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Study of Cellulase and Macerating Enzyme Activity and Effect of Catechin on Cellulase

Karishma Rajbhar[1*] and Himanshu Dawda[1]

[1]Plant Biotechnology Laboratory, Ramniranjan Jhunjhunwala College, Ghatkopar (West), Mumbai – 400086 (M.S.), India.

Authors' contributions

This work was carried out in collaboration between both authors. Author KR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors KR and HD managed the analyses of the study. Authors KR and HD managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: To study the activity of cellulase and macerating enzyme and effect of catechin on cellulose.
The cell walls of all higher plants primarily contain cellulose. In cellulose, the polysaccharides have linkages like β-1,4-linked homopolymer of glucose. Using sodium acetate buffer (0.1 M) optimum activity of cellulase and macerating enzyme activity was studied by DNSA method. The activity of the enzyme was also studied on dried tea leaf particles. Cellulases and macerating enzyme hydrolyse cellulose into glucose and other active ingredients like dextrose and fructose, thus improving the extraction rate by increasing the permeability of plant cell walls. The effect of catechin presence in the reaction mixture was studied as its inhibition is well documented. It was demonstrated that catechin took part in the process either by inhibiting or facilitating the reaction. The detailed mechanism is discussed in the article.

Keywords: Cell wall; cellulase; cellulose; macerating enzyme; catechin; DNSA; monosaccharides.

**Corresponding author: E-mail: karishmarajbhar@rjcollege.edu.in*

1. INTRODUCTION

Polysaccharides are of two types, firstly cellulose, hemicelluloses, pectin which is building blocks of plant cell wall and secondly, storage polysaccharides like starch, inulin and gums. They all are made of many different monomeric components, which are attached to each other by different linkages [1,2]. Cellulose is the most abundant carbohydrate in plants which provides structural integrity to cell wall which is formed of β-1,4-linkages homopolymer of glucose and can be degraded by enzyme cellulases [3,4]. D-glucose subunits are linked by β-1,4 glycosidic bonds forming cellobiose molecules which are linear polymer. These chains are linked together by hydrogen bonds and van der Waals forces [5]. Dry weight of wood is almost 45% made up of cellulose. Besides, there are small amounts of non-organized cellulose chains, which form amorphous cellulose. Enzymatic degradation of cellulose is more functional. Linkages like β-1,4 and occasionally β-1,3 glycosidic bonds are seen in between sugars molecules. Thus by enzymatic action, the cell wall can be degraded and inner plasma content can be easily extracted [6,7,5]. Hence, our works mainly focus on finding a simple and fastest method for maximum extraction of economical product.

Cellulase and macerating enzyme refers to a group of enzymes which acts together as hydrolyzing enzymes. Macerating enzyme is made up of cellulase and hemicellulase. It has been reviewed into two steps which involve degrading cellulose first, a pre-hydrolytic step wherein anhydroglucose chains are swollen or hydrated. Secondly, the hydrolytic cleavage of the susceptible polymers is either random or endwise. They also refer to a group of enzymes which work together for hydrolyzing cellulose including exoglucanase, endoglucanase and β-glucosidase (cellulase complex). Enzymes is subdivide into β-1,4-endoglucanases, β-1,4-cellobiohydrolases, β-glucosidase, β-mannanase and α-glucuronidase [6]. A true cellulase can convert crystalline, amorphous (i.e. native cellulose) and chemically derived celluloses to glucose very efficiently. It has been established that the system is multi-enzymatic, and plays an essential role in the overall process of converting cellulose to glucose [8,9]. Our hypothesis is that the degradation of this cell wall can be helpful in maximising the extraction of inner metabolite of plant cell.

Cellulase and macerating enzyme molecules generally have a similar structure, the catalytic domain, cellulose-binding domain and the connecting bridge (linker). As a result, cellulose can be degraded to glucose with this enzyme in a synergistic action. Cellulases hydrolyse cellulose into glucose and other active ingredients like dextrose and fructose, thus improving the extraction rate by increasing the permeability of plant cell walls [6,9].

