized twice with cold distilled water. After filtration, the pellet was mixed with 6 g of NaCl for 30-40 min at 4℃.Again it was filtered and centrifuged at 3500 g for 15 min at 4℃.The supernatant was used as an enzyme extract and it was shown in(fig:4.2.3.1).

Fig: 4.2.3.1 Enzyme extract obtained using NaCl

4.2.4 Extraction of PME using Acetone

The sweet lime peels were homogenized using acetone at -18℃.Afterfiltration the pellet was homogenized using cold distilled water.Again the collected pellet was mixed with 6g NaCl for 30-40 min at 4℃.The filtrate was centrifuged at 3500 g for 15 min at 4℃.The supernatant was used as an enzyme extract and it was shown in (fig4.2.4.1).

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Fig: 4.2.4.1 Enzyme extract obtained using Acetone**.**

4.2.5 Extraction of PME using PVPP

The sweet lime peels were homogenized twice using cold distilled water. After filtration, the pellet was mixed with 6g NaCl and 2g PVPP for 30-40 min at at4℃. The filtrate was centrifuged at 3500 g for 15 min at 4℃.The supernatant was used as an enzyme extract and it was shown in (Fig: 4.2.5.1).

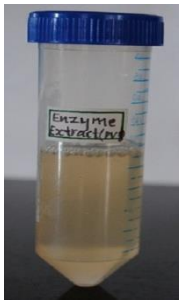

Fig: 4.2.5.1 Enzyme extract obtained using PVPP

4.3BromothymolBlue Assay

The presence of enzyme was identified by bromothymol blue assay. The decrease in absorbance was measured at 620nm.All the enzyme extract was analysed by this assay. The graph was plotted with OD against Time. The graph was shown in (Fig: 4.3.1).

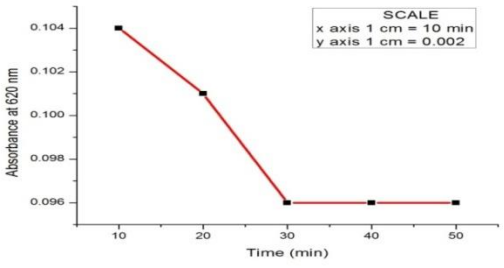

Fig: 4.3.1a) Bromothymol Blue Assay for extraction Procedure-1

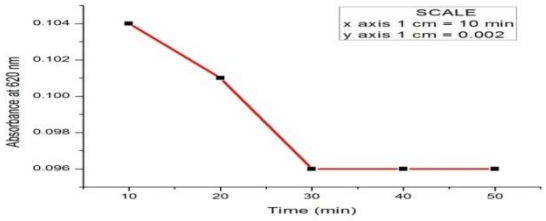

Fig: 4.3.1b) Bromothymol Blue Assay for extraction Procedure-2

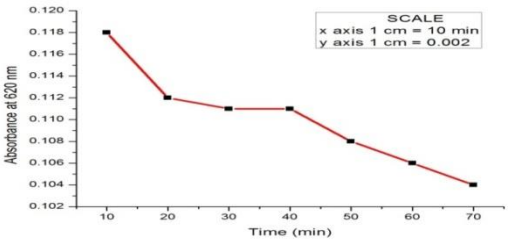

Fig: 4.3.1c) Bromothymol blue assay for extraction procedure-3

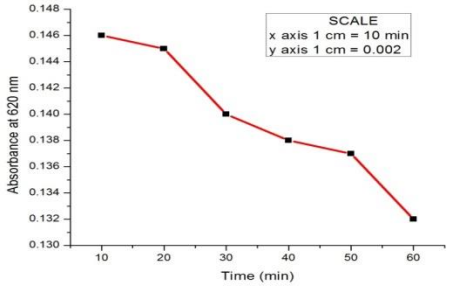

Fig: 4.3.1 d) Bromothymol blue assay for extraction procedure-4

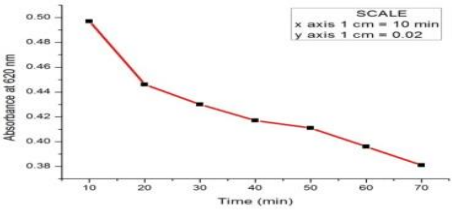

Fig: 4.3.1 Bromothymol Blue Assay for extraction procedure-5

4.4 Determination of PME Activity

The activity of enzyme was measured by pH titration method. The pH titration was performed for five different enzyme extract and the highest activity was achieved when treated with PVPP (42.5 IU/ml).Since it is a cell wall bound enzyme, it cannot be extracted by distilled water. But it can be achieved by high salt concentration. It was shown in (fig: 4.4.1)

