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Evaluation of the activity of hemicellulase and xylanase under different conditions of pH, temperature and catechin concentration

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Abstract

Plant cell wall contains hemicellulose which is degraded by the enzyme hemicellulase. Hemicelluloses are the second most abundant polysaccharides present in plants, after cellulose. Hemicellulose helps to cross-link the plant cell wall components to give the walls rigidity and strength, which serve as mechanical support to plants. Sodium acetate buffer (0.1 M) was used to check the optimum enzyme activity of the hemicellulase and xylanase enzymes by the DNSA method along with pH and temperature optimisation. Confirmatory activity check of the enzyme was carried out on dried leaves. Presence of catechin in the reaction mixture was studied as its inhibition is well documented. It was observed that catechin facilitated the reaction. The detailed mechanism is discussed in the article.

Keywords: Cell-Wall, Catechin, DNSA Method, Hemicellulose, Hemicellulase, Monosaccharides, Xylan and Xylanase.

1. Introduction

Hemicellulose is a complex carbohydrate polymer which is an essential component of primary and secondary plant cell walls and is present in all living plant tissue. Across the plant species on an average they make up for about 25% of the total plant dry biomass.[1] *Its molecular weight is less than that of cellulose. It* consists of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, and 4-*O*-methyl-glucuronic, D-galacturonic and D-glucuronic acids.[2,3]

Hemicellulose is mainly grouped into four classes depending upon the type of sugar residues present which are xylan, xyloglucan, mannans and mixed linkage glucans. Cell walls have xylan chains in the form of arabinoxylan polysaccharide which contains bonding of hydrogen to cellulose microfibril surfaces and connect several cellulose microfibrils to generate interconnected structures. Hemicellulose polymer helps to cross-link the plant cell wall components to give the walls rigidity, which serve as mechanical support to plants. They are polysaccharides with long chains of sugars that are major component of the plant cell wall. Their function plays a key role in the *loosening and tightening of cellulose microfibrils. This* process helps the cell to change its shape in growth and differentiation zones and to preserve its ultimate shape after cell maturation.[1,4]

Xylan can be degraded relatively easily than cellulose.[5] Xylan is a polysaccharide, which has a backbone of 1, 4-linked xylopyranosyl residues which can also be substituted by side chains of arabinosyl, glucuronosyl, methylglucuronosyl, acetyl, feruloyl and *p*-coumaroyl residues.[6] Xylans are only second to hemicellulose in proportion in plants. Xylan is mainly localised in the secondary cell wall with lignin, as it forms an amorphous matrix that includes and embeds cellulose microfibrils. Xylan interacts with lignin and cellulose via covalent and non-covalent linkages. Plant cell walls are important as they determine the quality of plant-based products. Fibre for textiles, pulp and paper manufacture, timber products and fuel are largely composed of plant cell walls.[6,7]

Microorganisms act as source for the extraction commercial enzymes. Various types of these enzymes have different applications in the food sciences. Enzymes are frequently used in combination for complete degradation of plant cell walls. Hemicellulase is an enzyme which degrades the plant cell wall polymer hemicellulose. Hemicellulases are well-known as glycan hydrolases which attack the backbone chain of the hemicellulose, although they are not responsible for cutting off side-branch sugar appendages of mono or oligosaccharides. They are best characterized as 1,4-β-D-xylanases and used in commercial products and in various other food preparation technologies.[8]

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Arabinoxylan, arabinoglucuronoxylan, arabino-4-*O*-methy-D-glucuronoxylan, 1,4-β-D-xylopyranosyl linkages of the 1,4-β-D-xylans and glucuronoxylan are hydrolysed by xylanase enzymes. Primary plant cell wall's mechanical properties are affected by xyloglucan, a type of acting enzyme. An increase in xyloglucan in cellulose microfibrils in plant cell walls increases the wall's rigidity. So when the xyloglucan endotransglucosylase or xyloglucanase acts on oligosaccharides, xylan degradation occurs. Hence, addition of xyloglucan endotransglucosylase fragments the oligosaccharides of plant tissue by increasing or decreasing the bonding-tension. The combined actions of different enzymes are vital to hydrolyse hemicellulose. That the arabinoglucuronoxylans can be mostly broken down by the synergistic action of exo-glycosidases is well documented. Exo-glycosidases can be like α-L-arabinosidases, α-D-glucuronidases and β-D-xylosidases and endo-acting xylanases can also be used to degrade it.[9]

