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Effect of tea fungal enzymes on the quality of black tea

G.S. Murugesan, J. Angayarkanni, K. Swaminathan*

Department of Biotechnology, Bharathiar University, Coimbatore- 641 046, Tamil Nadu, India

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Abstract

The cellulolytic enzymes, cellulases, pectinases and xylanases, isolated from the tea fungus (a symbiont of two yeast's *Pichia* sp. NRRL Y- 4810 and *Zygosaccharomyces* sp. NRRL Y- 4882 and the bacterium *Acetobacter* sp. NRRL B- 2357) and laccase from *Trametes versicolor*, were tried for the improvement of black tea quality. The effects of these enzymes on black tea quality parameters, i.e. theaflavin (TF), thearubigen (TR), high polymerised substances (HPS), total liquor colour (TLC), total soluble solids (TSS), caffeine (CAF) and dry matter content (DMC), were analysed. Purified cellulase amended with *Trametes versicolor* laccase in the ratio of 3:2 (v/v) was found to be most effective in enhancing tea quality. Teas processed in the conventional manner and fermented with commercial Biopectinase were maintained as controls. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Tea fungus; Cellulytic enzymes; Theaflavin; Thearubigan; Caffeine

1. Introduction

Tea is the most widely used stimulant throughout the world. It is generally processed from the tender shoots as two leaves and a bud of the tea plant (*Camellia sinensis*). Variations in the flavour and quality of tea are determined by the interplay of complex metabolic events that occur in the leaves during processing. During processing, the enzyme, polyphenoloxidase oxidises the polyphenols present in the tea leaves, resulting in the formation of black tea components. In native leaves, polyphenoloxidase is present in the epidermal cells and the vascular bundles, whereas the Substrate catechin is present inside the vacuoles of the palisade cells (Bhatia & Ullah, 1965). In the conventional method, withered tea leaves are processed by the CTC process (crush, tear and curl). The partial disruption of tea leaves during the CTC process reduces the amounts of peroxidase and polyphenoloxidase in the native leaves, thereby suppressing the formation of black tea components (Marimuthu, Manivel, & Kareem, 1997; Senthilkumar, Swaminathan, Marimuthu, & Rajkumar, 2000). Moreover, the cell wall polysaccharides act as a barrier for the enzyme substrate interaction. To overcome this problem, in the present study, the cellulolytic enzymes, cellulase, pectinase and xylanase obtained from the tea fungus and the polyphenoloxidase, laccase, from *Trametes versicolor* were exogenously added during tea fermentation to improve the tea quality. The quality parameters studied were theaflavin, thearubigen, high-polymerised substances, total liquor colour, total soluble solids, caffeine and dry matter contents.

2. Materials and methods

2.1. Tea fungus

The tea fungus (a symbiont of two yeast's, *Pichia* sp. NRRL Y-4810 and *Zygosaccharomyces* sp. NRRL Y-4882 and a bacterium *Acetobacter* sp. NRRL B-2357), was obtained from the tribal people of Kolli hills, Tamil Nadu. The fungus was grown in a tea medium (Hesseltine, 1965).

2.2. Enzyme production

The tea fungus (1 g mat) was inoculated and incubated in the minimal medium (Carter & Bull, 1969) for 8 days on an orbital shaker (120 rpm). For cellulase, pectinase and xylanase enzyme production, carboxymethylcellulose, pectin and xylan were used as substrates respectively. For laccase production, *Trametes versicolor* was grown in guaiacol amended minimal medium for four days. After the incubation period, the fungal mat was filtered and the filtrate used as crude enzyme.

* Corresponding author.

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2.3. Enzyme purification and assay

2.3.1. Cellulase

The culture filtrate was mixed with three volumes of chilled ethanol and kept overnight at 4 °C. The precipitate was collected, dried in a vacuum and dissolved in 10 ml of 0.1 M citrate buffer, pH 5.0. The concentrated enzyme was passed through a Sephadex G-100 column (1.5×45 cm), equilibrated with 0.1 M citrate buffer, pH 5.0 and eluted with the same buffer. Fractions of 5 ml/h were collected and the protein content (Lowry, Rosebrough, Farr, & Randall, 1951) and cellulase activity in each fraction were estimated. The cellulase activity was determined as the amount of enzyme releasing 1 μmol of reducing sugar as glucose in 1 min (Somogyi & Nelson, 1952). The active fractions were collected and pooled.

