A COMPREHENSIVE EVALUATION OF PECTINASE, PECTINMETHYLESTERASE AND PECTOLYASE ACTIVITY

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Abstract
Pectins polysaccharide has galacturonic acid with linear chains of α-(1–4)-linked D- galacturonic acid. Rhamnogalacturonan I pectins (RG-I) shows the presence of the repeating disaccharide 4-α-D-galacturonic acid-(1,2)-α-L- rhamnose, which act as a backbone. The sugars are mainly D-galactose, L-arabinose, and D-xylose with the types and proportions of neutral sugars varying with the origin of pectin. Pectinase, pectinmethylesterase, and pectolyase enzymes have important application in food, textile and agricultural industries. These enzymes play a key role in thebreak down of the central part of plant cell walls. Pectin forms the center of the plant cellwall. Pectin is a structural polysaccharide that's integral for the steadiness of plant cell walls. Citrate buffer 0.1 M is used to check optimum pH and temperature and it is standardised for pectinase, pectolyase, and pectinmethylesterase enzyme by DNSA method. Confirmatory activity check of an enzyme is done on plant leaves dried particles. The effect of catechin presence in enzyme reaction was also studied. Plant polysaccharide was degraded by this enzyme and there was a significant increase in galacturonic acid quantity also. The highest release of polyphenols was found due to pectolyase followed by pectinmethylesterase and pectinase. Pectinmethylesterase effect showed the highest release of flavonoids followed by pectinase and pectolyase which was remarkable.

Keywords: Plant Cellwall, Pectin, Catechin, Dnsa Method, Monosaccharides.

Received: 25.07.2020 Reviewed: 04.12.2020 Accepted: 21.12.2020

INTRODUCTION

Pectin is a biopolymer of D-*galacturonic acid,* which is structural *heteropolysaccharide* in the primary cell wall. *Pectins* polysaccharide has several galacturonic acids with linear chains of α-(1–4)-linked D-galacturonic acid. *Rhamnogalacturonan I pectins (RG-I) shows the presence of the repeating disaccharide 4-α-D-galacturonic acid-(1,2)-α-L- rhamnose, which act as a backbone.* Besides *Rhamnogalacturonan I* another structure with highly branched but less frequent complex *polysaccharide* of pectin is of *rhamnogalacturonan II (RG-II*). Its backbone is entirely made up of D-galacturonic acid units *(Caffall et al.,2009 and Voragen et al.,2009).*

Plant pectin varies in its amount, structure, and chemical composition in various parts. Studies have revealed that pectinshelp in primary cell wall extension and plant growth. Pectin is also a major component of the middle lamellae; it helps to bind cells together and also found in primary cell walls. During the process of fruit ripening, pectin is broken down by the set of enzymes, in which the fruit becomes softer as the middle lamellae are broken down leading in the detachment of cells from each other. A similar process of cell separation caused by the breakdown of pectin occurs in the abscission region of petioles of the deciduous plant at the time of leaf fall (Voragen *et al.*, 2009 and Mollet *et al.*,2013).

Pectin forms the center of the plant cell wall. Hence, pectinase, *pectinmethylesterase, and pectolyase enzymes* have important applications in food, textile, and agricultural industries. Other moleculeslike celluloseare embedded in it. Plant cell walls stability is integral due to pectin structural polysaccharide. Pectinase, pectinmethylesterase, and pectolyase are a group of an enzyme that breaks down a central part of plant cell walls. The glycosidic bonds of the long carbon chains are broken down by pectolyase. They proceed towards the substrate at a random way and also catalyze the substrate cleavage from the non-reducing end. These enzymes are of prime importance for plants as they help in cell-wall extension and

softening of some plant tissues during maturation and storage. The random cleaving of the pectin is chieflyseen near the high esterified regions of pectin which produces unsaturated methyloligogalacturonates through trans-elimination of glycosidic linkages (Jayani et al., 2005 and Pedrolli et al., 2009).

It is found that pectolyase mostly cleaves glycosidic linkages on polygalacturonic acid, which forms an unsaturated product called 4, 5-D-galacturonate through trans-elimination reaction (Jayani et al., 2005). Pectinase is a well known unique group of complex enzymes composed of pectinesterase and polygalacturonase which ensures a high level of decomposition of pectic substances. Enzymes catalyze the degradation of pectic polymers present in the plant cell walls. It functions to hydrolyze pectin into polygalacturonic acids and ultimately to galacturonic acid (Kashyap et al., 2001). The catalyzation is of random hydrolysis of 1-4-α-D galacturonic acid linkages with the introduction of water across the oxygen bridge in the smooth region of pectin. They are involved in the hydrolysis of all pectic substances. Pectinaseenzymes act only on pectin with a degree of esterification of less than 50-60% (Jayani et al., 2005 and Aehle, 2007). Pectinmethylesterase is pectinesterases, which catalyzes de-esterification of the methoxyl group of pectin forming pectic acid by demethylation of pectin. The enzyme acts mainly on a methyl ester group of galacturonate units which is next to a non-esterified galacturonate unit (Kashyap et al., 2001). Preparations of all these pectin degrading enzymes have been widely used in the clarification of fruit juices and wines (Pedrolli et al., 2009).