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The formula to find enzyme activity:

$$\text{PME units/ml} = \frac{(\text{ml of NaOH for test} - \text{ml of NaOH for blank}) * \text{Molarity of NaOH}}{\text{Time} * \text{ml of enzyme used}}$$

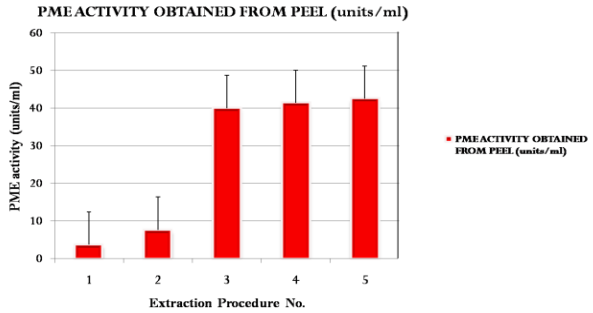

Fig: 4.4.1 e) PME activity obtained from sweet lime peels

4.5 Analysis of PME

Hydrolysis of ester was qualitatively analyzed using Thin Layer Chromatography(TLC) which used a solvent phase as Petroleum ether : Ethyl acetate (7:3) in a silica gel plate the samples were loaded and TLC was performed and then developed using Potassium permanganate which showed yellow coloured spots which was shown in (fig:4.5.1).

Fig: 4.5.1a)P-Nitrophenol
b) P-NPA + Enzyme extract

4.6 Effect of pH on Galacturonic acid production

Enzymatic assay was analyzed for enzyme extracts and maintained at various pH ranging from 3.0- 5.0 and the concentration of Galacturonic acid was found maximum in pH 3.5.The graph was plotted with concentration of Galacturonic acid against pH. The graph was shown in (fig: 4.6.1)

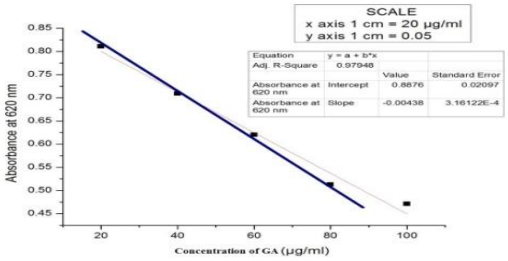

Fig: 4.6 Standardgraph for Galacturonic acid

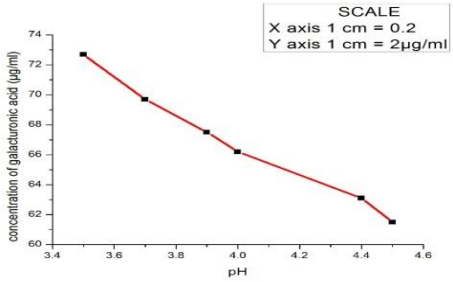

Fig 4.6.1 Effect of pH on Galacturonic acid production

4.7 Effect of Temperature on Galacturonic acid production

With various temperatures as reaction temperature the enzymatic assay was performed and the concentration of Galacturonic acid was found maximum in temperature 37℃. The graph was plotted with concentration of galacturonic acid against temperature. The graph was shown in (fig: 4.7.1).

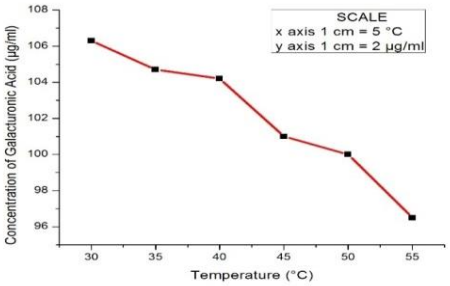

Fig: 4.7.1 Effect of Temperature on Galacturonic acid production

4.8 Effect of$CaCl_2$ on Galacturonic acid production

Bromothymol blue assay was performed with 1-50 mM $CaCl_2$ for enzyme extracts and the concentration of Galacturonic acid was found maximum in 1mM $CaCl_2$ .The graph was plotted with concentration of Galacturonic acid against molarity of $CaCl_2$.The graph was shown

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in(fig:4.8.1).