In this article, optimum pH and temperature for cellulase and macerating enzyme activity was determined by using Miller's DNSA method (1972) and Rajbhar et al. [11]. In addition the presence of dose effect of catechin on cellulase activity was also considered for its mechanism in enzymatic reaction.

2. MATERIALS AND METHODS

Optimum activity of an enzyme in different pH and temperature was determined by estimating sugar products formed. Activity of enzyme on standard cellulose was studied and standard curve was made. By using Rajbhar et al. [11] modified DNSA method the optimum pH and temperature were measured. In this article, the activity optimisation was made by calculating the amount of sugar product released in equivalence to glucose, dextrose and fructose against a known amount of macromolecules cellulose.

2.1 Chemicals & Instrument Used

Dinitrosalicylic acid (DNSA) and crystalline phenol were obtained from HI-Media (India). Cellulose, potassium sodium tartarate (Rochelle salt), sodium sulphite and sodium hydroxide were obtained from Loba Chemie (India). Cellulase and macerating enzyme were supplied by Sigma (India) and Novozyme (India). Instrument used were water bath (Equitron), microwave and Jasco V-530 spectrophotometer.

2.2 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 1% NaOH 100 mL, and was stored at 4°C. The reagent deteriorates due to sodium sulphite so it was added at the time of use to enable prolonged storage, prior to the addition of 40% Rochelle salt solution (Potassium sodium tartarate).

Cellulose was dissolved in distilled water by preparing mg/ml solution. The solution was heated for 5 min at 25°C on a heating mantle until a clear substrate solution is formed. Enzyme stock solution was prepared by mg/ml solution in distilled water and later in sodium acetate buffer of respective pH buffer.

2.3 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 mins at 40°C. Subsequently 0.5 ml DNSA reagent was added and the mixture kept in waterbath 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. This reaction mixture was cooled and absorbance of the coloured complex formed was measured at 456 nm, 455 nm and 453 nm in terms of glucose, dextrose and fructose equivalence using a Jasco V-530 spectrophotometer [12,11,13,14]. Standard graph was plotted with monosaccharide equivalence concentration (microgram) on Y-axis against respective parameter on X-axis.

2.4 Standardisation of Optimum pH and Optimum Temperature

Substrate cellulose reaction with cellulase and macerating enzymes at different pH of 0.1 M sodium acetate buffer and at different temperature in pH 6.0 and pH 3.8 sodium acetate buffer (0.1 M) was prepared and product sugars estimated by DNSA method in glucose equivalent (456 nm), dextrose equivalent (455 nm) and fructose equivalent (453 nm) [11,13].

2.5 Preparation of Reaction Mixture for Effect of Catechin on Enzyme

A solution containing 0.1 ml enzyme, 0.1 ml of substrate and catechin solution varying concentration from 25 µl to 300 µl finally make up total volume of 2 ml with respective buffers. The reaction mixture was incubated at room temperature for 30 mins at respective temperature. Subsequently 0.5 ml DNSA reagent was added and the mixture is made and kept in waterbath 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. For better result reagent colour correction and catechin colour correction was also checked. Reaction mixture was cooled and absorbance of the coloured complex formed was measured at 460 nm in terms of arabinose equivalence, 458 nm in terms of xylose equivalence, 456 nm in terms of glucose equivalence, 455 nm in terms of dextrose equivalence, 453 nm in terms of fructose equivalence and 430 nm of galacturonic acid equivalence using a Jasco V-530 spectrophotometer [12,13]. Graphs were plotted with monosaccharide equivalents concentration released on Y-axis against catechin concentration on X- axis.

2.6 Cellulase & Macerating Enzyme Activity on *Camellia sinensis* Dried Leaves Particles

The 0.1 gram *Camellia* leaf particles was used as a substrate for cellulase and macerating enzyme was used to check the activity on *Camellia* leaf particles. Leaf polysaccharide decoction was prepared with 0.1 gram of *Camellia* leaf particles before and after treatment with enzymes diluted with 5 ml distilled water under 1 minute microwave condition for polyphenols and flavonoids estimation by [10,11,13,15,16,17]. The enzyme effect is visible in Fig. 5.