2.3.2. Pectinase

For pectinase, the ethanol precipitate was dissolved in 0.1 M acetate buffer, pH 5.0, and eluted through a Sephadex G-100 column, using the same buffer with a flow rate of 5 ml/h. In each fraction, the pectinase activity was estimated (Sherwood, 1966) and expressed as increase in OD per hour per mg protein. The active fractions were pooled.

2.3.3. Xylanase

In xylanase purification, the ethanol precipitate was dissolved in 0.01 M sodium phosphate buffer, pH 8.0 and fractionated by Sephadex G-100 column chromatography, using the same buffer as eluant with a flow rate of 5 ml/h. The xylanase activity was estimated as amount of reducing sugars released from oat spelt xylan (Somogyi & Nelson, 1952). The activity was expressed as amount of enzyme releasing 1 μmol of reducing sugar as xylose in 1 min.

2.4. Characteristics of enzymes

The enzyme properties, i.e. optimum pH, temperature and substrate concentration for maximum enzyme activity (V_{max} and K_m), were determined. The molecular weights of the enzymes were estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970), using a Phast Gel gradient 10–15 medium. Pharmacia high molecular weight calibration kit proteins were used as markers. The methods proposed in the Phast system^{TM} separation technique file No. 110, Pharmacia LKB Biotechnology, Uppsala, Sweden, were followed.

2.5. Tea processing

Tea clone, representing 'Assam' (UPASI-3) cultivar, was selected for the study. Tea shoots, consisting of an apical bud and two leaves, were harvested and withered for 18 h. The withered leaves were made into cut dhool by the CTC (crush, tear and curl) process and four cuttings were performed. The crude and purified enzymes of cellulase, pectinase, xylanase and biopectinase, diluted (1:25 v/v) with 0.1 M acetate buffer of pH 5.0, were sprayed individually on cut dhools; cut dhools sprayed with 0.1 M acetate buffer pH 5.0 were maintained as controls. The spray volume was 25 ml for 750 g of tea leaves for all the treatments. At this volume, the enzyme

Table 1
Purification of cellulolytic enzymes from the culture filtrates of tea fungus

Sample	Volume (ml)	Activity (IU/ml)	Protein (mg/ml)	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Yield (%)	Purification fold
Cellulase								
Culture filtrate	200	0.55	2.34	110	468	0.23	100	1
Ethanol precipitation	10	0.65	0.78	6.50	7.80	0.83	5.91	3.61
Column chromatography Sephadex G 100								
Fraction 3	5	0.65	0.02	3.25	0.10	32.50	2.95	141
6	5	0.77	0.02	3.85	0.10	38.50	3.50	167
9	5	0.82	0.02	4.10	0.10	41.00	3.73	178
Pectinase								
Culture filtrate	200	0.65	2.14	130.00	428	0.30	100	1
Ethanol precipitation	10	0.85	0.96	8.50	9.60	0.88	6.54	2.93
Column chromatography Sephadex G 100								
Fraction 8	5	0.96	0.03	4.80	0.15	32.00	3.69	107
Xylanase								
Culture filtrate	200	0.66	2.10	132.00	420	0.31	100	1
Ethanol precipitation	10	0.75	0.86	7.50	8.60	0.87	5.68	2.81
Column chromatography Sephadex G-100								
Fraction 9	5	0.84	0.03	4.20	0.15	28	3.18	90.3

activities were 0.80, 0.96 and 0.83 IU/ml respectively, for cellulase, pectinase and xylanase. The dhool cut was allowed to ferment for 45 min at room temperature. The fermented dhool was dried at 120 °C for 20 min in an oven and then sifted and graded. The quality parameters of the black tea, theaflavin, thearubigen, high polymerised substances, caffeine, dry matter contents and total liquor colour, were analysed by the solvent extraction method (Takeo & Osawa, 1976); total soluble solids content was estimated by ISS (1973) method. The data on quality parameters were analysed statistically by DMRT (Duncan, 1955) and the best treatment for tea quality improvement was identified. In the next set of experiments, the enzyme preparation yielding best quality tea was mixed in various proportions with the polyphenoloxidase enzyme, laccase, obtained from the wood rotting fungi *Trametes versicolor* (Selvam, 2000). Effect of this enzyme mixture on tea quality were analysed.