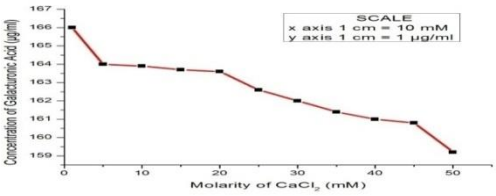

Fig: 4.8.1 Effect of $CaCl_2$on Galacturonic acid production

4.9 Effect of Substrate Concentration on Galacturonic Acid Production

With various percentage of substrate concentration, the reaction mix was subjected to enzymatic assay. All the enzyme extracts were added with various percentage of substrate concentration. The concentration of galacturonic acid was found maximum in 0.2% pectin at different time intervals. The graph was plotted with concentration of Galacturonic acid against percentage of substrate. The graph was shown in (fig: 4.9.1).

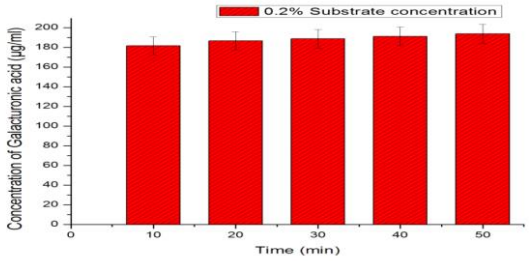

Fig:4.9.1 a) Galacturonic acid production at 0.2% substrate concentration

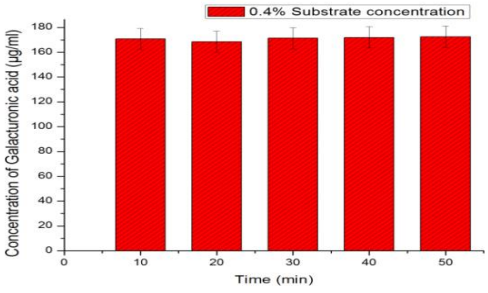

Fig:4.9.1 b) Galacturonic acid production at 0.4% substrate concentration

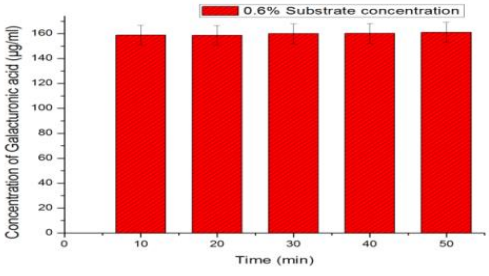

Fig:4.9.1c)Galacturonic acid production at 0.6% substrate concentration

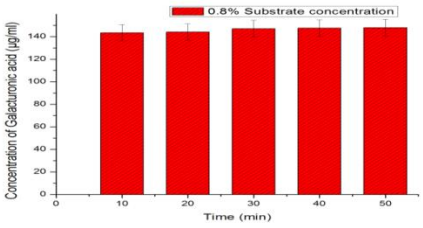

Fig:4.9.1 d) Galacturonic acid production at 0.8% substrate concentration

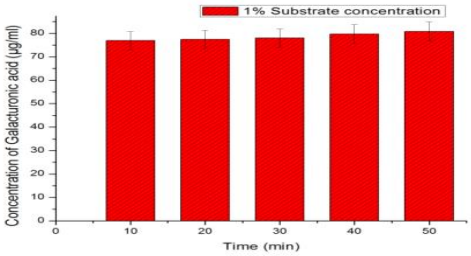

Fig:4.9.1 e) Galacturonic acid production at 1% substrate concentration

V. CONCLUSION

Potent Pectin Methyl Esterase (PME) producing source was identified as sweet lime peels (*citrus limetta*). PME was extracted by five different extraction methods (Procedures). Bromothymol blue was used to confirm the presence of PME spectrophotometrically at 620 nm. Based on the pH titration method, it was found that maximum PME activity was observed 42.5 IU/ml in PVP extraction method (Procedure). Since PME is a cell wall bound enzyme, it cannot be extracted by distilled water. So that it can be done by high salt concentration. Phenolic compounds in the extract was removed by phenolic scavengers like PolyVinylPyrrolidone(PVP),Acetone. Hydrolysis of ester using PME was analysed qualitatively byThin Layer Chromatography (TLC). The effect of pH, temperature, $CaCl_2$ and substrate concentration on the production of Galacturonic acid was identified. The optimum pH was 3.5 at which maximum concentration of Galacturonic acid was produced. The optimum temperature was 35℃ at which maximum concentration of Galacturonic acid was produced. At 1 mM $CaCl_2$, maximum concentration of Galacturonic acid was produced. At minimum substrate concentration, the yield of Galacturonic acid produced was high compared to maximum substrate concentration.

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