Camellia sinensis leaf was shade dried and powdered. The polyphenols were extracted from dried powder till no traces of polyphenol were seen; powder was dried again for future process. The enzyme effect is visible in Figs. 6 and 7.

2.7 Statistical Analysis

Statistical analysis of the data for significance and error removal will be conducted using ANOVA with the help of SPSS version 22. Running of Analysis of Variance would give results which will tell the difference of means. Duncan's Multiple Range Test (DMRT) is a post hoc test to measure specific differences between the pairs of data means. DMRT study helps in avoiding error with $P<0.05$. Required coding and other parameter would be used according to need.

3. RESULTS

Cellulase showed optimum activity in 0.1 M sodium acetate buffer at pH 6.0 at room temperature with cellulose and the breakdown reducing sugars product was quantified in glucose equivalence (GE), dextrose equivalence (DE) and fructose equivalence (FE). Statically

given codes (A, a and A) for pH 5.9 and 6.0 are same but 6.0 shows high value of GE, DE & FE, the significance of pH is seen in Fig. 1 for Cellulase activity. (Note: Statistic code in ascending order).

Macerating enzyme showed optimum activity in 0.1 M sodium acetate buffer at pH 3.8 at room temperature with cellulose and the breakdown reducing sugars product was quantified in glucose equivalence (GE), dextrose equivalence (DE) and fructose equivalence (FE). Statically given codes (A, a and A) for pH 3.8 shows high value of GE, DE & FE, the significance of pH is seen in Fig. 2 for macerating enzyme activity. (*Note: - Statistic code in ascending order).

Cellulase showed optimum activity in 0.1 M sodium acetate buffer pH 6.0 at 40°C with cellulose and the breakdown reducing sugars product was quantified in glucose equivalence (GE), dextrose equivalence (DE) and fructose equivalence (FE). Statically given codes A, a and M shows the significance of temperature effect where 40°C is best suited for cellulase activity. (Note: - Statistic code in ascending order).

Macerating enzyme showed optimum activity in 0.1 M sodium acetate buffer pH 3.8 at 40°C with cellulose and the breakdown reducing sugars product was quantified in glucose equivalence (GE), dextrose equivalence (DE) and fructose equivalence (FE). Statically given codes (A, a and M) shows the significance of temperature effect where 40°C is best suited for macerating enzyme activity. (Note: - Statistic code in ascending order).

3.1 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 was performed, multiple comparisons were made using Duncan's Multiple Range Test (DMRT). Glucose equivalents (GE), dextrose equivalents (DE) and fructose equivalents (FE) for reducing sugar and gallic acid equivalents (GAE), catechin equivalents (CE), quercetin equivalents (QE), rutin trihydrate equivalents (RTE) and ascorbic acid equivalents (AAE) series have been assigned groups using upper case letters (A>B >C…) (M>N>O...) as well as lower case (a>b>c...) as per requirement in graphs. Highest value reported in ascending wayas A>AB>ABC>ABCD>....>B>BC>BCD>..... same with lower case alphabets also as used for differentiation purpose. In a given series, mean assigned the same letter(s) are not significantly different from each other $P<0.05$.

4. DISCUSSION

Cellulase hydrolyses the cellulose polysaccharides into glucose, fructose and dextrose. Our study help in quantifying the amount of degraded product at various pH of sodium acetate buffer (SAB) as 10 µl cellulase activities on 100 mcg cellulose release of 0.0326±0.0002 mcg of glucose, 0.0312±0.0013 mcg of dextrose and 0.0473±0.0008 mcg of fructose equivalence at room temperature in pH 6.0 of sodium acetate buffer 0.1 M at room temperature as shown in Fig. 1. Optimum cellulase activity in SAB pH 6.0 was then seen at 40°C which showed the release of 0.0426±0.0006 mcg of glucose, 0.0507±0.0012 mcg of dextrose and 0.0538±0.0002 mcg of fructose equivalence (Fig. 3).