In this article, optimising of pH and temperature for pectinase, pectinmethylesterase, and pectolyaseenzyme activity are studied by using the Miller's method of DNSA, 1972 and Rajbhar et al., 2015 and presence of catechin effect in various dosage was also considered for enzymatic reaction action mechanism. The amount of reducing sugar (D-galacturonic acid) which was released in supernatant measured for determination of pectinase, pectinmethylesterase, and pectolyase activity.

MATERIAL AND METHODS

Optimum activity of an enzyme in different pH and temperature is checked by estimating sugars product i.e galacturonic acid formed due to activity of the enzyme on standard macromolecules i.e. pectin. In this article the optimum pH and temperature were be optimised and standardization by calculating the amount of sugar product equivalent to galacturonic acid released by using a known amount of macromolecules pectin.

Chemicals used

Dinitrosalicylic acid (DNSA) and crystalline phenol were obtained from HI-Media (India), potassium sodium tartrate (Rochelle salt), sodium sulphite and sodium hydroxide were obtained from Loba Chemie (India). Pectin, pectinase, pectinmethylesterase, and pectolyase were supplied by Sigma (India) and Novozymes (India). An instrument used were water bath (Equitron) and Jasco V-530 spectrophotometer.

Preparation of DNSA reagent, substrate solution, and enzyme solution

Dinitrosalicylic Acid Reagent (DNSA Reagent) was prepared by dissolving 1 g DNSA, 200 mg crystalline phenol and 50 mg sodium sulphite in 100 mL 1% NaOH and was stored at 4° C. The reagent deteriorates because of sodium sulphite so it was added at the time of use to enable prolonged storage, before the addition of 40% Rochelle salt solution (Potassium sodium tartrate).

Pectin was dissolved in distilled water by preparing an mg/ml solution. The solution was heated for 5 min at 20°C on a heating mantle until a clear substrate solution was formed. Enzyme stock solution of pectinase, pectinmethylesterase, and pectolyase were prepared by mg/ml solution in distilled water and later in citrate buffer of the respective buffer.

Preparation of reaction mixture

A total volume of 2 ml solution with enzyme volume 0.1 ml suspended in respective buffer

making a volume of 1.9 ml followed by 0.1 ml of substrate solution was prepared. The reaction mixture is incubated at room temperature for 30 mins at 40°C. Addition of 0.5 ml DNSA reagent to the mixture was made and kept in water bath at 80°C to 85°C for 15 min. When the reaction mixture of the tubes were still heated, 0.5 mL of 40% Rochelle salt was added. After cooling the reaction mixture at room temperature the absorbance of the coloured complex formed was quantified at 430nm in terms of galacturonic acid equivalence; employing a Jasco V-530 spectrophotometer (Milleret al.,1972; Sadasivamet al.,1996 and Rajbhar et al.,2015). The standard graph was plotted with monosaccharide equivalence of galacturonic acid concentration in micrograms on the Y-axis against the respective parameter of buffer pH and temperature on the X-axis.

Optimisation of pH and temperature for pectinase, pectinmethylesterase, and pectolyase

Reaction on substrate pectin with pectinase, pectinmethylesterase, and pectolyase enzymes was studied at different pH of citrate buffer (0.1 M) and later on at different temperatures in pH 6.0, pH 5.5 and pH 5.4 respectively after standardisation. Product sugar was estimated by a modified DNSA method in galacturonic acid equivalent (430nm) (Rajbhar et al.,2015).

Effect of pectinase, pectinmethylesterase and pectolyase activity on Camellia sinensis dried leaves particles

Camellia sinensis (Plant) leaf was shade dried and powdered. The polyphenols were extracted from dried powder till no traces of polyphenol were detected; the powder was dried again for future process. It was considered as treated (control) sample and the test sample was with polyphenol present in leaf particles. The 0.1 gram Camellia leaf particles were used as a substrate for pectinase, pectinmethylesterase, and pectolyase enzyme was used to check their activity on Camellia leaf particles. Decoction of leaf polysaccharide was prepared with 0.1 gram of Camellia leaf particles; before and after treatment with enzymes diluted with 5 ml distilled water microwaved for 1 minute followed by polyphenols and flavonoids estimation. Estimation of polyphenols, flavonoids, and galacturonic acid (monosaccharide) was done before and after treatment by the method as discussed in Rajbhar et al., 2016, and Rajbhar et al.,2015 respectively.