This article shows optimum pH and temperature for degradation of hemicellulose by enzymatic activity of hemicellulase and xylanase which was determined by using Miller's DNSA method[10] and the method of Rajbhar *et al.*[11] Optimum activity of the enzyme at different pH and temperature conditions was determined by estimating the sugar products formed due to the action of the enzyme on standard xylan. The optimum pH and temperature was derived and standardised by calculating the amount of sugar product equivalent to xylose and arabinose released against known amount of xylan. In addition, the effect of catechin on hemicellulase and xylanase activity in various dosages was also considered for the enzymatic mechanism of action.

2. Materials and methods

Chemicals and instruments used

Dinitrosalicylic acid (DNSA) and crystalline phenol were obtained from Hl-Media (India), xylan, potassium sodium tartrate (Rochelle salt), sodium sulphite and sodium hydroxide were obtained from Loba Chemie (India). Hemicellulase and xylanase enzyme were supplied by Sigma (India) and Novozyme (India). Water bath of Equitron, microwave of LG and Jasco V-530 spectrophotometer instruments were used.

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, prior to the addition of 40% Rochelle salt (potassium sodium tartrate) solution.

Xylan was dissolved in distilled water by preparing mg/ml solution. The solution was heated for 5 min at 45 °C on a heating mantle until a clear substrate solution was formed. Enzyme stock solutions of hemicellulase and xylanase were prepared in distilled water and later in sodium acetate buffers of the respective pH as desired.

Preparation of reaction mixture

A total volume of 2 mL solution with 0.1 mL enzyme volume suspended in respective buffer making a volume of 1.9 mL followed by 0.1 ml of substrate solution was prepared. The reaction mixture was incubated at room temperature for 30 min at 60 °C. Subsequently 0.5 ml DNSA reagent was added and the mixture was incubated in the water bath at 85 °C for 15 min. When the contents of the tubes were still warm, 0.5 mL of 40% Rochelle salt solution was added. The reaction mixture was cooled and the absorbance of the coloured complex formed was measured at 460 nm and 458 nm of arabinose and xylose equivalence respectively using a Jasco V-530 spectrophotometer.[10-12] Standard graph was plotted with monosaccharide equivalence i.e., arabinose and xylose concentration in microgram on the Y-axis against the respective parameters of buffer pH and temperature on the X- axis.

Optimisation of pH and temperature

Substrate xylan reaction with hemicellulase and xylanase enzymes at different pH of 0.1 M sodium acetate buffer and at different temperature was performed and product sugars were estimated by the modified DNSA method in arabinose equivalents (460 nm) and xylose equivalents (458 nm).[11]

Hemicellulase and xylanase activity on *Camellia sinensis* dried leaves particles

Camellia sinensis leaf was shade dried and powdered. The polyphenols were extracted from dried powder till no traces of polyphenol were detected; powder was dried again for further process. It was considered as treated (control) sample. The test sample was with polyphenol present in the *Camellia* leaf particles. The 0.1 g sample of *Camellia* leaf particles were used as the substrate for hemicellulase and xylanase enzymes and were used to check the activity on leaf particles. *Camellia* leaf polysaccharide decoction was prepared using microwave for 1 min with 0.1 g of leaf particles before and after treatment with

enzymes diluted with 5 mL distilled water for polyphenols and flavonoids estimation. Estimation of polyphenols and flavonoids was done by the methods of Khatiwora *et al*,[13] Singleton *et al*,[14] Sultana *et al*,[15] and Vladimir *et al*,[16] and of galacturonic acid (monosaccharide) was done before and after the treatment by the method as discussed in Rajbhar *et al.*, 2015[11] and Rajbhar *et al.*, 2016[17] respectively.

Results

The observed results were collected and analysed to obtain optimum pH and temperature for the optimum activities of hemicellulase and xylanase.

