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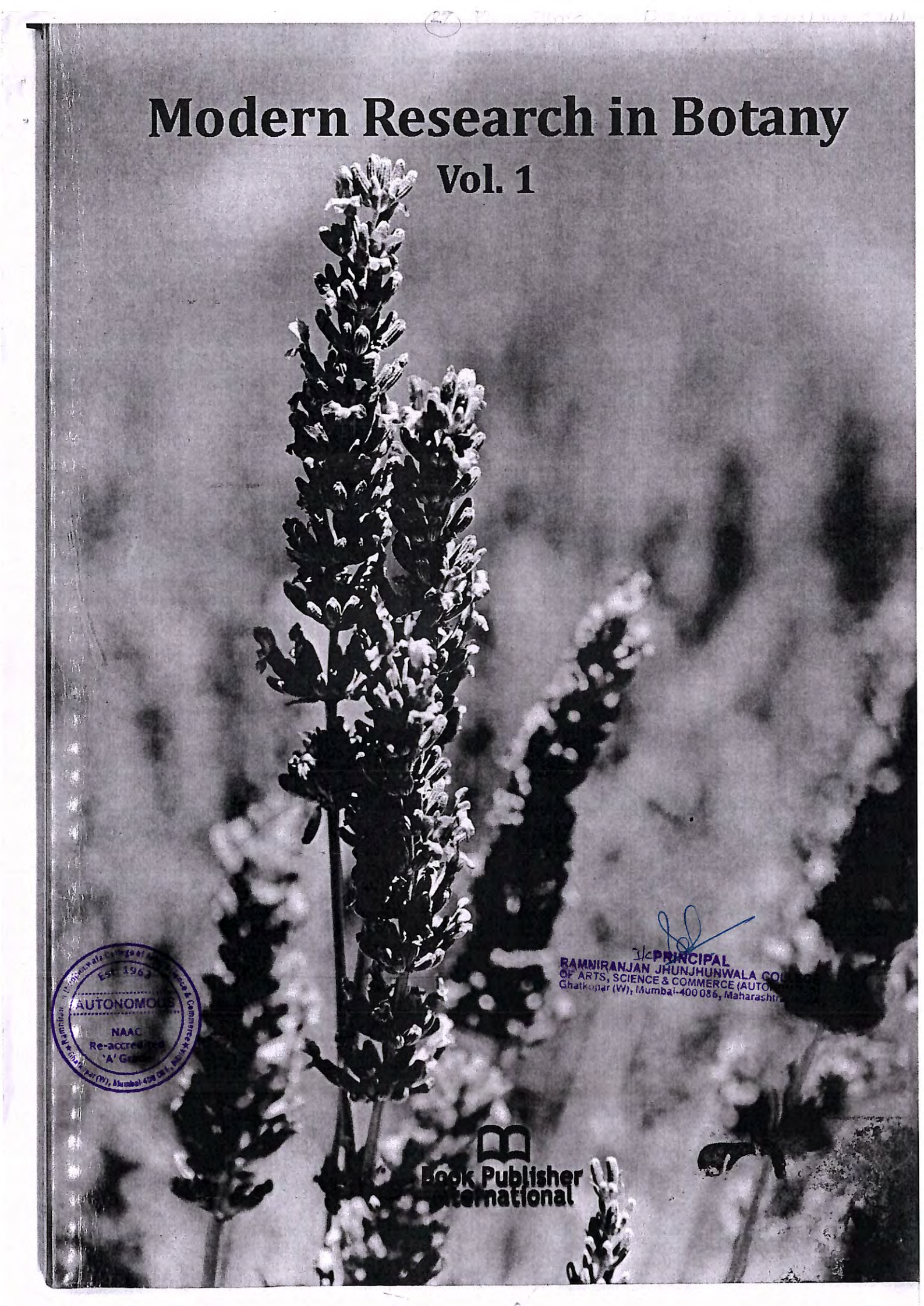