Macerating enzyme is a combination of cellulase with a small part of hemicellulase. It is clearly shown in graphs 1.1, 1.2 and 2.1, 2.2 that macerating is different with cellulase activity. The synergic effect is all together different as shown in Fig. 2 i.e. 100 µl of macerating enzyme activities on 100 mcg cellulose as the highest activity in 0.1 M sodium acetate buffer in pH 3.8 at room temperature by releasing 0.2041±0.008 mcg of glucose, 0.2066±0.0064 mcg of dextrose and 0.1877±0.0079 mcg of fructose equivalence at room temperature in pH 3.8 of sodium acetate buffer 0.1 M at room temperature. Optimum macerating activity in SAB pH 3.8 was obtained at 40°C with the release of 1.2786±0.0224 mcg of glucose, 1.1408±0.0520 mcg of dextrose and 1.5681±0.039 mcg of fructose equivalence as shown in Fig. 4.

This enzyme degraded plant polysaccharide and there was a significant increase in monosaccharide quantity and as well in polyphenols and flavonoids content. The β-1,4 glycosidic bonds are mostly broken due to enzymatic reaction. As shown in Fig. 5, polyphenol content in gallic acid equivalence (GAE) & catechin equivalence (CE) was 39.24±0.88 mcg & 41.63±1.15 mcg in control; which increased to 45.06±1.77 mcg & 47.92±2.52 mcg after cellulase treatment and 52.16±3.0 mcg & 56.28±3.63 mcg after macerating enzyme treatment.