Figure 1.1: Hemicellulase enzyme activity on xylan in sodium acetate buffer

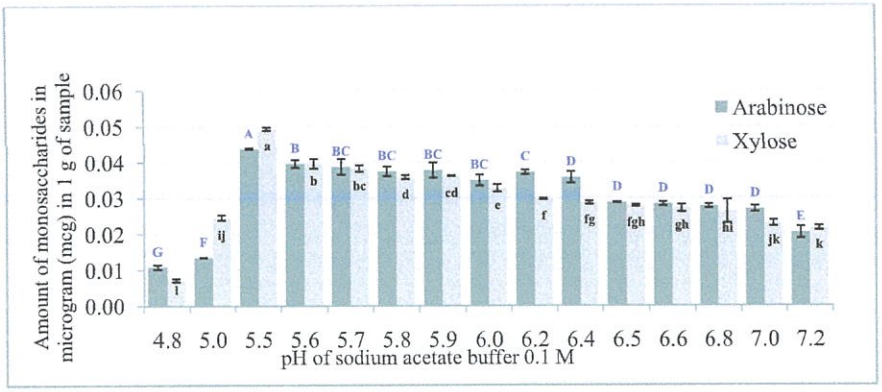

Figure 1.2: Xylanase enzyme activity on xylan in sodium acetate buffer

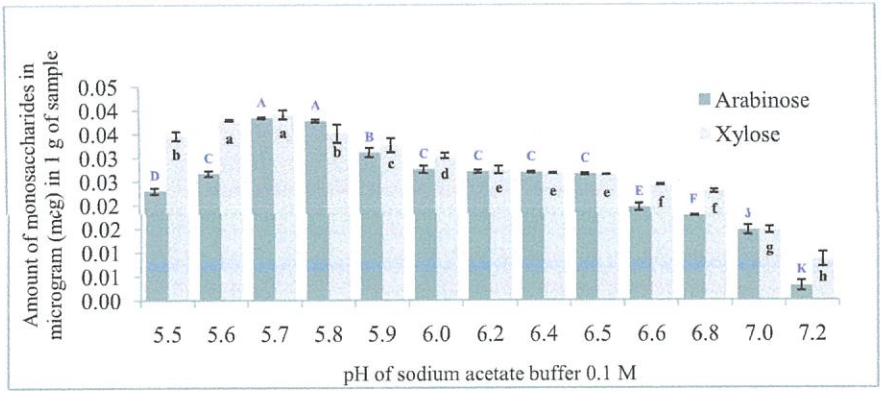

Figure 2.1: Hemicellulase enzyme activity on xylan in sodium acetate buffer pH 5.5

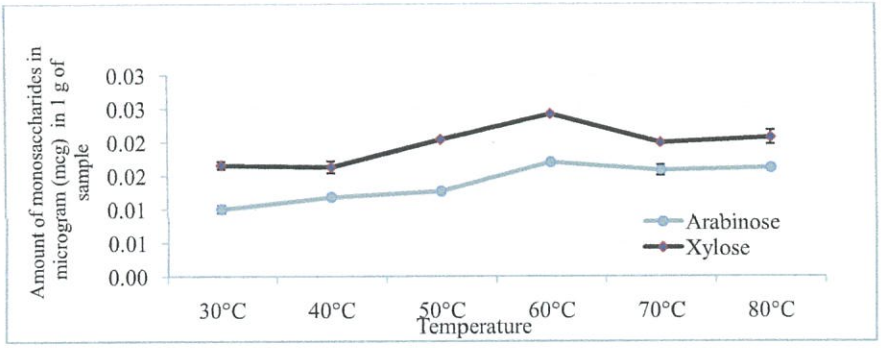

Figure 2.2: Xylanase enzyme activity on xylan in sodium acetate buffer pH 5.7

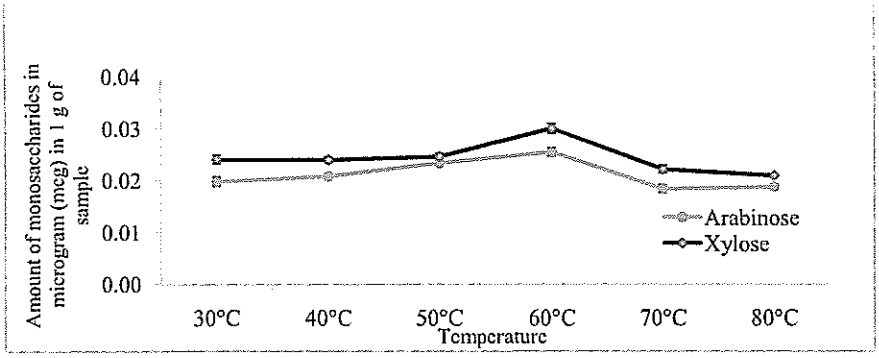

Figure 3.1: Hemicellulase and xylanase activity on *Camellia* leaf polyphenols and flavonoids