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Contents

Preface

This book covers several areas of biological science. The contributions by the authors include Cellulose activity, macerating enzyme, monosaccharides, seed germination, rose cultivars, hormones, urban solid waste compost, crude fiber & crude protein, pharmacology, ethnobotany, micropropagation, genetic fidelity, bioactive compounds, proteomic variations, antioxidants, osmotic stress, tissue culture, shoot induction, nucleic acid isolation, plants genomic DNA, aerosol particulates, harmful algal blooms, red tide, blue-green algae, geoengineering, particulate pollution, biological efficiency etc. This book contains various materials suitable for students, researchers and academicians in the field of biological sciences.

Similar structure is found in the catalytic domain of cellulase and macerating enzyme molecules like cellulose-binding domain and domain bridge connecter. Thus, cellulose can be broken down to glucose with the help of these enzymes in a synergistic action. Cellulases degrade cellulose into glucose, dextrose and fructose which helps in improving inner metabolite extraction by hydrolysing the plant cell walls [6,9].

With the help of product estimation at different temperature and pH, the enzyme activity of cellulase and macerating enzyme was determined by using Miller's DNSA method (1972) and Rajbhar et al. [10]. Also, the effect of catechin dose on cellulase activity was studied in the enzymatic reaction.

2. MATERIALS AND METHODS

Optimal activity of both the enzyme in different pH and temperature was determined by estimating sugar products like glucose, dextrose and fructose. Standard substrate was cellulose for enzyme study, collected data was used for linear curve plotting. By using Rajbhar et al. [10] modified DNSA method the optimal pH and temperature was studied. Thus, the activity optimisation was calculated by the amount of sugar product released in equivalence to glucose, dextrose and fructose against a known amount of substrate cellulose.

2.1 Chemicals & Instrument Used

Dinitrosalicylic acid (DNSA) and crystalline phenol were used in the study was obtained from HI-Media (India); Cellulose, potassium sodium tartarate (Rochelle salt), sodium sulphite and sodium hydroxide from Loba Chemie (India). Cellulase and macerating enzyme used for the study was from Sigma (India) and Novozyme (India). Instrument used were water bath (Equitron), microwave (LG) and Jasco UV-VIS V-530 spectrophotometer.

2.2 DNSA Reagent, Substrate Solution and Enzyme Solution Preparation

Dinitrosalicylic Acid Reagent (DNSA Reagent) was prepared by using 1 g DNSA, 200 mg crystalline phenol and 50 mg sodium sulphite in 1% NaOH 100 mL, and shelf life was increased by storing at 4°C. The reagent deteriorates due to sodium sulphite, so it was added at the time of use to qualify prolonged storage, prior to the addition of 40% Potassium sodium tartrate (Rochelle salt solution).

Cellulose was solubilised in luke warm distilled water for preparation of mg/ml clear substrate solution. Enzyme stock solution was prepared by mg/ml solution in distilled water and later in sodium acetate buffer of respective pH buffer.

2.3 Reaction Mixture Preparation

A total volume of 2 ml solution with 0.1 ml enzyme volume suspended in corresponding buffer making a volume of 1.9 ml followed by 0.1 ml of substrate solution. Incubation of 30 mins at 40°C was given to reaction mixture at room temperature. Later, 0.5 ml DNSA reagent was added and the mixture kept in 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. 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 [11,10,12,13]. 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

Preparation of substrate cellulose with cellulase and macerating enzymes for reaction mixture, 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) resulted to sugars product estimation by DNSA method in glucose equivalent (456 nm), dextrose equivalent (455 nm) and fructose equivalent (453 nm) [10,12].