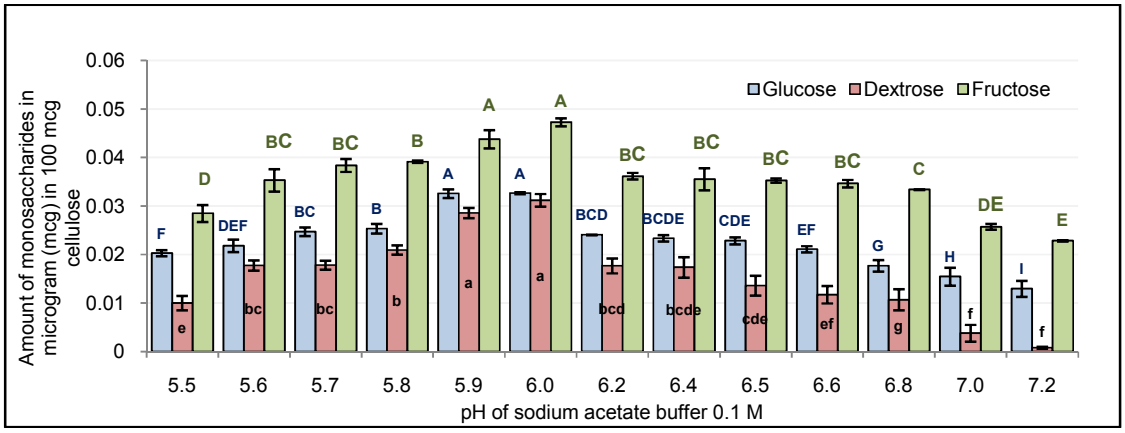

Fig. 1. Cellulase enzyme activity on cellulose in sodium acetate buffer

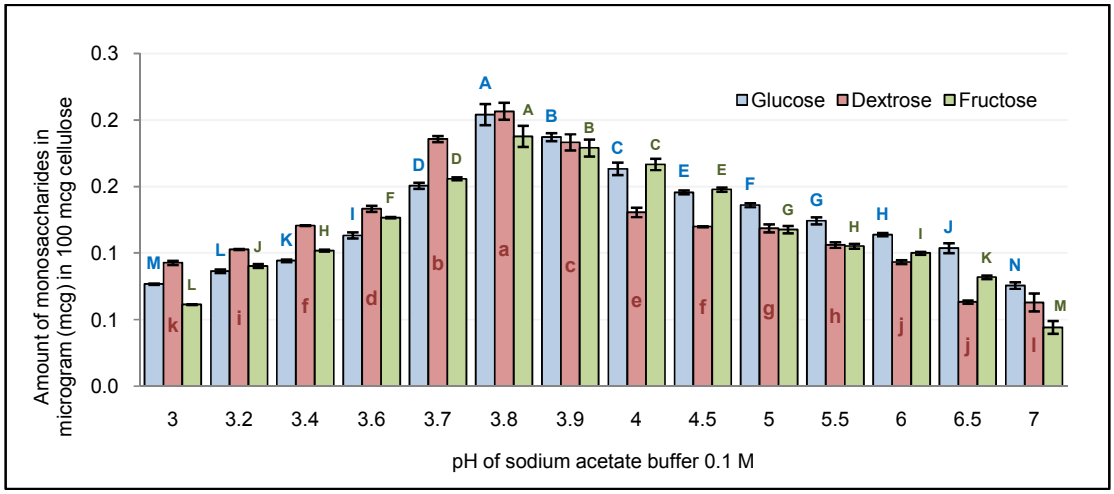

Fig. 2. Macerating enzyme activity on cellulose in sodium acetate buffer

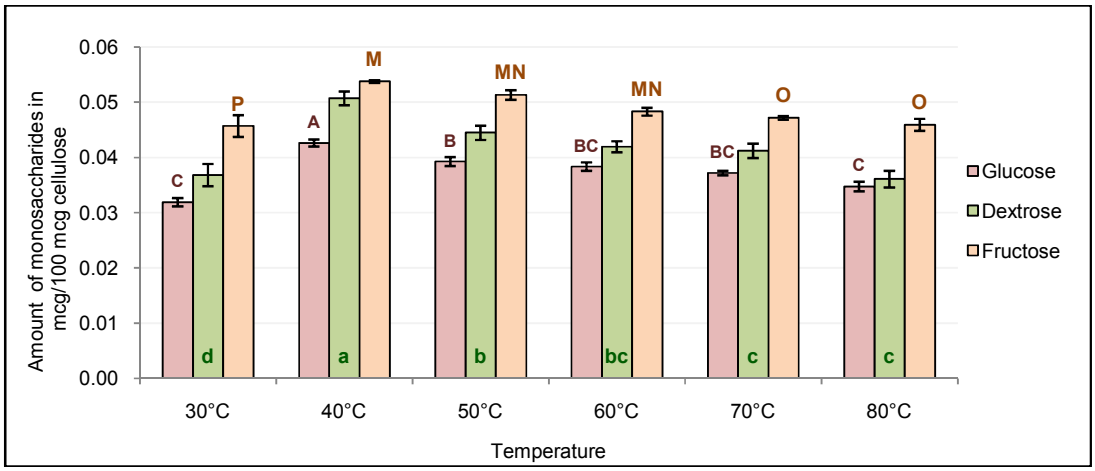

Fig. 3. Cellulase enzyme activity on cellulose in sodium acetate buffer pH 6.0 at different temperature

