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Microbial polyphenol oxidases in tea catechin oxidation: A novel approach to tea biotransformation

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ABSTRACT

Black tea processing is a complex biochemical process influenced by both plant-derived and microbial enzymes, during which catechins undergo enzymatic oxidation to form compounds such as theaflavins and thearubigins - key contributors to tea's colour, flavour, and health benefits. While endogenous polyphenol oxidases have traditionally been regarded as the primary agents of catechin oxidation, emerging evidence highlights the significant role of bacterial species in modulating tea quality through enzymatic transformations. In this study, bacterial communities were isolated from various stages of black tea processing and screened for extracellular polyphenol oxidase (PPO) activity. Among 43 isolates, *Alcaligenes faecalis* exhibited the highest PPO activity. Enzyme profiling of *A. faecalis* revealed peak laccase and catechol oxidase activities at 36 h (12.6 U/mL and 3.6 U/mL, respectively), while peroxidase activity peaked earlier at 24 h (4.2 U/mL) in nutrient broth. High-performance liquid chromatography (HPLC) analysis showed a concentration-dependent decline in epigallocatechin gallate (EGCG) from 816.24 mg/L to 333.33 mg/L, accompanied by the formation of gallic acid (up to 29.81 mg/L), epigallocatechin, gallocatechin, gallocatechin gallate, and tea pigments. These results confirm the enzymatic degradation and transformation of EGCG into key tea polyphenols, closely mimicking traditional black tea oxidation. A proposed bioconversion pathway outlines the microbial transformation of EGCG into tea pigments. These findings demonstrate the functional contribution of tea-processing-associated bacteria and propose microbial enzymes as a novel biocatalytic tool to enhance black tea fermentation and improve product quality. Future research should focus on enzyme purification and industrial scalability to integrate microbial biotransformation into tea production.

1. Introduction

Tea, sourced from the terminal leaves and buds of *Camellia sinensis*, ranks among the most popular beverages worldwide (Abudureheman et al., 2022; Casanova et al., 2019; Govindarajan et al., 2021). Depending on the processing methods, tea is generally divided into four primary categories: unfermented (green tea), semi-fermented (oolong tea), fully fermented (black tea), and post-fermented (dark tea) (Abudureheman et al., 2022; Theuma and Attard, 2020). Among these, black tea production relies on enzymatic oxidation, transforming catechins into theaflavins (TFs) and thearubigins (TRs) through endogenous plant enzymes (Aaqil et al., 2023).

These oxidizing enzymes, including polyphenol oxidases (PPOs) such as laccases (EC 1.10.3.2) and catechol oxidases (EC 1.10.3.1), as well as peroxidases (EC 1.11.1.7), are vital to the development of the characteristic taste, colour, and aroma of black tea (Li et al., 2013; Zhang et al., 2019). However, plant PPO activity varies by cultivar and processing conditions, limiting consistency in catechin transformation (Chen et al., 2022; Zou et al., 2024). While plant-mediated oxidation is well-characterized, alternative biocatalysts, such as microbial enzymes,

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offer potential for controlled polyphenol biotransformation (Fang et al., 2019; Govindarajan et al., 2021).

Recent studies highlight microbial contributions in black tea processing environments, with bacteria like *Pantoea* and *Pseudomonas* amplifying PPO and peroxidase activity (Wang et al., 2023), and genera such as *Sphingomonas* enhancing enzyme activity (Tong et al., 2021). These microbes, found on leaves and equipment (e.g., withering trays, rollers, and oxidation beds), act as co-catalysts with plant enzymes under high-oxygen conditions, influencing catechin biotransformation (Wang et al., 2023). Fungal PPOs are extensively studied for dark tea production, but bacterial PPOs, particularly extracellular enzymes, remain underexplored (Govindarajan et al., 2021; Liang et al., 2022; Nurmilah et al., 2022). Bacterial PPOs offer advantages for industrial applications, including rapid growth rates, ease of large-scale production, and suitability for genetic modification (Muniraj et al., 2021). However, despite documented microbial diversity in black tea processing (Karunaratne et al., 2024; Liu et al., 2023), screening of bacterial species for PPO activity to identify potential strains for microbial oxidation remains limited (Wang et al., 2023; Nurmilah et al., 2022).