Statistical analysis of data

Statistical analysis of the data obtained from the studies was performed using SPSS version 22. The reported values are mean ±SD (n=3). The results of the analysis were obtained for $p<0.05$. In cases where ANOVA has been performed, multiple comparisons were made using Duncan's Multiple Range Test (DMRT). Galacturonic acid equivalence (GaE) for reducing sugar and gallic acid equivalents, catechin equivalents, quercetin equivalents, rutin trihydrate equivalents, and ascorbic acid equivalents series have been assigned groups using upper case letters series have been assigned groups using upper case letters (A>B >C…) as well as the lower case (a>b>c...) as per requirement in graphs. The highest value reported in ascending way in the format of A>AB>ABC>ABCD>....>B>BC>BCD>..... same with P>Q>R>S>.... and L>M>N>O>....In addition, lower case alphabets are also used for distinction purpose. In a given series, mean assigned the same letter(s) are not significantly different from each other $p<0.05$.

RESULTS AND DISCUSSION

Pectinase, pectinmethylesterase, and pectolyase enzymes activity on dried leaf powdered of *Camellia sinensis* shown in the above graphs has the highest activity in 0.1 M citrate buffer in pH 6.0, 5.5 & 5.4 at 50°C, 40°C & 50°C respectively by modified DNSA method. The leaf powder was treated with enzymes and polyphenols, flavonoids, and antioxidant activity were estimated. There was an increase in amount after treatment as compared to control. Plant polysaccharide was degraded by this enzyme and there was a significant increase in monosaccharide quantity i.e. Galacturonic acid. Release of polyphenols and flavonoids under 1-minute microwave was estimated before and after enzymes treatment.

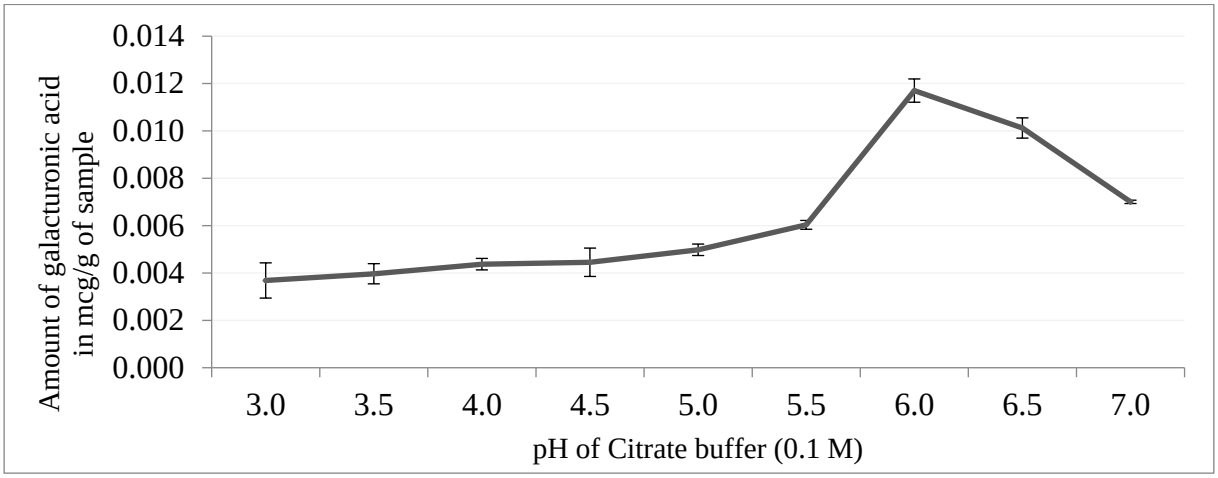

Figure 1.1: Pectinase enzyme activity on pectin in term of galacturonic acid equivalence