2.5 Reaction Mixture Preparation for the Study of Catechin Effect on Enzyme

A reaction solution with 0.1 ml cellulase enzyme, 0.1 ml of substrate and catechin solution varying concentration from 25 µl to 300 µl with makeup 2 ml total volume with respective buffers. Later followed by incubation at room temperature for 30 mins at respective temperature. Afterward, 0.5 ml DNSA reagent was added and the mixture was kept in 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. For better result reagent colour correction and catechin colour correction was also checked. Reaction mixture was brought to room temperature 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 UV-VIS V-530 spectrophotometer [11,12]. Graphs were plotted with product monosaccharide equivalents concentration released on Y-axis against catechin concentration on X- axis.

2.6 Cellulase & Macerating Enzyme Activity on *Sinensis* Dried Leaves Particles

The 0.1 gram leaf particles were used as a substrate for cellulase and macerating enzyme was used to check the activity on leaf particles. Leaf polysaccharide decoction was prepared with 0.1 gram of 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 several authors [14,10,12,15, 16,17]. The enzyme effect is visible in Fig. 5.

Leaf was shade dried and powdered. The polyphenols were extracted from leaf 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 as per the need.

3. RESULTS

Cellulase showed optimal activity in 0.1 M sodium acetate buffer at pH 6.0 at RT with cellulose and the broken-down 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 optimal activity in 0.1 M sodium acetate buffer at pH 3.8 at RT 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 optimal 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 as 40°C is best suited for cellulase activity. (Note: - Statistic code in ascending order).

Macerating enzyme showed optimal 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 was at 40°C which is best suited for macerating enzyme activity. (Note: - Statistic code in ascending order).

3.1 Statistical Analysis of Data

SPSS version 22 was used for statistical analysis of the data obtained. The reported values are the of mean n (n=3) ±SD. The results of the analysis were obtained for $P<0.05$. In cases where ANOVA was performed, multiple assessments 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 to groups using upper case letters (A>B >C...) (M>N>O...) as well as lower case (a>b>c...) as per the needed requirement in graphs. Highest value reported in ascending way as A> AB> ABC> ABCD> ....> B>BC>BCD>..... same method with lower case alphabets were 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 degrade cellulose polysaccharides into glucose, fructose and dextrose. Thus, quantification of the amount of degraded product at various pH of sodium acetate buffer (SAB) by using 10 µl cellulase on 100 mcg cellulose have released product in 0.033±0.0002 mcg of glucose, 0.031±0.0013 mcg of dextrose and 0.047±0.0008 mcg of fructose equivalence at room temperature in pH 6.0 of sodium acetate buffer 0.1 M at RT as shown in Fig. 1. Optimal cellulase activity in SAB pH 6.0 was observed at 40°C which showed the product release of 0.043±0.0006 mcg of glucose, 0.051±0.0012 mcg of dextrose and 0.054±0.0002 mcg of fructose equivalence (Fig. 3).

Macerating enzyme is a combination of cellulase and hemicellulase. It is well observed in graphs 1.1, 1.2 and 2.1, 2.2 that macerating is unlike with cellulase activity. The synergic effect is all together diverse as shown in Fig. 2 i.e. 100 µl of macerating enzyme activity on 100 mcg cellulose as the highest activity in 0.1 M sodium acetate buffer in pH 3.8 at room temperature by releasing 0.204±0.008 mcg of glucose, 0.207±0.0064 mcg of dextrose and 0.188±0.0079 mcg of fructose equivalence at RT in pH 3.8 of sodium acetate buffer 0.1 M at room temperature. Optimal macerating activity in SAB pH 3.8 was obtained at 40°C with the release of 1.279±0.0224 mcg of glucose, 1.141±0.0520 mcg of dextrose and 1.568±0.039 mcg of fructose equivalence as observed in Fig. 4.