To address this gap, our study aimed to identify bacteria with high PPO activity from the black tea processing environment. Here, we focused on *Alcaligenes faecalis* because of its robust extracellular PPO activity identified in the preliminary screenings. Further, *Alcaligenes faecalis* was selected for its strong extracellular PPO activity observed in preliminary screenings and its previously reported laccase production involved in phenolic degradation (Abdelgalil et al., 2020; Mehandia et al., 2020). We hypothesize that *A. faecalis* PPOs could efficiently catalyse catechin biotransformation, offering a novel microbial approach distinct from plant-mediated oxidation. Our objectives were to isolate and characterize PPO-producing bacteria, examine PPO expression across growth phases, assess epigallocatechin gallate (EGCG) biotransformation using crude enzyme filtrates, and propose a mechanistic pathway for microbial oxidation. By elucidating bacterial PPO mechanisms, this research seeks to develop microbial starters for innovative tea production and explore applications in food biotechnology and bioremediation, advancing sustainable polyphenol modification.

2. Methodology

Abbreviations used in the manuscript are defined in Table 1

2.1. Chemicals and instruments

Nutrient Agar (NA), Tryptic Soy Agar (TSA), and Nutrient Broth (NB) were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, Maharashtra, India). High-purity standards for (+)–catechin (C), (–)–epicatechin (EC), (+)–gallocatechin (GC), (–)-epigallocatechin (EGC), (+)–catechin gallate (CG), (–)–epicatechin gallate (ECG), (+)-gallocatechin gallate (GCG), and (–)–epigallocatechin gallate (EGCG), and gallic acid (GA) were sourced from Sigma-Aldrich (St. Louis, MO, USA), while standards for theaflavin (TF), theaflavin-3-gallate (TF3G), theaflavin-3′-gallate (TF3'G), and theaflavin-3–3′-digallate (TF3–3'G) were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). All standards had ≥ 98 % purity and were stored at −20 °C under dry conditions. The reagents, syringaldazine, catechol, ethylenediaminetetraacetic acid (EDTA), guaiacol, L-ascorbic-ascorbic acid, and hydrogen peroxide (30 % w/w) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were of analytical grade and stored as per the manufacturer's instructions.

Solvents and mobile phases such as formic acid (LC-MS grade, ≥98 %), acetonitrile (HPLC and LC-MS grades, ≥99.9 %), ethyl acetate (analytical grade, ≥99.5 %), and n-butyl alcohol (analytical grade, ≥99 %) were procured from Thermo Fisher Scientific (Waltham, MA, USA). Milli-Q water (18.2 MΩ·cm, Millipore, Billerica, MA, USA) was used for preparing all solutions and mobile phases. All chemicals and reagents were handled according to safety data sheets, and solutions were prepared fresh or stored at 4 °C for no longer than one week to ensure stability.

Key instruments included a Microplate Spectrophotometer (Multiskan SkyHigh, Thermo Fisher Scientific, Waltham, MA, USA) for bacterial growth monitoring (OD_{600}) and enzyme activity assays at 420 nm, 530 nm and 470 nm, a Vanquish high-performance liquid chromatography (HPLC-DAD) system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with diode-array detector (DAD) for analyzing EGCG oxidation products at 210 nm and 280 nm, a spectrophotometer (UV-2450, Shimadzu Corporation, Japan) for tea pigment quantification at 380 nm, a centrifuge (Sorvall ST1 Plus, Thermo Fisher Scientific, Waltham, MA, USA) for preparing crude enzyme supernatants (at 10,000 × g), and an orbital shaker incubator (Solaris, Thermo Fisher Scientific, Waltham, MA, USA) for bacterial cultivation (37 °C, 150 rpm). The chemical structures referenced in the study were drawn using the chemical sketch tool available at https://www.rcsb.org/chemical-sketch, provided by the Research Collaboratory for Structural Bioinformatics (RCSB).

2.2. Isolation, screening, and identification of PPO-positive bacterial isolates

Tea leaf samples (*Camellia sinensis* (L.) Kuntze) were collected from the Somerset Tea Estate, Talawakelle, Sri Lanka. To minimize contamination, workers collected two leaves and a bud while wearing gloves. Samples were transported in aerated nylon bags and processed into black tea using a modified orthodox method involving withering, rolling, rotovane cutting, oxidation, and firing.