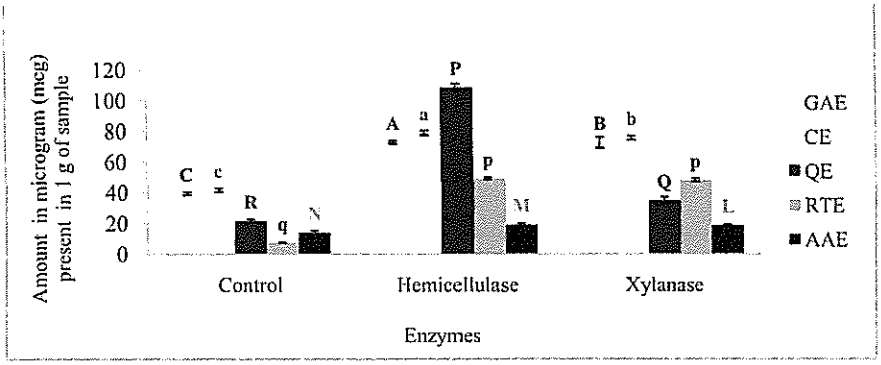

Figure 3.2: *Hemicellulase activity on Camellia leaf in equivalence of sugar by* DNSA method

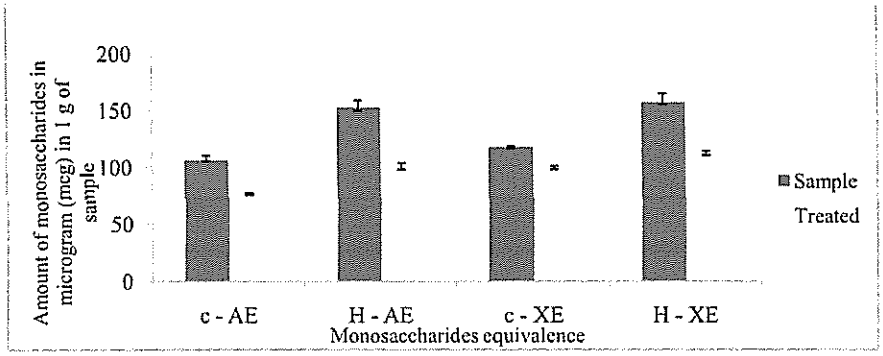

Figure 3.3: Xylanase activity on *Camellia* leaf in equivalence of sugar by DNSA method

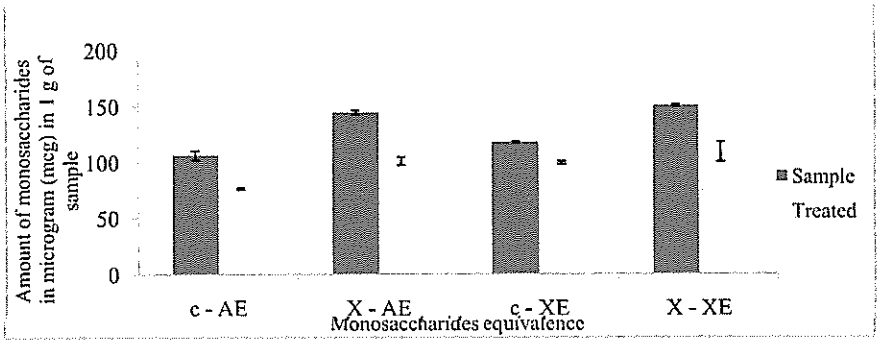

Figure 4.1: Effect of catechin on hemicellulase activity on xylan in arabinose and xylose equivalence