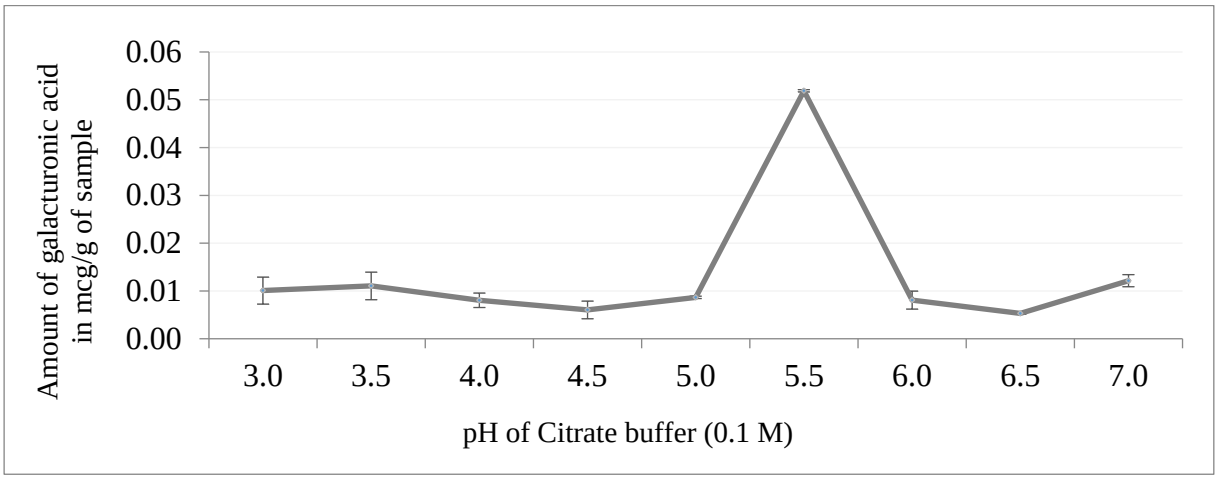

Figure 1.2: Pectinmethylesterase enzyme activity on pectin in term of galacturonic acid equivalence

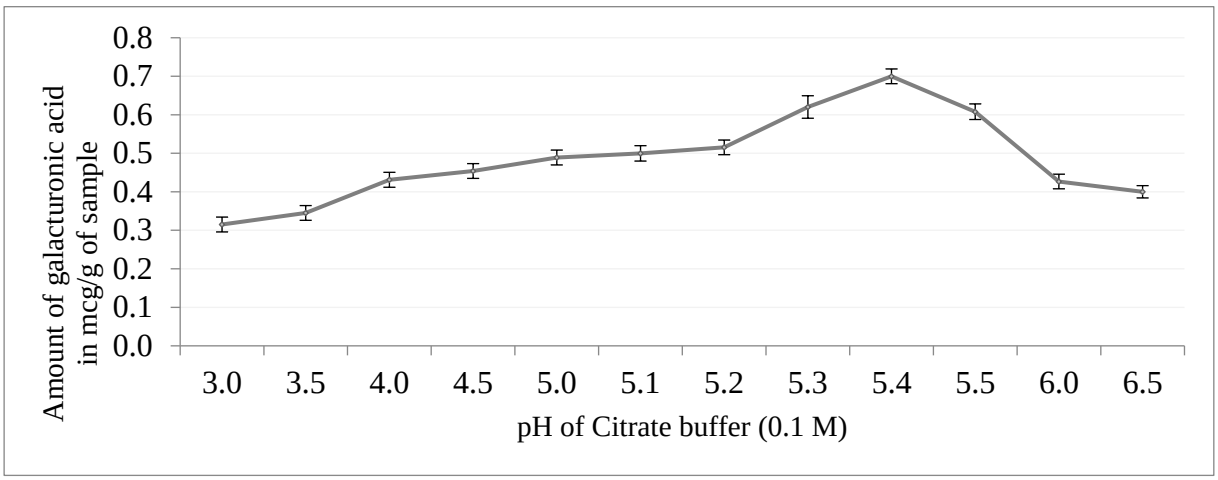

Figure 1.3: Pectolyase enzyme activity on pectin in term of galacturonic acid equivalence

Substrate (mg/ml)	Enzymes (mg/ml)	Buffer (0.1 M)	Optimum pH
Pectin	Pectinase	Citrate buffer	6.0
Pectin	Pectinmethylesterase	Citrate buffer	5.5
Pectin	Pectolyase	Citrate buffer	5.4

Table 1: Optimisation of pH at 40°C (Figure 1.1, 1.2 & 1.3)

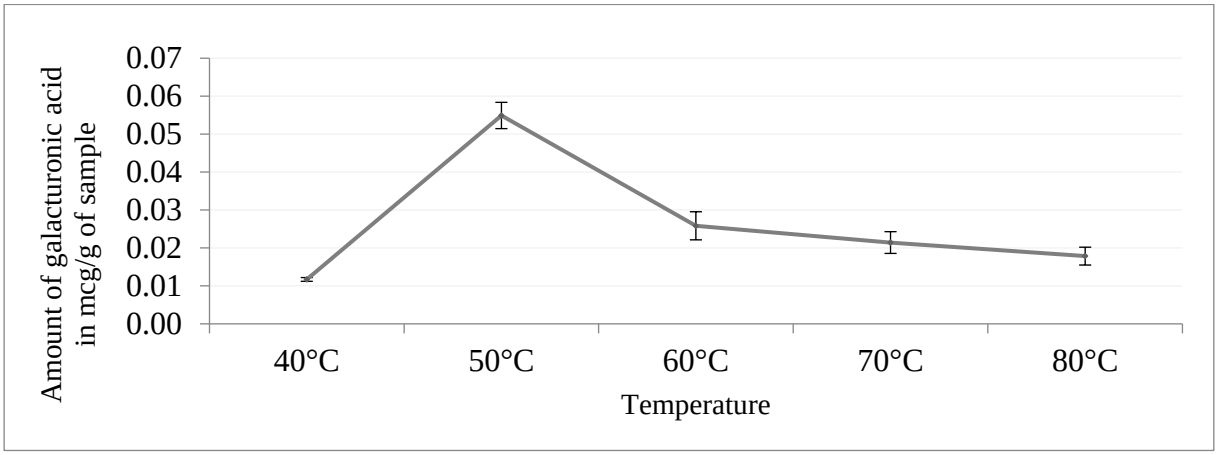

Figure 2.1: Pectinase enzyme activity on pectin in citrate buffer pH 6.0 in term of galacturonic acid equivalence