Bacterial isolation was conducted from both fresh tea leaves and various stages of tea processing. Epiphytic bacteria were isolated by swabbing leaf surfaces with sterile cotton swabs moistened in 0.85 % NaCl, vortexed in 10 mL sterile saline, serially diluted (10^{-1} to 10^{-6}), and plated on TSA and NA. Plates were incubated at 37 °C for 24–48 h, and colonies were sub-cultured for purity. For endophytic bacteria, leaves were surface-sterilized with 75 % (v/v) ethanol (1 min) and 4 % (w/v) sodium hypochlorite (3 min), rinsing thrice with sterile Milli-Q water, and sectioned (Thambugala et al., 2021). Sections were then plated on TSA and NA and incubated at 37 °C for 48 h. Tea samples from processing stages (withered, rolled, oxidized, fired) were suspended in sterile Milli-Q water, vortexed, diluted, and plated on NA and TSA. Environmental samples were collected by swabbing withering trays, rollers, and oxidation beds with sterile swabs, processed similarly, and plated.

Isolates were preserved short-term on agar slants at 4 °C under sterile mineral oil and long-term in 15 % v/v glycerol at –80 °C. Phenotypic characterization included colony morphology, Gram staining, and microscopic observation. For PPO screening, isolates were cultured in TSB at an optical density (OD_{600}) of 0.2, incubated at 37 °C with 100 rpm agitation for 48 h. Cultures were centrifuged at 10,000 x g for

Table 1
Abbreviations used in the manuscript with their definitions.

Abbreviation	Definition
ANOVA	Analysis of Variance
DAD	Diode-Array Detector
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin Gallate
HPLC	High-performance Liquid Chromatography
HSD	Honestly Significant Difference
NA	Nutrient Agar
NB	Nutrient Broth
OD_{600}	Optical Density at 600 nm
PPO	Polyphenol Oxidase
ROS	Reactive Oxygen Species
TB	Theabrownin
TF	Theaflavin
TR	Thearubigin
TSA	Tryptic Soy Agar

10 min at 4 °C, and supernatants were assayed for extracellular PPO activity assays using 5 mM catechol as the substrate (Nichols-Orians, 1991). Absorbance at 420 nm was measured, and one unit (U) of PPO activity was defined as a 0.001 absorbance increase per min.

Selected isolates with PPO activity underwent molecular characterization at Genetech Molecular Diagnostics, Sri Lanka. Identification was based on the 16S ribosomal RNA (rRNA) gene, which was amplified using primers 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'-TACGGYTACCTTGTTACGACTT-3'). Sequencing was performed by Macrogen (Seoul, South Korea), and sequences were deposited in GenBank. A BLAST analysis against the NCBI database identified taxa with $\geq$ 95 % similarity at the genus level. Multiple sequence alignment refined classification. The species with the highest PPO activity (*A. faecalis*) was selected for further study. Due to the inability to import the Sri Lankan strain of *A. faecalis* into New Zealand, a New Zealand strain of *A. faecalis* NZRM 836 was instead used for the remainder of the experiments.

2.3. Determination of polyphenol-oxidising enzymatic potential of the *A. faecalis*

A. faecalis NZRM 836 was cultured in nutrient broth at 37 °C and 150 rpm using an orbital shaker (Solaris, Thermo Fisher Scientific, USA) as per Kumar and Azmi (2016). Growth was monitored every 12 h for 72 h by measuring its OD_{600} value using a UV-Spectrophotometer. At each interval, 20 mL of culture was centrifuged at 10,000 × g for 10 min at 4 °C to obtain crude culture enzyme supernatants.

Catechol oxidase activity was determined spectrophotometrically by monitoring the formation of benzoquinone at 420 nm, following the method of Eicken et al. (1998) with slight modifications. The reaction mixture (1.0 mL total volume) contained 950 µL of 50 mM phosphate buffer (pH 6.5), 20 µL of 100 mM catechol (final 2 mM), and 30 µL of enzyme extract. The reaction was initiated by enzyme addition, and the increase in absorbance was recorded for 2 min at 25 °C. One unit (U) of enzyme activity was defined as the amount required to produce 1 µmol of benzoquinone per min.