3. Results and discussion

3.1. Purification and properties of cellulolytic enzymes

Sephadex G-100 column chromatography of cellulase enzymes revealed three active fractions. The first fraction showed a purification fold of 141 with a specific activity of 32.5 IU/mg protein. In the second fraction, the purification was 167-fold and specific activity was 38.5 IU/mg protein and in the third fraction, the purification fold and specific activity were 178 and 41.0 IU/mg protein, respectively. The enzyme yield (%) was in the range 2.95–3.73. In pectinase and xylanase purifications, the enzymes showed only one fraction on Sephadex G-100 chromatography. In pectinase fractions, the purification fold was 107, specific activity was 32.0 IU/mg protein and the yield was 3.69%. In xylanase, the purification fold was 90.3, specific activity was 28 IU/mg protein and the yield was 3.18% (Table 1 and Fig. 1).

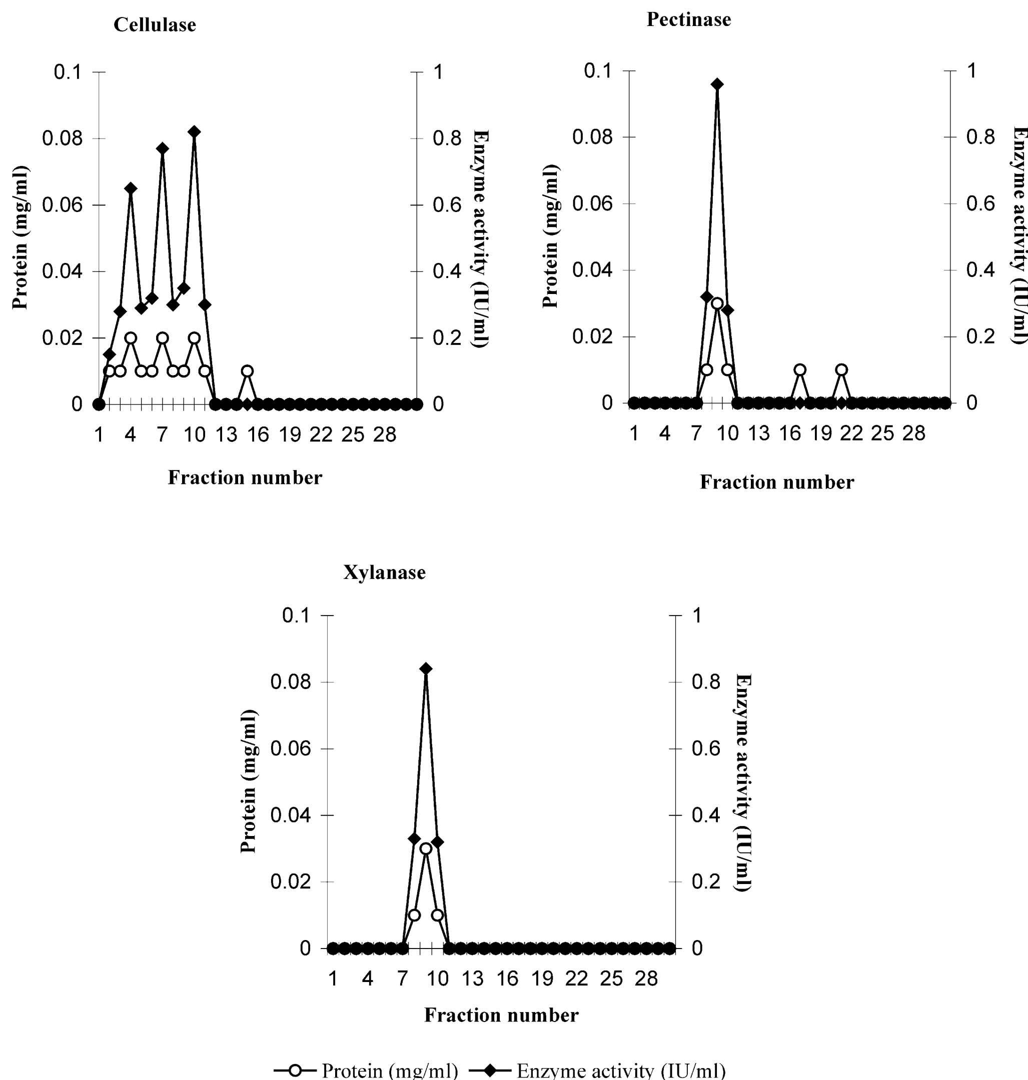

Fig. 1. Purification of cellulolytic enzymes by Sephadex G-100 column.

Table 2
Properties of tea fungal enzymes

Properties	Cellulase	Pectinase	Xylanase
Optimum pH	7.0	6.0	6.0
Optimum temperature, °C	50	40	50
V_{max}, IU/mg protein	32.00	31.00	30.00
K_m, mg/ml	2.50	7.50	2.50
Molecular weight, KDa (SDS-PAGE)	35, 40 & 42	20	45

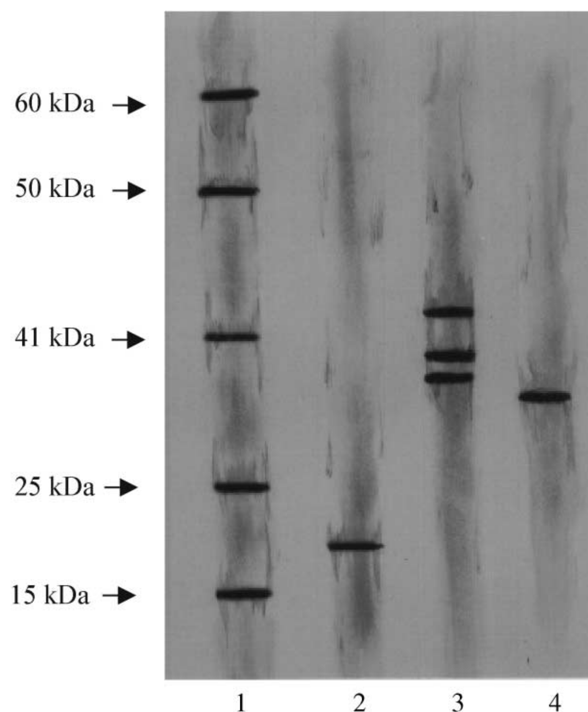

Fig. 2. SDS-PAGE for cellulase, pectinase and xylanase.