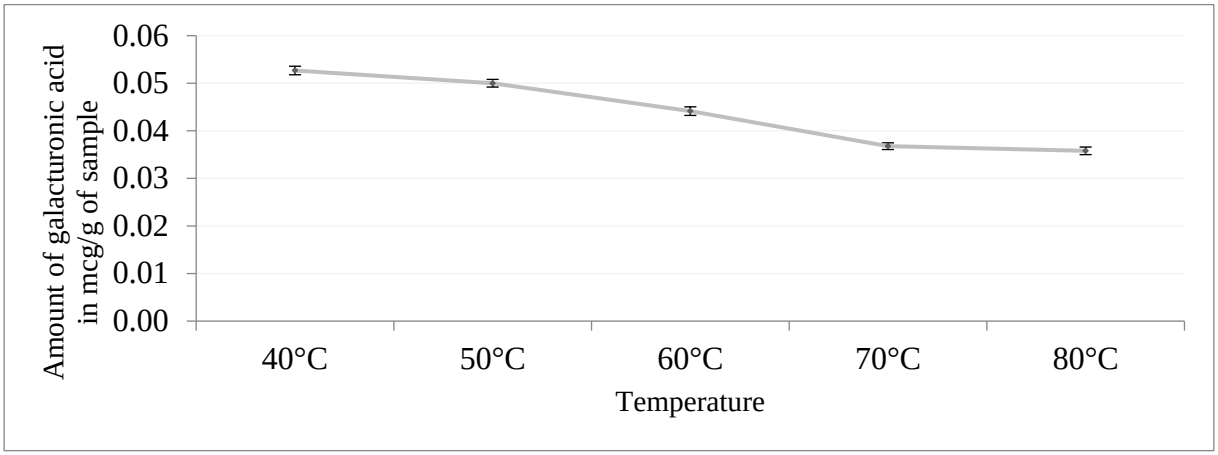

Figure 2.2: Pectinase enzyme activity on pectin in citrate buffer pH 5.5 in term of galacturonic acid equivalence

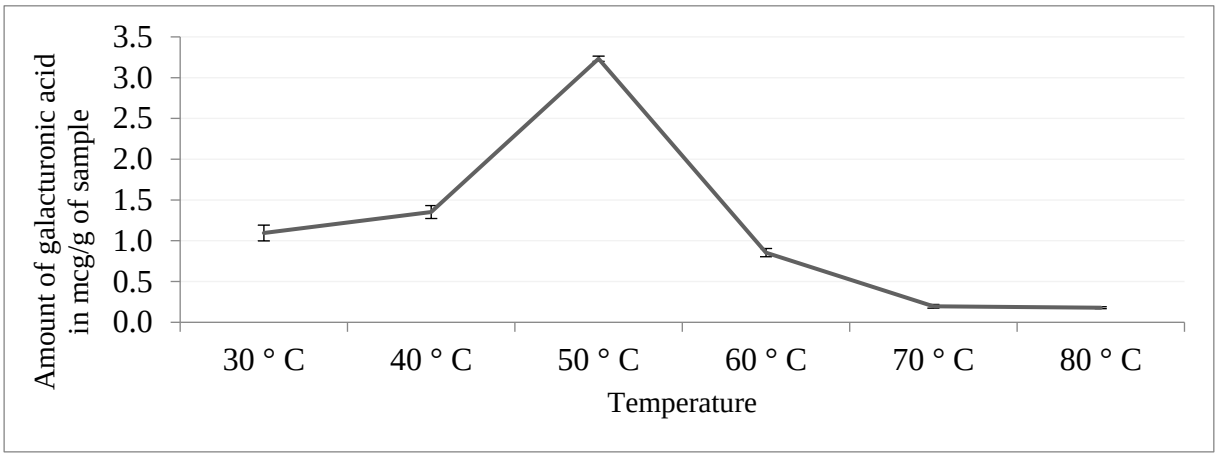

Figure 2.3: Pectolyase enzyme activity on pectin in citrate buffer pH 5.4 in term of galacturonic acid equivalence

Substrate (mg/ml)	Enzymes (mg/ml)	Citrate Buffer (0.1 M)	Optimum temperature
Pectin	Pectinase	pH 5.5	50°C
Pectin	Pectinmethylesterase	pH 6.0	40°C
Pectin	Pectolyase	pH 5.4	50°C

Table 2: Optimisation of temperature (Figure 2.1, 2.2 & 2.3)

The graph result shows released total polyphenols in gallic acid and catechin equivalence i.e GAE and CE. Total flavonoids in equivalence of quercetin and rutin trihydrate i.e QE and RTE, while total antioxidant activity in terms of ascorbic acid equivalence AAE. There was a significant effect of the enzyme on plant intercalating polysaccharide as the amount released of polyphenols and flavonoids after enzymes treatment is quite high.