Laccase activity was determined spectrophotometrically by monitoring the oxidation of syringaldazine at 530 nm (Holm et al., 1998). The reaction mixture (1.0 mL total volume) contained 700 µL of 100 mM phosphate buffer (pH 6.5), 100 µL of 10 mM syringaldazine (final 0.5 mM), and 200 µL of enzyme extract. The reaction was initiated by enzyme addition, and the increase in absorbance was recorded for 3 min at 30 °C. One unit (U) of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of syringaldazine per min.

Peroxidase activity was determined spectrophotometrically by monitoring guaiacol oxidation at 470 nm, following Tonami et al. (2004) with minor modifications. The reaction mixture (1.0 mL total volume) contained 850 µL of 50 mM phosphate buffer (pH 6.5), 50 µL of 400 mM guaiacol (final 20 mM), 50 µL of 240 mM H_2O_2 (final 12 mM), and 50 µL enzyme extract. The reaction was initiated by enzyme addition, and the linear absorbance change was recorded for 1 min at 25 °C. One unit (U) of enzyme activity was defined as the amount producing 1 µmol of tetraguacol per min under these conditions. All assays were performed in triplicate.

2.4. Oxidation of EGCG standard solution using crude enzyme of *A. faecalis*

A highly abundant catechin of tea, EGCG, was employed as the substrate for oxidation. A substrate mixture of 1 mL (20 mM) EGCG in 4 mL phosphate buffer (50 mM, pH 6.5) and with varying volumes of 36-h *A. faecalis* enzyme filtrate (5.00, 2.50, 1.25, 0.625, 0.3125 mL) was prepared as in Section 2.3. The final volume was adjusted to 10 mL with buffer. Controls used autoclaved enzyme (121 °C, 15 min) to assess non-enzymatic changes. Reactions were incubated at 60 °C for 2 h, stopped by placing in an ice bath, and filtered (0.22 µm syringe filter). Aliquots (500 µL) were stored at −20 °C for HPLC-DAD analysis, and the remaining samples were used for tea pigment quantification. Experiments were conducted in triplicate.

2.5. Analysis of EGCG oxidation products

Oxidation products were analyzed using an HPLC-DAD system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with a Hypersil GOLD 2.6 µm C_{18} column (150 × 2.1 mm, Thermo Scientific, Waltham, MA, USA) and a diode-array detector (DAD) at 210 nm and 280 nm. The mobile phase consisted of solvent A (0.1 % v/v formic acid in Milli-Q water) and solvent B (100 % acetonitrile, Thermo Fisher Scientific). The column was maintained at 25 °C, with a 10 µL injection volume and 0.3 mL/min flow rate. The gradient elution was: 8–11 % B (0–5 min), 11–20 % B (5–10 min), 20 % B (10–16 min), 20–28 % B (16–25 min), 28–30 % B (25–30 min), 30–35 % B (30–34 min), 35-90% (35–36 min)90 % B (36–39 min), followed by 6 min re-equilibration.

Tea pigments (theaflavins (TFs), thearubigins (TRs), and theabrownins (TBs) were quantified using a modified Wang et al. (2011) method adapted for EGCG oxidation products. A 5 mL reaction mixture was processed via sequential solvent partitioning to prepare four solutions (A–D):

Solution A: A 2 mL sample was extracted with 2 mL ethyl acetate (Thermo Fisher Scientific), shaken for 5 min, and the organic layer was separated. A 0.2 mL aliquot was diluted to 1 mL with 95 % ethanol.

Solution B: 1 mL of the ethyl acetate layer (from Solution A) was extracted with 1 mL 2.5 % (w/v) sodium bicarbonate (Sigma-Aldrich) for 30 s; 0.2 mL organic layer was diluted to 1 mL with 95 % ethanol.

Solution C: 1 mL of the original sample was mixed with 1 mL n-butyl alcohol (Thermo Fisher Scientific); 0.2 mL aqueous layer was combined with 0.2 mL saturated oxalic acid (Sigma-Aldrich), 0.6 mL Milli-Q water, and 1 mL 95 % ethanol.

Solution D: 1 mL of the aqueous layer (from ethyl acetate extraction) was mixed with 0.2 mL saturated oxalic acid, 0.6 mL Milli-Q water, and 1 mL 95 % ethanol.