The cellulase enzymes of tea fungus had an optimum pH of 7.0 and temperature of 50 °C. The V_{max} against carboxymethylcellulose was 32 IU/mg protein and K_m was 2.50 mg/ml. The molecular weights of the three fractions were 35, 40 and 42 kDa. The optimum pH for pectinase activity was 6.0 and temperature was 40 °C. V_{max} and K_m values were calculated against apple pectin, which were 31.0 IU/mg protein and 7.50 mg/ml, respectively. The molecular weight was 20 kDa. For xylanase, the optimum pH was 6.0 and temperature was 50 °C; the V_{max} and K_m values against oat spelt xylan were 30.0 IU/mg protein and 2.50 mg/ml, respectively and the molecular weight was 32 kDa (Table 2 and Fig. 2).

3.2. Tea processing

Digestion of cell wall components and release of coloured phenolic compounds are two important requisites in tea manufacture. The enzymes peroxidase (PO) and polyphenoloxidase (PPO) play an important role in oxidative reactions, governing the distribution of pigments responsible for tea quality (Marimuthu, Kumar, Balasubramanian, Rajkumar, & Christie, 2000). Polyphenoloxidase oxidises polyphenol bodies to orthoquinones. Subsequently, orthoquinones, by a process known as dimerisation, condense to bis-flavonols and these in turn condense rapidly into theaflavins, which are golden yellow substances (Bhatia, 1963; Bhatia & Ullah, 1965; Sanderson, 1965). Further oxidation results in transformation of theaflavins into thearubigens (reddish brown pigments), which is an enzyme-independent process. An ideal fermentation result in a proper balance of theaflavins and thearubigens (Sanderson, 1965).