It was shown in figure 3.1 that polyphenol in GAE and CE in control was 39.24±0.88 mcg and 41.63±1.15 mcg was increased to 53.44±1.24 mcg and 56.13±2.39 mcg in pectinase treatment and 57.12±2.7 mcg and 61.47±2.43 mcg in pectinmethyl esterase enzyme treatment. While pectolyase showed the increased to 73.15±3.25 mcg and 77.93±3.25 mcg which is almost double of control. Total Flavonoids in QE and RTE in control 21.49±0.88 mcg and 7.24±0.13 mcg was increased to 59.9±0.7 mcg and 8.53±0.83 mcg in pectinase treatment and 68.57±1.62 mcg and 11.94±0.55 mcg in pectinmethyl esterase enzyme treatment. While pectolyase showed the increased to 43.75±1.77 mcg and 13.59±0.55 mcg which is almost double of control. Antioxidant activity of sample control 13.67±1.27 mcg of AAE was increased to 17.88±0.09 mcg in pectinase, 15.46±0.95 mcg in pectinmethyl esterase and 16.36±0.78 mcg in pectolyase enzyme treatment as seen in figure 3.2 showed an increase in galacturonic acid before and after treatment confirming disruption of cell wall structure resulting in optimum extraction. SPSS ANOVA coding states that pectolyase enzyme treatment shows the highest release of polyphenols in gallic acid

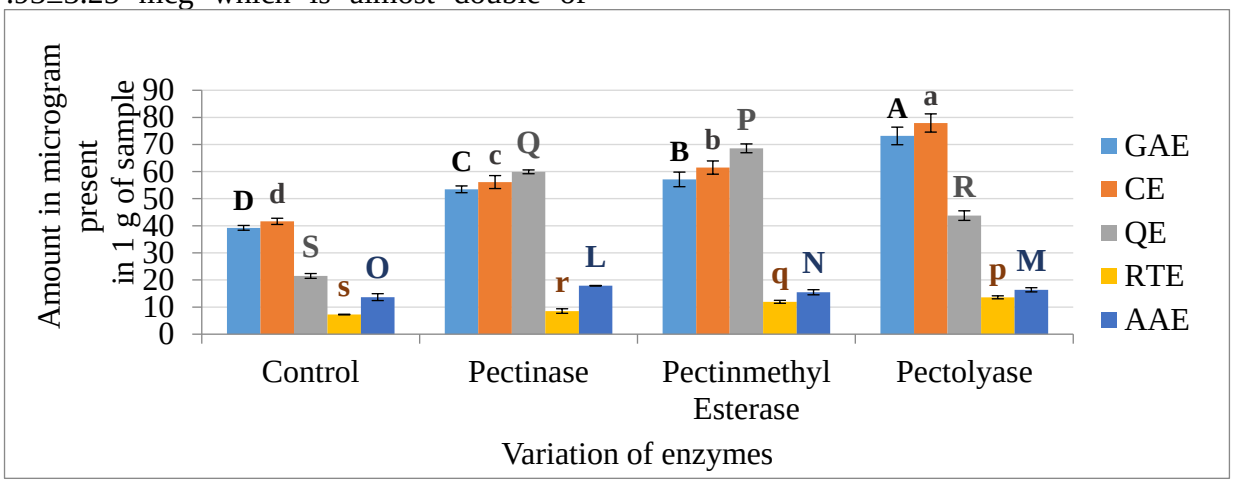

Figure 3.1: Pectinase, pectinmethylesterase and pectolyase enzymes activity on leaves polyphenols and flavonoids of *Camellia sinensis*