Absorbance of solutions A–D (E_A, E_B, E_C, and E_D) was measured at 380 nm using a spectrophotometer set to a 1-cm path length. Total concentrations of TFs (%), TRs (%), and TBs (%) were calculated using standard Eqs. 1–3 (Wang et al. 2011), normalized to EGCG dry weight equivalent. All analyses were performed in triplicate.

$$TFs(\%) = \frac{E_C \quad \times \quad 2.25}{\text{Dry weight of EGCG}(\%)} \times 100 \tag{1}$$

$$TRs(\%) = \frac{7.06 \times (2E_A + 2E_D - E_C - 2 \quad E_B)}{\text{Dry weight of EGCG}(\%)} \times 100 \tag{2}$$

$$TBs(\%) = \frac{2E_B \quad \times \quad 7.06}{\text{Dry weight of EGCG}(\%)} \times 100 \tag{3}$$

2.6. Statistical analysis

Experiments were conducted in triplicate, and data are reported as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's Honestly Significant Difference (HSD) test was used to compare means across enzyme volume treatments. Regression analysis assessed the relationship between enzyme activity and EGCG oxidation. Pearson correlation evaluated associations between enzyme activity and oxidation product concentrations. Statistical analyses were performed using Minitab 21.0 software (Minitab LLC, State College, PA, USA), with significance level set at $p < 0.05$.

3. Results and discussion

Bacterial isolates from black tea oxidative processing exhibited diverse enzymatic capabilities. This section details the identification,

PPO activity, and biotransformation potential of these isolates, highlighting their role in enhancing tea quality.

3.1. Screening and identification of PPO-positive isolates

Of the 43 bacterial isolates from fresh *C. sinensis* leaves, processing stages (withered, rolled, oxidized, fired), and equipment, 27 exhibited extracellular PPO activity (Table 1). The 16 isolates with negligible activity (<1 U/mL) included genera such as *Kocuria*, *Staphylococcus*, *Brevundimonas*, and *Klebsiella*, suggesting minimal roles in catechin oxidation. 16S rRNA sequencing identified active isolates with 88–100 % sequence similarity, revealing a diverse microbiota (Table 2).

A. faecalis was the most prevalent PPO-active species, particularly isolate B12 from oxidized leaves, which showed the highest activity (132.57 ± 0.65 U/mL, $p < 0.05$). Other active isolates included *Microbacterium testaceum* (13.2 ± 0.2 U/mL) from rolled leaves and *Alcaligenes* sp. from fired tea (87.5 ± 0.5 U/mL). Isolates from later processing stages (oxidation, firing) consistently displayed higher PPO activity, likely due to microbial adaptation to phenolic-rich environments or enrichment during processing (Nurmilah et al., 2022). The presence of *A. faecalis* on equipment surfaces suggests potential transmission via contact with humans, soil, or water while picking or handling, while processing, influencing microbial succession (Tong et al., 2021).

Among the various microbes identified, *A. faecalis*, a bacterium with diverse metabolic capabilities, has shown significant promise in facilitating the oxidation of polyphenols. Its enzymatic functions could provide new avenues for altering phenolic compounds similar to tea flavanols, potentially enhancing their stability and functional attributes (Rehfuss and Urban, 2005).

A. faecalis produces enzymes like laccases (Abdelgalil et al., 2020), which are recognized for their ability to break down lignin and decolorize dyes (Mehandia et al., 2020). Moreover, certain *A. faecalis* strains have demonstrated antimicrobial properties and the capacity to degrade environmental pollutants (Chen et al., 2020; El-Sayed et al., 2024). Additionally, some strains of *A. faecalis* exhibit advantageous probiotic traits, underscoring their potential to support gut health and overall well-being (Gutiérrez-Falcón et al., 2021; Wang et al., 2020).

3.2. Polyphenol-oxidizing enzymatic potential of A. faecalis

The enzymatic profile of *A. faecalis* was characterized across its growth in nutrient broth (Fig. 1). Optical density (OD_{600}) followed a typical bacterial growth curve: lag phase (0–12 h), exponential phase (12–36 h), stationary phase (36–60 h), and decline (60–72 h). Extracellular enzyme activities peaked at distinct phases, reflecting metabolic regulation. This pattern aligns with previous reports on *A. faecalis* and related species (He et al., 2023).