Theaflavins (TF), which include theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate and theaflavin-3–3′-digallate are keys to the colour and taste and account for 2–6% of the dry weight in brewed black tea (Yang, Chung, Yang, Chhabra, & Lee, 2000). Thearubigens (TR), such as TR-1, TR-2 and TR-3 formed during manufacture of black tea, contribute largely towards the total colour (Dix, Fairley, Millin, & Swaine, 1981). Thearubigens start forming right from plucking and continue until drying of the tea particles. TR increase linearly during fermentation and attains their maximum in over-fermented leaves. The role of black tea components in tea liquor was analysed as theaflavins for colour, taste, brightness and briskness and as thearubigens for colour, total depth and strength and caffeine for the stimulation of body and mind (Roberts, 1962).

The tea polyphenols are antioxidants and have the ability to sequester metal ions and scavenge reactive oxygen species (Wiseman, Balentine, & Frei, 1997). Catechins, theaflavins and caffeine are reported to inhibit cancer formation in animal models (Yang et al., 2000). The theaflavins in black tea are reported to inhibit lung and oesophageal carcinogenesis (Morse et al., 1997; Yang et al., 1997). Yang, Liao, Kim, Yurkow, and Yang (1998) reported that the black tea polyphenol, theaflavin-3–3′-digallate, inhibits the growth of Ha-ras-transformed 21BES cells. Hence, any increase in tea phenolic compounds will increase the therapeutic value of tea.

In the present study, the cellulolytic enzymes produced by the tea fungus were used to increase the maceration of plant tissues so that high amounts of phenolic substrates, catechins and the oxidising enzymes are released, resulting in increased product formation. Moreover, to enhance the oxidation of phenolic compounds, the phenoloxidase, laccase, obtained from the wood rot fungi *Trametes versicolor* (Selvam, 2000) was exogenously supplied during tea fermentation. The results revealed that exogenous application of cellulase, pectinase and xylanase increased the black tea components, theaflavins (TF), thearubigens (TR), caffeine (CAF), high polymerised substances (HPS), total liquor colour (TLC), total soluble solids (TSS) and dry matter