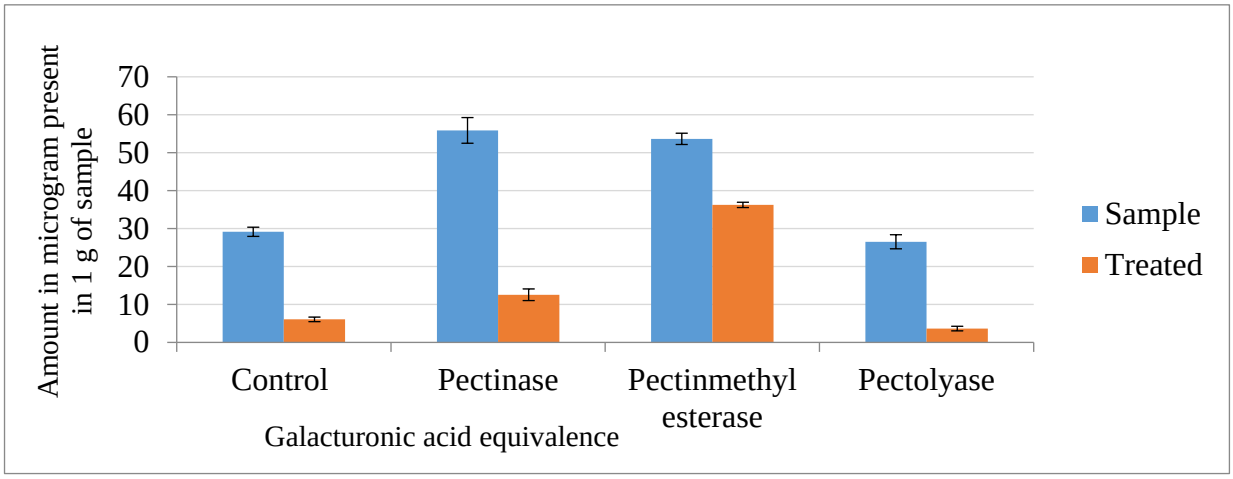

Figure 3.2: Pectinase, pectinmethyl esterase and pectolyase enzymes activity on leaf in equivalence of sugar

GAE and catechin CE equivalence while pectinase and pectinmethyl esterase enzyme shows highest flavonoids released in form of quercetin equivalence QE followed by rutin trihydrate RTE, and also total antioxidant activity (AAE) was found maximum released in pectinase treatment.

Catechin effect on pectinase, pectinmethylesterase and pectolyase enzymes activity

Comparative graph figures 4.1, 4.2, and 4.3 showed a prominent difference of catechin effect when visually observed, significance was well displayed by catechin inhibitory effect on the enzyme. Graph of galacturonic acid equivalence when showed in a comprehensive way the figure states a gradual decrease in the amount of galacturonic acid equivalence in the presence of catechin ranging from 25 micrograms to 300 micrograms. Thus, as the amount of catechin presence in reaction mixture,it inhibits the enzyme activity, which is justified by biostatistical coding saying the effect is significant.

CONCLUSION

Plant polysaccharide was degraded by this enzyme and there was a significant increase in galacturonic acid quantity also. The Pectolyase effect showed the highest release of polyphenols followed by pectinmethylesterase and pectinase. Pectinmethylesterase effect showed the highest release of flavonoids followed by pectinase and pectolyase which was remarkable. Inhibition of catechin has been well documented and the study showed that as the amount of catechin presence increases from range 25 µg to 300 µg it decreases the enzyme activity reaction by showing a negative effect.

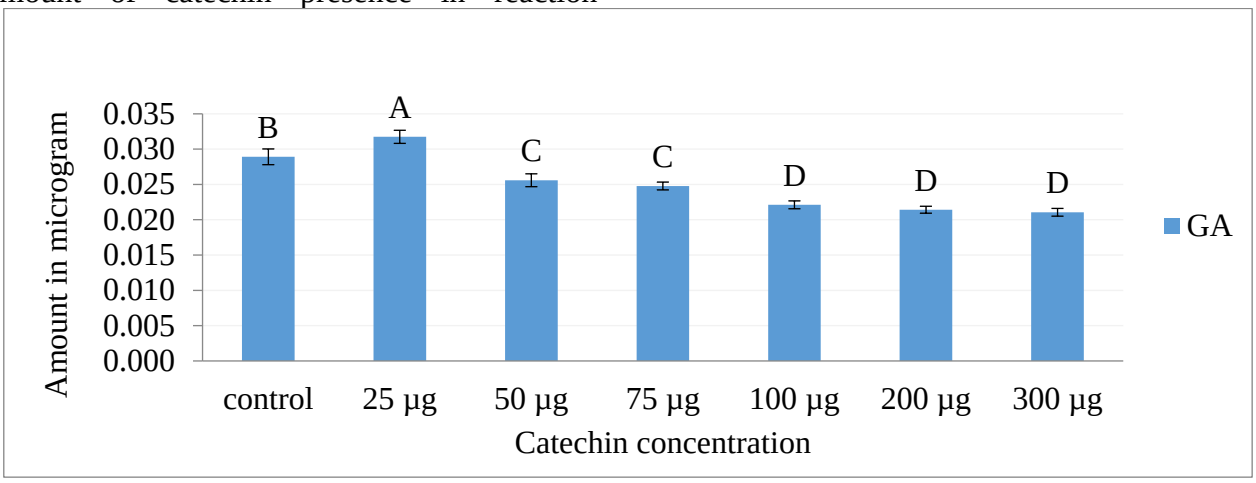

Figure 4.1: Effect of Catechinon pectinase activity on pectin in galacturonic acid equivalence