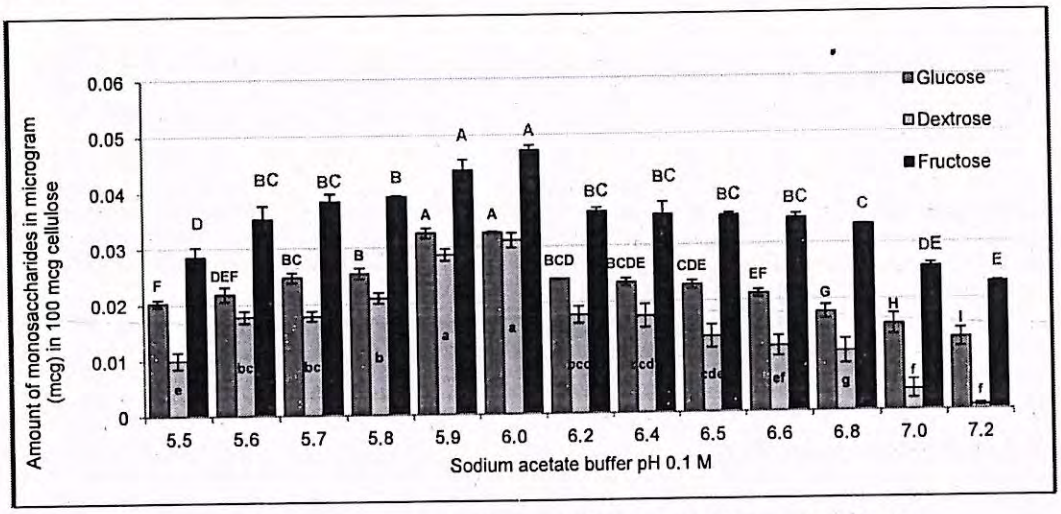

Fig. 1. Cellulase enzyme activity on cellulose in sodium acetate buffer

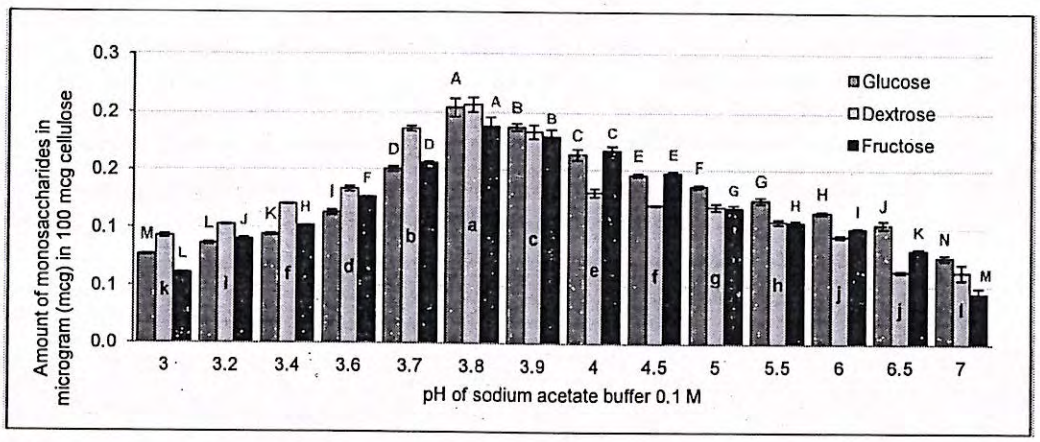

Fig. 2. Macerating enzyme activity on cellulose in sodium acetate buffer

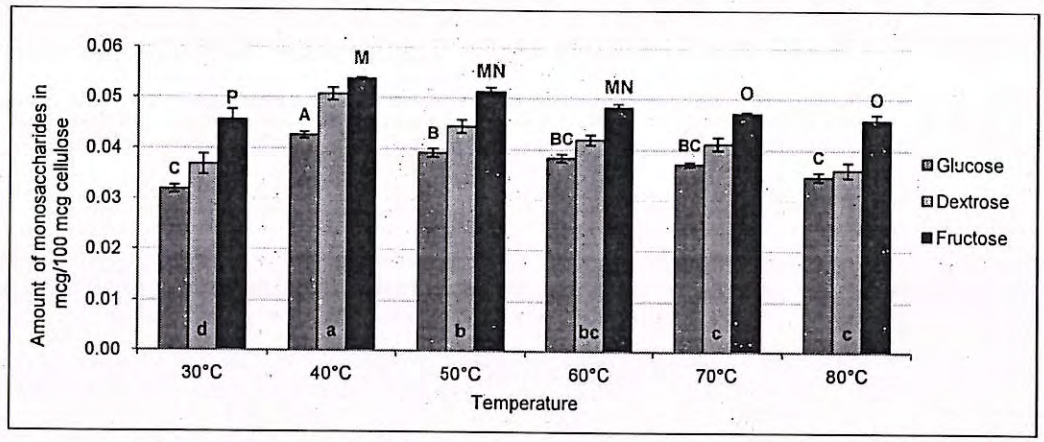

Fig. 3. Cellulase enzyme activity on cellulose in sodium acetate buffer pH 6.0 at different temperature