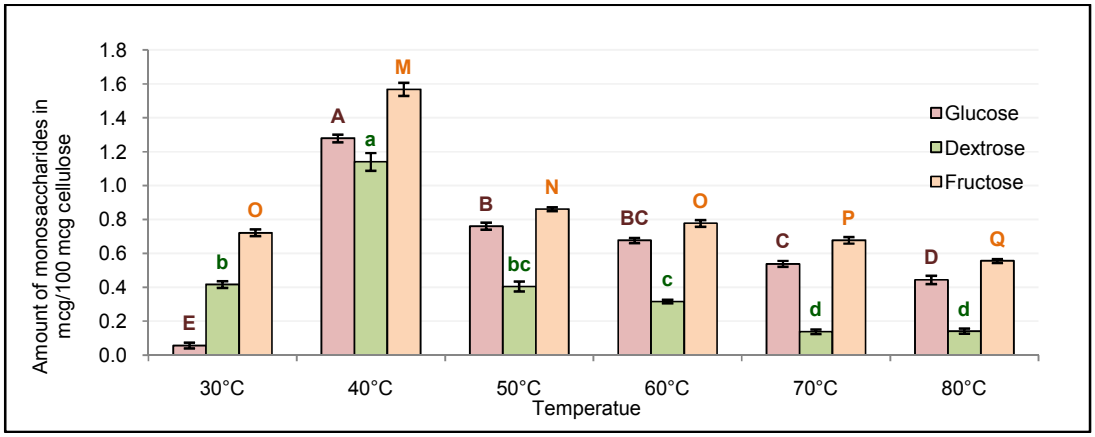

Fig. 4. Macerating enzyme activity on cellulose in sodium acetate buffer pH 3.8 at different temperature

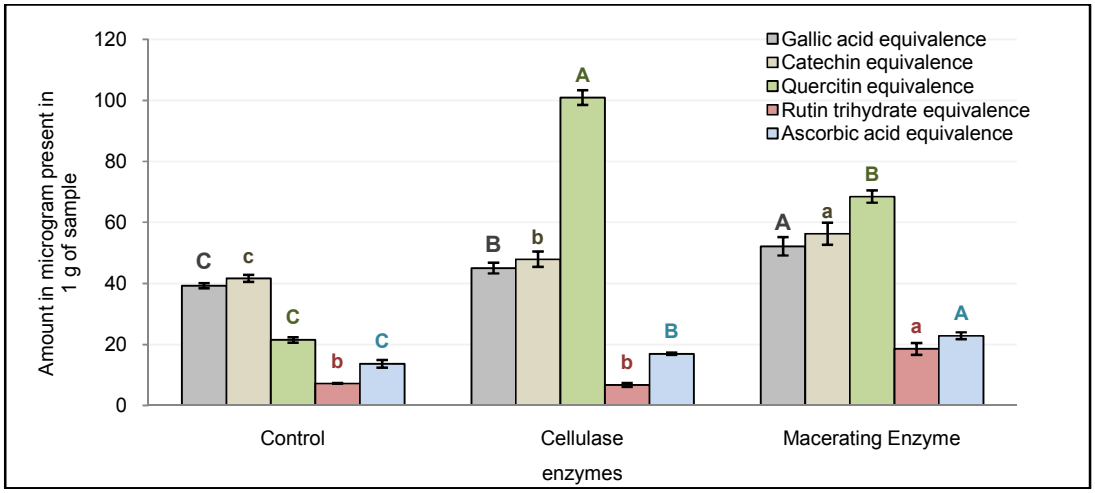

Fig. 5. Cellulase and macerating activity on enzymes on leaves polyphenols and flavonoids of *Camellia sinensis*

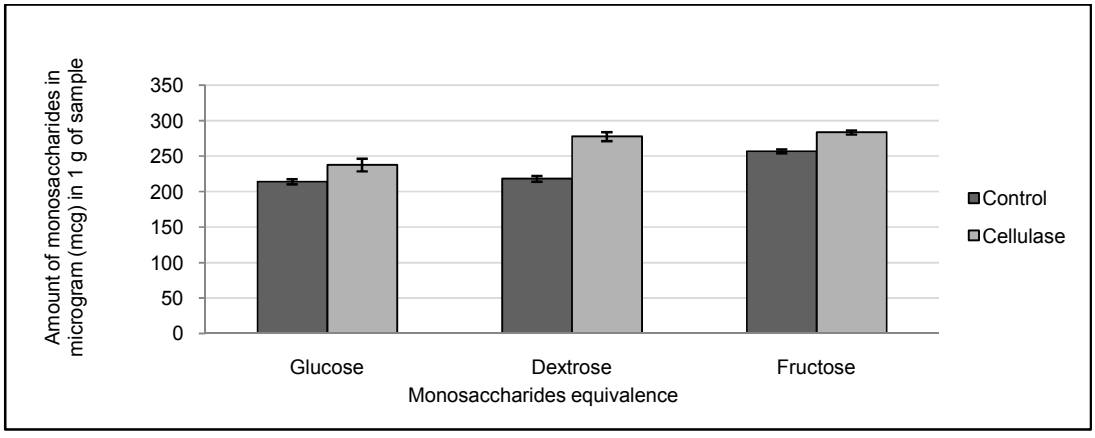

Fig. 6. Cellulase activity on leaf (without polyphenol) in equivalence of sugar by DNSA method