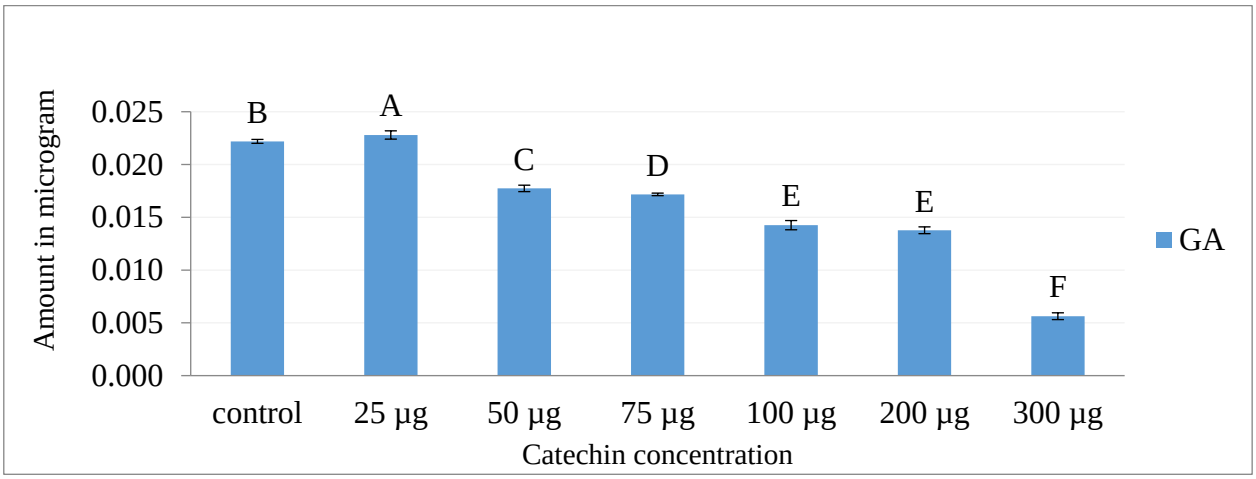

Figure 4.2: Effect of Catechinon pectolyase activity on pectin in galacturonic acid equivalence

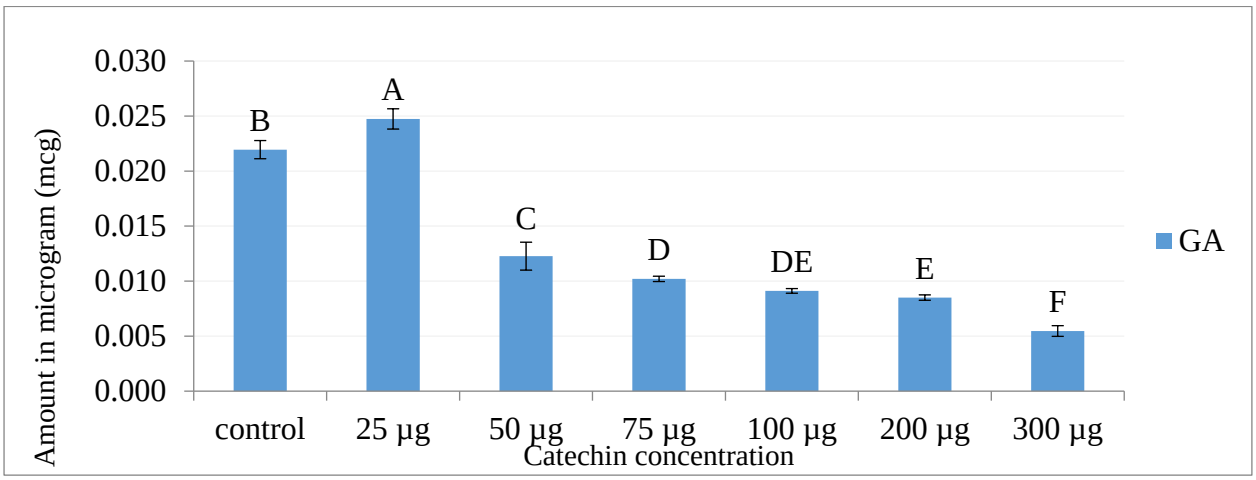

Figure 4.3: Effect of Catechinon pectinmethylesterase activity on pectin in galacturonic acid equivalence

Acknowledgment

The authors would like to thank HUL (Hindustan Unilever) for financial assistance for the collaborated research project and the fellowship provided to the first author. We are also thankful to DST for their financial support to the department of botany under the DST FIST program.

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