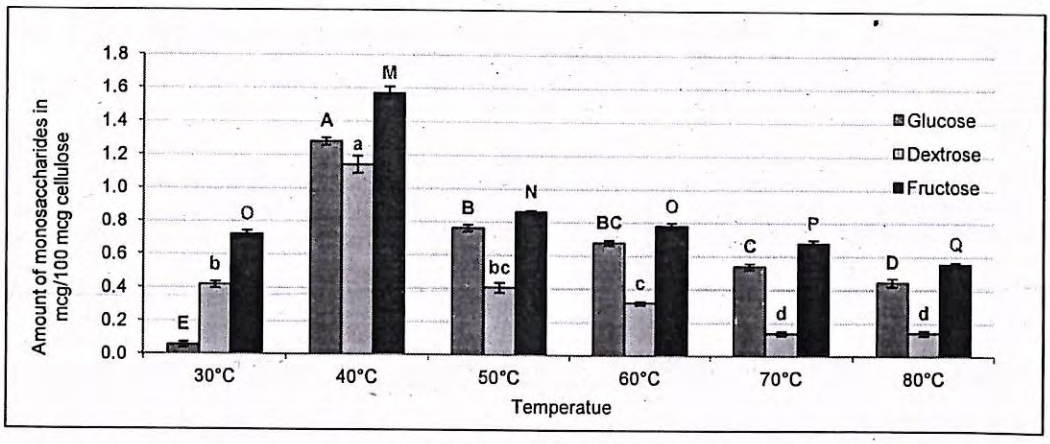

Fig. 4. Macerating enzyme activity on cellulose in sodium acetate buffer pH 3.8 at different temperature

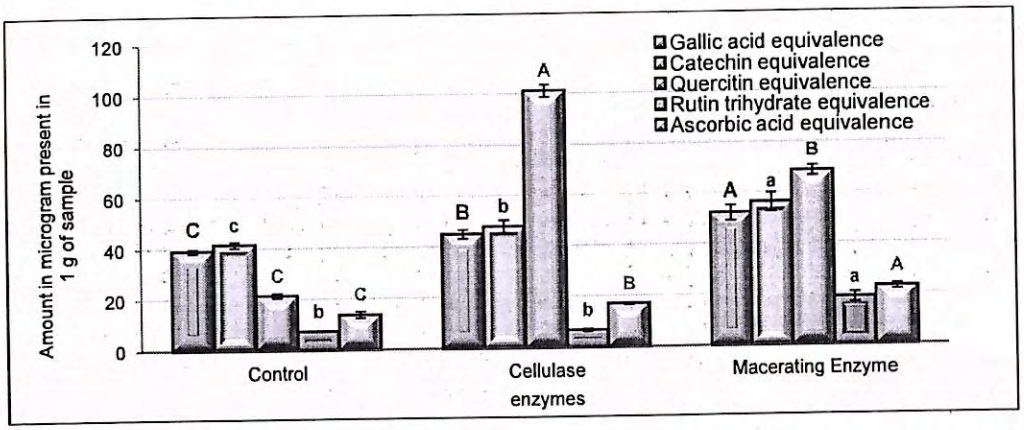

Fig. 5. Cellulase and macerating activity on enzymes on leaves polyphenols and flavonoids of *sinensis*