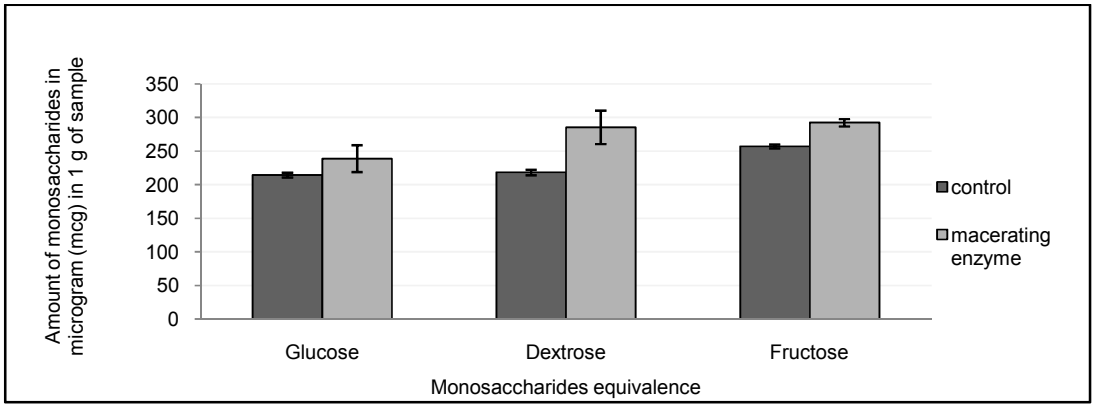

Fig. 7. Macerating activity on leaf (without polyphenol) in equivalence of sugar by DNSA method

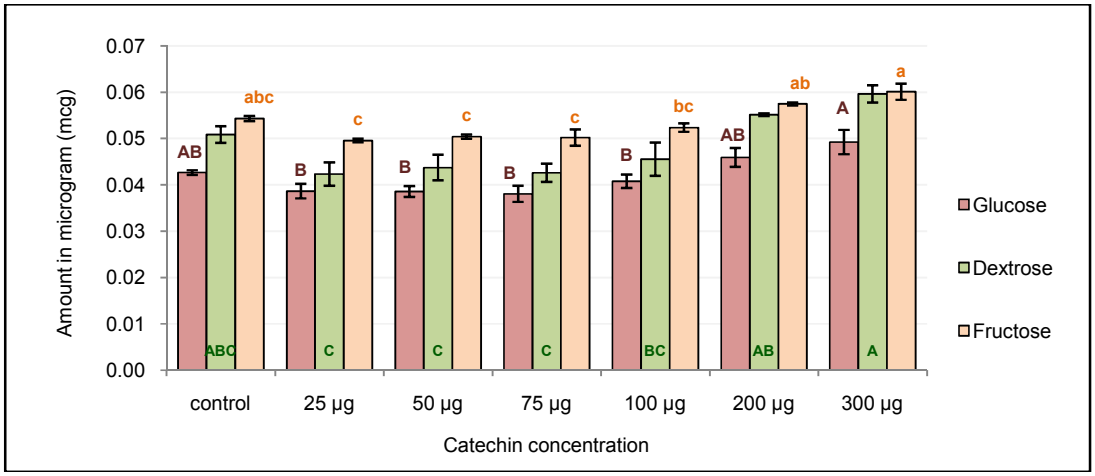

Fig. 8. Dose effect of catechin on cellulase activity on cellulose in glucose, dextrose and fructose equivalence

Table 1. Optimum pH at room temperature (Figs. 1 & 2)

Substrate	Enzymes	Buffer (0.1 M)	Optimum pH
Cellulose (mg/ml)	Cellulase (0.1 mg/ml)	Sodium acetate	6.0
Cellulose (mg/ml)	Macerating (mg/ml)	Sodium acetate	3.8