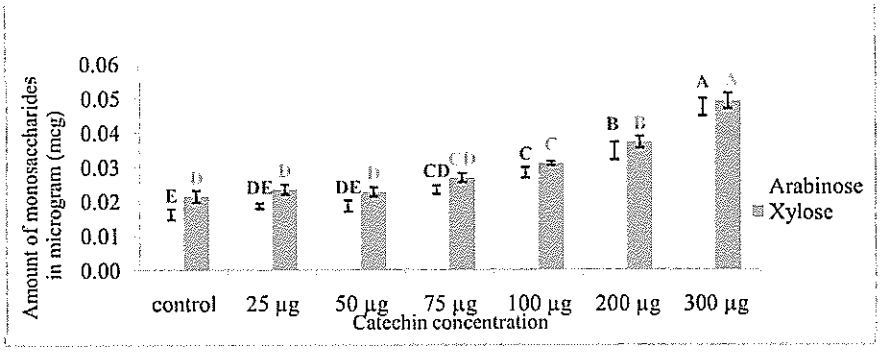

Figure 4.2: Effect of catechin on xylanase activity on xylan in arabinose and xylose equivalence

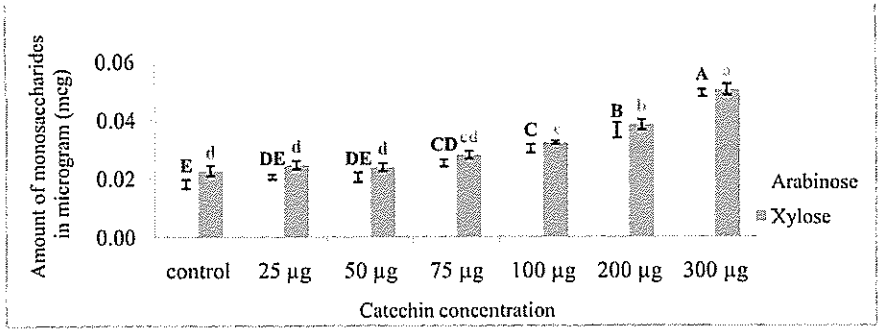

Table 1: Optimisation of pH at 40°C (Fig 1.1 and 1.2)

Substrate (mg/ml)	Enzymes (mg/ml)	Buffer (0.1 M)	Optimum pH
Xylan	Hemicellulase	Sodium acetate	5.5
Xylan	Xylanase	Sodium acetate	5.7

Table 2: Optimisation of temperature (Fig 2.1 and 2.2)

Substrate (mg/mL)	Enzymes (mg/mL)	Sodium acetate Buffer (0.1 M)	Optimum temperature
Xylan	Hemicellulase	pH 5.5	60 °C
Xylan	Xylanases	pH 5.7	60 °C

Statistical analysis

Statistical analysis of the experimental data 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 the cases where ANOVA has been performed, multiple comparisons were made using Duncan's Multiple Range Test (DMRT). Arabinose equivalents and xylose equivalents for reducing sugars and gallic acid equivalents, catechin equivalents, quercetin equivalents, rutin trihydrate equivalents and ascorbic acid equivalents have been assigned to groups using upper case letter series (A>B >C...) as well as lower case (a>b>c...) as per the requirement in graphs. Highest value reported in ascending way as A>AB>ABC>ABCD>....>B>BC>BCD>..... same with L>M>N..... and P>Q>R.... along with lower case alphabets are used for differentiation purpose. In a given series, mean assigned the same letter(s) are not significantly different from each other $p<0.05$.

Discussion

Hemicellulase 100 µL activities on 100 µg xylan as shown in Figure 1.1, there is release of 0.0438±0.0002 microgram of arabinose and 0.0493±0.0002 microgram of xylose equivalence at room temperature in pH 5.5 of sodium acetate buffer 0.1 M (RT). Optimum hemicellulase activity in SAB pH 5.5 was seen at 60 °C (Figure 2.1) with the release of 0.0510±0.0002 microgram of arabinose and 0.0726±0.00001 microgram of xylose equivalence.

Xylanase 100 microlitre activities on 100 microgram xylan as shown in Figure 1.2, there is release of 0.0384±0.0002 microgram of arabinose and 0.0391±0.0002 microgram of xylose equivalence at room temperature in pH 5.7 of sodium acetate buffer 0.1 M (RT). Optimum hemicellulase activity in SAB pH 5.7 was seen at 60°C (Figure 2.2) with the release of 0.0510±0.0002 microgram of arabinose and 0.0726±0.00001 microgram of xylose equivalence.