Table 3
Effect of cellulase, pectinase and xylanase on the quality of black tea

Treatment	TF (%)	DMRT rank	TR (%)	DMRT rank	HPS (%)	DMRT rank	TLC (%)	DMRT rank	CAF (%)	DMRT rank	TSS (%)	DMRT rank	DMC (%)	DMRT rank
Conventional	1.05	h, 10	9.00	k, 11	8.28	k, 11	3.12	h, 11	2.56	k, 11	37.53	g, 7	96.9	b, 2
Biopectinase	1.20	c, 3	9.52	c, 3	9.25	e, 5	3.56	c, 3	2.79	d, 4	37.81	b, 2	96.8	e, 5
	(14.29)		(5.78)		(11.7)		(14.10)		(8.98)		(0.008)		(−0.001)	
Cellulase														
Crude	1.22	b, 2	9.56	b, 2	9.90	c, 3	3.68	b, 2	2.82	c, 3	37.68	c, 3	96.9	c, 3
	(16.2)		(6.22)		(19.6)		(18.0)		(10.2)		(0.005)		(−0.0002)	
Eth.ppt.	1.18	d, 4	9.37	e, 5	9.98	b, 2	3.55	c, 4	2.84	b, 2	37.43	i, 9	96.9	d, 4
	(12.4)		(4.11)		(20.5)		(13.8)		(10.9)		(−0.003)		(−0.0005)	
Purified	1.60	a, 1	9.80	a, 1	10.33	a, 1	3.75	a, 1	2.90	a, 1	38.02	a, 1	97.0	a, 1
	(52.4)		(8.89)		(24.8)		(20.2)		(13.3)		(1.31)		(0.0010)	
Pectinase														
Crude	1.15	e, 5	9.48	d, 4	9.65	d, 4	3.29	d, 5	2.76	e, 5	37.64	d, 4	96.7	fg, 7
	(9.52)		(5.33)		(16.6)		(5.45)		(7.81)		(0.003)		(−0.0018)	
Eth. ppt.	1.09	fg, 7	9.32	f, 6	9.15	f, 6	3.20	e, 7	2.70	f, 6	37.55	f, 6	96.65	i, 10
	(3.81)		(3.56)		(10.5)		(2.56)		(5.47)		(0.001)		(−0.0024)	
Purified	1.06	h, 9	9.14	g, 7	9.09	g, 7	3.16	g, 9	2.68	g, 7	37.33	j, 10	96.56	j, 11
	(0.01)		(1.56)		(9.78)		(1.28)		(4.69)		(−0.005)		(−0.0033)	
Xylanase														
Crude	1.10	f, 6	9.10	h, 8	9.03	h, 8	3.21	e, 6	2.59	j, 10	37.58	e, 5	96.70	gh, 8
	(4.76)		(1.11)		(9.06)		(2.88)		(1.17)		(0.001)		(−0.0019)	
Eth.ppt.	1.08	g, 8	9.08	i, 9	8.96	i, 9	3.18	f, 8	2.61	i, 9	37.55	f, 6	96.69	h, 9
	(2.86)		(0.009)		(8.21)		(1.92)		(1.95)		(0.001)		(−0.0020)	
Purified	1.06	h, 9	9.06	j, 10	8.79	j, 10	3.13	h, 10	2.65	h, 8	37.50	h, 8	96.72	f, 6
	(0.01)		(0.007)		(6.16)		(0.003)		(3.52)		(−0.001)		(−0.0017)	
CV (%)		0.9		0.1		0.1		0.3		0.4		0.1		0.1
P		0.010		0.010		0.010		0.010		0.010		0.010		0.010
SED		0.008		0.007		0.008		0.006		0.008		0.007		0.008
LSD (%)		0.017		0.018		0.017		0.018		0.023		0.016		0.017

TF, theaflavin; TR, thearubigen; HPS, high polymerised substances; TLC, total liquor colour; CAF, caffeine; TSS, total soluble solids; DMC, dry matter content; Eth. ppt., ethanol precipitate. Means followed by a common letter are not significantly different at 5% level by DMRT; Values in parentheses are per cent change over control.

Table 4
Effect of purified cellulase, amended with various proportions of laccase, on the quality of black tea