Table 2. Standardisation of optimum temperature (Figs. 3 & 4)

Substrate	Enzymes	Sodium acetate buffer (0.1 M)	Optimum temperature
Cellulose (mg/ml)	Cellulase (0.1 mg/ml)	pH 6.0	40°C
Cellulose (mg/ml)	Macerating (mg/ml)	pH 3.8	40°C

Flavonoids content in quercetin equivalence (QE) & rutin trihydrate equivalence (RTE) was 21.49±0.88 mcg & 7.24±0.13 mcg in control. It was increased to 100.95±2.41 mcg QE which is almost 5-fold increase & 6.71±0.61 RTE mcg after cellulase treatment and 68.47±2.0 mcg QE & 18.54±1.94 RTE mcg almost double compared to control after macerating enzyme treatment.

Antioxidant activity of sample control was 13.67±1.27 mcg of ascorbic acid equivalence (AAE) which increased to 16.92±0.38 mcg after cellulase treatment and 22.83±1.14 mcg after

macerating enzyme treatment. Figs. 6 and 7 shows increase in GE, DE & FE before and after treatment confirming disruption of cell wall structure can result in optimum extraction of polyphenols and flavonoids.

4.1 Catechin Effect on Cellulase

The comparative graph (Fig. 8) shows a notable effect of catechin on enzyme activity. Catechin has a significant inhibitory effect on an enzyme. Graph of glucose, dextrose and fructose equivalents shows an initial decrease in the amount of sugar equivalents when 25 to 75 mcg catechin is present in the reaction mixture. Even though the amount of enzyme and substrate is same in a reaction mixture, there is a gradual increase in amount of sugar equivalents in the presence of catechin (100 mcg to 300 mcg). Low amounts of catechin present in reaction mixture lower the activity of an enzyme. Higher amount of catechin facilitates the activity of the enzyme, which gradually increases the reaction between enzyme and substrate.

5. CONCLUSION

Cellulase and macerating enzymes activities on dried leaf powdered of *Camellia sinensis* can be seen in Figs. 5, 6 & 7. Amount of sugar in glucose, dextrose and fructose equivalence is shown in Figs. 6 & 7 before treatment i.e. control (c-GE, c-DE & c-FE). When plant material was treated with enzymes there was gradual increase in glucose, dextrose and fructose equivalence. Thus, enzymes work best on leaf polysaccharide at 40°C in sodium acetate buffer, in pH 6 for cellulase and in pH 3.8 for macerating enzyme. Release of polyphenols and flavonoids with 1 minute microwave-assisted extraction was estimated before and after enzyme treatment. It was seen that macerating gave better result than cellulase enzyme. The graph result shows released total polyphenols in gallic acid and catechin equivalence i.e. GAE & CE. It was seen that there was a rise of 1.14 and 1.15 fold in cellulase and 1.32 and 1.35 fold after macerating enzyme treatment respectively. Total flavonoids in equivalence of quercetin and rutin trihydrate i.e. QE & RTE, it was also seen that there was rise of 4.69 and 0.92 fold in cellulase and 3.18 and 2.56 fold after macerating enzyme treatment respectively; while total anti-oxidant activity in terms of ascorbic acid equivalence AAE was risen by 1.23 fold in cellulase and 1.67 in macerating enzyme respectively. There was a significant effect of enzyme on plant polysaccharide as the amounts of released polyphenols and flavonoids after enzymes treatment were quite remarkable. SPSS ANOVA coding states that cellulase enzyme treatment resulted highest release of flavonoids in quercetin equivalence while macerating enzyme showed highest polyphenol release in GAE & CE, as well as the total antioxidant activity (AAE).

Inhibitory effect of catechin is well documented in literature but during the analysis there it was observed that the lower amount of catechin presence resulted in negative effect when concentration ranges from 25 mcg to 75 mcg. However, when the concentration was increased from 100 mcg to 300 mcg it favoured and facilitates the reaction by showing positive increase. Hence, presence of catechin in lower amount inhibits the reaction while higher amount facilitates the enzyme activity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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