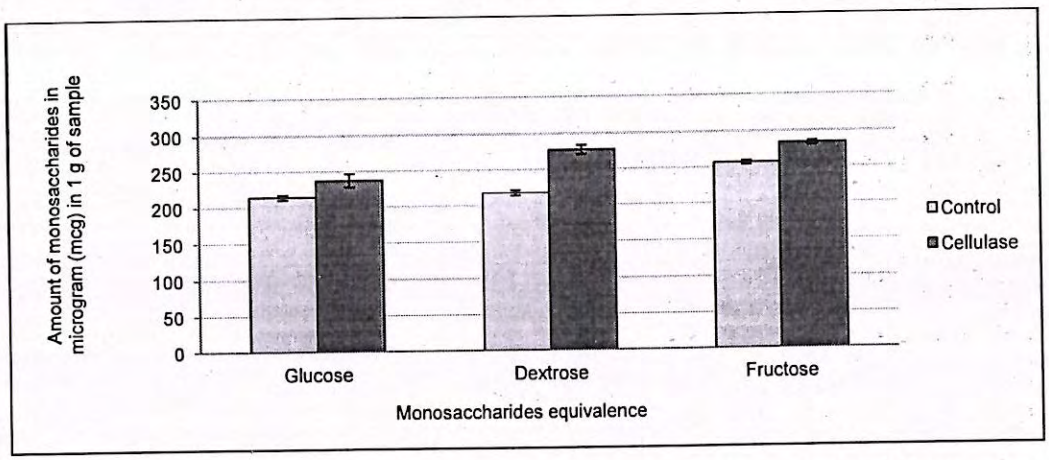

Fig. 6. Cellulase activity on leaf (without polyphenol) in equivalence of sugar by DNSA method

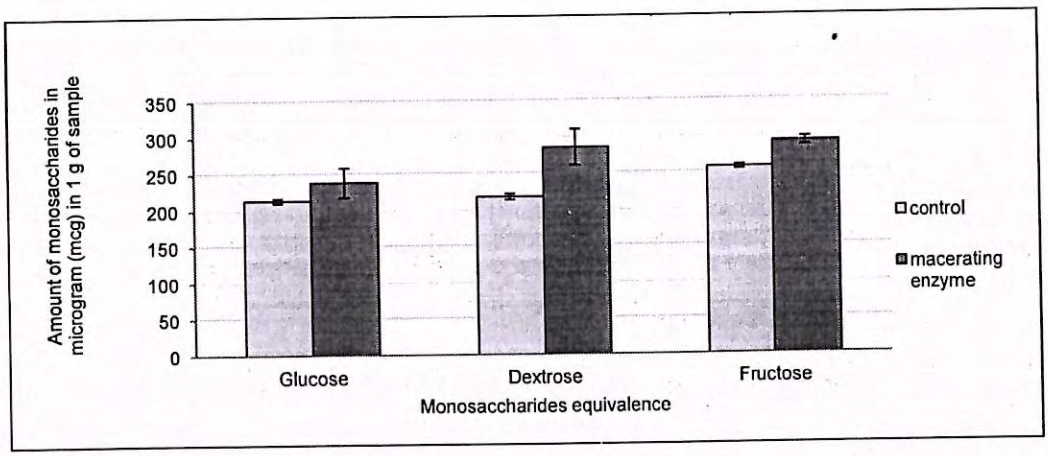

Fig. 7. Macerating activity on leaf (without polyphenol) in equivalence of sugar by DNSA method