Plant polysaccharide was degraded by this enzyme and there was significant increase in monosaccharide quantity, polyphenols and flavonoids after treatment, as SPSS gave different group for the treatment in between and within class with error probability less than 0.05. As shown in figure 3.1 that polyphenol in GAE and CE in control was 39.24±0.88 microgram and 41.63±1.15 microgram was increased to 72.57±0.95 microgram and *78.75±1.51 microgram in hemicellulase treatment* and 72.38±3.62 microgram and 75.45±1.35 microgram in xylanase enzyme treatment. Flavonoids in QE and RTE in control 21.49±0.88 microgram and 7.24±0.13 microgram was increased to 108.31±2.5 microgram almost 5-fold increase and 49±0.79 microgram in hemicellulase treatment and 34.56±2.35 microgram and 47.85±1.03 microgram almost doubled compared to control in xylanase enzyme treatment.

Antioxidant activity of sample control 13.67±1.27 microgram of AAE was increased to 18.9±0.74 microgram in hemicellulase and 18.40±0.52 microgram in xylanase enzyme treatment. Fig 3.2 and 3.3 show increase in AE and XE before and after treatment confirming disruption of cell wall structure, which results in optimum extraction of polyphenols and flavonoids.

Enzymes activity on dried leaf powdered of Camellia sinensis is shown in Fig 3.1, 3.2 and 3.3. Amount of sugar in xylose and arabinose equivalence is shown in Fig 3.2 and 3.3 before treatment i.e. control (c-XE and cAE). When plant material was treated with

enzymes there was gradual increase in xylose and arabinose equivalence. Thus, enzymes best work on leaf polysaccharide at 60 °C in sodium acetate buffer pH 5.5 for hemicellulase and pH 5.7 for xylanase enzyme. Release of polyphenols and flavonoids under 1 min microwave was estimated before and after the enzyme treatment. The degradation of this structure causes the walls to loosen. Degradation occurs through the breakdown of xyloglucan oligosaccharides because of the direct hydrolysis action of hemicellulases and xylanases. The synergistic action of a multitude of both the enzymes is essential to hydrolyze a particular hemicellulose. The 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 anti-oxidant activity in terms of ascorbic acid equivalence AAE. There was significant effect of enzyme on plant polysaccharide as amount released of polyphenols and flavonoids after enzymes treatment was quite remarkable. Hemicellulase enzyme treatment showed highest release of flavonoids in quercetin equivalence while xylanase shows highest polyphenols release in GAE and CE, and also total antioxidant activity (AAE) was released to its optimum level.

Catechin effect on hemicellulase and xylanase

Comparative graphs (Fig 4.1 and 4.2) show a distinct effect of catechin. Catechin significantly inhibits hemicellulase and xylanase enzyme activity. Graph of arabinose and xylose equivalence when showed in comprehensive way the figure states a gradual increase in amount of sugar equivalence in presence of catechin 25 microgram to 300 microgram. As the amount of catechin in the reaction mixture increases it might stabilise the hemicellulase enzyme in the reaction mixture. A gradual increase in the sugar equivalents was observed even though the amount of enzyme and substrate was constant. Catechin presence in reaction mixture may increase, facilitate and also stabilise the hemicellulase and xylanase enzyme activity which shows catechins are catalysing the reaction mixture.

Conclusion

Hemicellulase and xylanase showed the highest activity in 0.1 M sodium acetate buffer at pH 5.5 and 5.7 respectively at 60 °C by the modified DNSA method. Plant polysaccharide was degraded by this enzyme and there was significant increase in the monosaccharide quantity. Hemicellulase effect showed highest release of polyphenols followed by flavonoids and was notable. Xylanase activity resulted in an increase in the polyphenol amount after the enzyme treatment.

Inhibition by catechin has been well documented in the literature but the analysis of catechin dose-effect on the activity of the enzymes showed that while lower catechin concentration ranges (25 to 50 µg) showed neutral effect, increased concentration ranges (100 to 300 µg) favoured the reaction by showing an increased activity. Thus, presence of catechin in lower amounts does not affect the reaction whereas higher amounts of it facilitates the enzyme activity more effectively.

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