Treatment	TF (%)	DMRT rank	TR (%)	DMRT rank	HPS (%)	DMRT rank	TLC (%)	DMRT rank	CAF (%)	DMRT rank	TSS (%)	DMRT rank	DMC (%)	DMRT rank
Conventional	1.05	j, 11	9.00	j, 11	8.28	k, 11	3.12	i, 9	2.56	j, 11	37.53	i, 10	96.9	i, 10
PC + C-Lac (ml)														
20 + 5	1.56	d, 4	9.48	f, 7	9.65	f, 6	3.25	e, 5	2.68	f, 6	37.75	d, 5	97.0	c, 3
	(48.6)		(5.33)		(16.6)		(4.17)		(4.69)		(0.006)		(0.0010)	
15 + 10	1.58	c, 3	9.53	e, 5	9.68	e, 5	3.30	d, 4	2.70	e, 5	37.80	c, 4	96.9	d, 4
	(50.5)		(5.89)		(16.9)		(5.77)		(5.47)		(0.007)		(0.0006)	
10 + 15	1.46	e, 6	9.30	g, 8	9.43	h, 8	3.23	f, 6	2.63	g, 7	37.68	e, 6	96.9	de, 5
	(39.1)		(3.33)		(13.9)		(3.53)		(2.73)		(0.004)		(0.0005)	
5 + 20	1.30	g, 8	9.23	h, 9	9.27	i, 9	3.18	g, 7	2.59	h, 9	37.65	f, 7	96.9	ef, 6
	(23.8)		(2.56)		(12.0)		(1.92)		(1.17)		(0.003)		(0.0004)	
0 + 25	1.08	i, 10	9.12	i, 10	8.62	j, 10	3.15	h, 8	2.57	i, 10	37.60	g, 8	96.9	gf, 8
	(2.86)		(1.33)		(4.11)		(0.01)		(0.004)		(0.002)		(0.0002)	
PC + P-Lac (ml)														
20 + 5	1.62	b, 2	9.83	b, 2	10.4	b, 2	3.80	b, 2	2.95	b, 2	38.05	b, 2	97.0	b, 2
	(54.3)		(9.22)		(25.4)		(21.8)		(15.2)		(0.010)		(0.0012)	
15 + 10	1.65	a, 1	9.85	a, 1	10.4	a, 1	3.83	a, 1	2.98	a, 1	38.15	a, 1	97.05	a, 1
	(57.1)		(9.44)		(25.7)		(22.8)		(16.4)		(0.020)		(0.0018)	
10 + 15	1.56	d, 5	9.78	c, 3	10.3	c, 3	3.60	c, 3	2.78	c, 3	37.81	c, 3	96.9	d, 4
	(48.6)		(8.67)		(22.3)		(15.4)		(8.59)		(0.006)		(0.0006)	
5 + 20	1.38	f, 7	9.69	d, 4	9.98	d, 4	3.02	j, 10	2.75	d, 4	37.68	e, 6	96.91	fg, 7
	(31.4)		(7.67)		(20.5)		(−0.032)		(7.42)		(0.004)		(0.0003)	
0 + 25	1.10	h, 9	9.52	e, 6	9.52	g, 7	2.86	k, 11	2.60	h, 8	37.56	h, 9	96.9	hi, 9
	(4.76)		(5.78)		(15.0)		(−0.083)		(1.56)		(0.001)		(0.0001)	
CV (%)		0.7		0.1		0.1		0.3		0.4		0.0		0.0
P		0.010		0.010		0.010		0.010		0.010		0.010		0.010
SED		0.008		0.008		0.008		0.008		0.008		0.008		0.008
LSD (%)		0.023		0.023		0.023		0.023		0.023		0.023		0.023

TF, theaflavin; TR, thearubigen; HPS, high polymerised substances; TLC, total liquor colour; CAF, caffeine; TSS, total soluble solids; DMC, dry matter content; PC, purified cellulase; C-Lac, crude laccase; P-Lac, purified laccase. Means followed by a common letter are not significantly different at 5% level by DMRT. Values in parentheses are per cent change over control.

content over conventionally prepared and biopectinase-fermented tea to a significant level (Table 3). Among all the treatments, the purified cellulase treatment was observed to be most effective in improving tea quality. The conventionally treated tea contained 1.05% of TF, 9.00% of TR, 10.3% of HPS, 3.12% of TLC, 2.56% of CAF and 37.35% of TSS. The biopectinase-treated tea contained slightly higher values (except that of HPS where the HPS content was slightly decreased). However, in the purified cellulase treatment, the TF content was increased by 52.4%, TR content by 8.89%, HPS by 24.8%, TLC by 20.2%, CAF by 13.3%, TSS by 1.31% and DMC by 0.001%. The enhancement of the tea quality in cellulase treated samples might be due to the solubilisation of cellulosic materials present in the cell walls, leading to the mixing up of polyphenoloxidase and the substrate polyphenol (Marimuthu et al., 1997; Selvendran & Perera, 1971).

In the second set of experiments the exogenous application of peroxidase enzyme, laccase, obtained from the wood rotting fungi *Trametes versicolor*, was tried for tea quality improvement, hoping that it may trigger oxidation of polyphenols in addition to the native enzymes. In this trial, the purified cellulase of tea fungal enzyme was mixed with laccase enzyme in various proportions and sprayed onto the cut dhools. The trial revealed that the mixture of purified cellulase and laccase, in the ratio of 3:2 (v/v), yielded better quality tea than the purified cellulase. The tea processed by this treatment increased TF by 57.1%, TR by 9.44%, HPS by 25.7%, TLC by 22.8%, CAF by 16.4%, TSS by 0.02% and DMC by 0.002% (Table 4).

The present study revealed that the exogenous application of purified cellulase of tea fungus and laccase of *Trametes versicolor*, mixed in the ratio of 3:2 (v/v), could increase the quality of black tea and, thereby, its market value. The enzyme-fermented tea may have more therapeutic value than conventional tea.

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