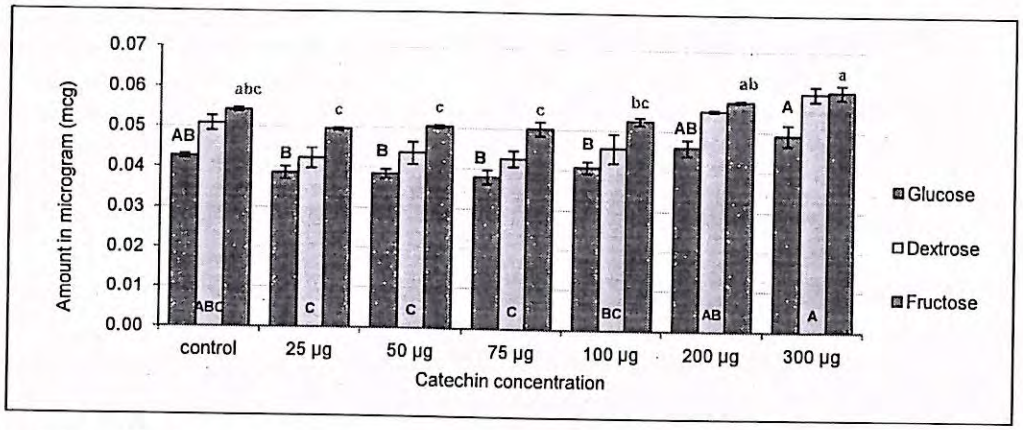

Fig. 8. Catechin dose effect on cellulase activity on cellulose in glucose, dextrose and fructose equivalence

Plant polysaccharide was degraded by enzymes and there was a significant increase in monosaccharide product quantity and as well in polyphenols and flavonoids content. The β-1,4 glycosidic bonds are frequently broken due to enzymatic reaction. As observed in Fig. 5, polyphenol content in gallic acid equivalence (GAE) & catechin equivalence (CE) was 39.2±0.9 mcg & 41.6±1.2 mcg in control; which increased to 45.1±1.8 mcg & 47.9±2.5 mcg after cellulase treatment and 52.2±3.0 mcg & 56.3±3.6 mcg after macerating enzyme treatment.

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

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

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

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

Control showed presence of flavonoids content in quercetin equivalence (QE) & rutin trihydrate equivalence (RTE) which was 21.5±0.9 mcg & 7.2±0.1 mcg. It was increased to 100.9±2.4 mcg QE which is almost 5-fold increase & 6.7±0.6 RTE mcg after cellulase treatment and 68.5±2.0 mcg QE & 18.5±1.9 RTE mcg almost double when compared to control after macerating enzyme treatment.

Antioxidant activity of sample control was 13.7±1.3 mcg of ascorbic acid equivalence (AAE) which was increased to 16.9±0.4 mcg after cellulase treatment and 22.8±1.1 mcg after macerating enzyme treatment. Figs. 6 and 7 displays the increase in GE, DE & FE before and after treatment confirming disruption of cell wall structure can result in optimal extraction of polyphenols and flavonoids.

4.1 Catechin Effect on Cellulase

The comparative graph (Fig. 8) shows a prominent effect of catechin on enzyme activity. Catechin has a significant inhibitory effect on an enzyme is well documented. 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 product sugar equivalents in the presence of catechin (100 mcg to 300 mcg). Thus, low amounts of catechin present in reaction

mixture lower the activity of an enzyme. Higher amount of catechin can 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 can be observed in Figs. 5, 6 & 7. Amount of product 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 consistent 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 microwave-assisted extraction for 1 minute was estimated before and after enzyme treatment. It was realised 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 observed and analysed 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 ascorbic acid equivalence (AAE) terms 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 notable. SPSS ANOVA coding states that cellulase enzyme treatment resulted highest release of flavonoids in quercetin equivalence whereas macerating enzyme showed highest polyphenol release in GAE & CE, as well as the total antioxidant activity (AAE).

Catechin inhibitory effect is well documented in literature but during the analysis there it was observed that the lower amount of catechin presence resulted in negative effect during the concentration range of 25 mcg to 75 mcg. However, when the concentration of catechin was increased to 100 mcg to 300 mcg it favoured and facilitates the reaction by showing positive increase in product amount. 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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