Laccase activity increased during the exponential phase, peaking at 12.6 ± 0.25 U/mL at 36 h ($p < 0.01$), then declined to 4.8 ± 0.10 U/mL by 72 h (Fig. 1). Catechol oxidase activity followed a similar trend, reaching 3.6 ± 0.07 U/mL at 36 h ($p < 0.01$) before dropping to 1.1 ± 0.022 U/mL by 72 h. Peroxidase activity peaked earlier at 24 h (4.2 ± 0.08 U/mL, $p < 0.01$), declining to 2.2 ± 0.04 U/mL by 72 h. These patterns suggest laccase and catechol oxidase are secondary metabolites linked to phenolic degradation, while peroxidase supports oxidative stress during rapid growth (El-Sayed et al., 2024). The 36-h peak for laccase and catechol oxidase was selected for subsequent EGCG oxidation experiments to maximize catalytic efficiency. Enzyme units (U/mL) are assay-specific (e.g., laccase: syringaldazine oxidation; catechol oxidase: benzoquinone formation; peroxidase: tetraguacol formation), limiting direct comparisons but reflecting substrate-specific activity (Eicken et al., 1998; Holm et al., 1998; Tonami et al., 2004).

Based on the activity profiles, the optimal time for harvesting extracellular enzymes for catechin oxidation in nutrient broth was determined to be at 36 h, when both laccase (12.6 U/mL) and catechol oxidase (3.6 U/mL) activities peaked. Crude enzyme extracts from this time point were subsequently used for experiments aimed at enhancing tea flavour and aroma via microbial-assisted oxidation.

3.3. Oxidation of EGCG by the enzyme filtrate of A. faecalis

Crude enzyme filtrates from *A. faecalis* (36 h) effectively oxidized

Table 2
Molecular identification and polyphenol oxidase (PPO) activity of bacterial isolates recovered from different stages of black tea processing.

Isolate Code	*Isolation Source	Ref. GenBank No.	Closest Match	% Identity	% Coverage	GenBank Accession No.	PPO Activity (U/mL)
B12	OTL	KY744644.1	*Alcaligenes faecalis*	99	97	PP346132	132.57 ± 0.65
B23	OTL	MK617289.1	*A. faecalis*	99	100	PP346140	87.5 ± 0.5
B21	WTL	MZ068206.1	*Brevundimonas diminuta*	95	100	PP346138	67 ± 0.2
B35	WT	OM293496.1	*A. faecalis*	100	100	PP346146	66.95 ± 0.05
B30	RS	EF011115.1	*A. faecalis*	99	100	PP346144	60.05 ± 4.15
B18	FTL	MH133190.1	*A. faecalis*	98	100	PP346136	41 ± 2.1
B08	WTL	MT378145.1	*A. faecalis*	100	100	PP346129	33.1 ± 0.3
B05	OTL	KR782275.1	*Brevibacillus parabrevis*	98	100	PP346126	31 ± 1.1
B33	FB	MN216192.1	*A. faecalis*	99	100	PP346145	28.8 ± 0.3
B24	OTL	KJ729607.1	*Alcaligenes* sp.	92	100	PP346141	23.25 ± 0.25
B20	FTL	MH793409.1	*A. faecalis*	99	100	PP346137	18.25 ± 0.75
B29	OTL	MN578054.1	*A. faecalis*	100	100	PP346143	18.05 ± 0.45
B15	ROL	MT605456.1	*Microbacterium testaceum*	100	100	PP346135	13.2 ± 0.2
B36	ROL	MH029146.1	*Alcaligenes faecalis*	97	100	PP346147	11.5 ± 0.5
B10	RTL	MZ295250.1	*Mammaliicoccus sciuri*	95	100	PP346130	11.35 ± 0.35
B26	OTL	MT378145.1	*Alcaligenes* sp.	90	92	PP346142	10 ± 0.54
B03	FTL	LC507944.1	*B. diminuta*	100	100	PP346124	5.75 ± 0.75
B01	WTL	KM203630.1	*B. parabrevis*	99	100	PP346122	4.55 ± 0.15
B11	OTL	CP125088.1	*Klebsiella pneumoniae*	100	100	PP346131	3.35 ± 0.85
B14	FTL	OQ780604.1	*Brevibacterium* sp.	99	100	PP346134	2.25 ± 0.15
B06	ROL	KR047782.1	*Brachybacterium rhamnosum*	100	100	PP346127	2.2 ± 0
B04	ROL	KT779213.1	*Staphylococcus sciuri*	100	100	PP346125	1.28 ± 0.58
B07	ROL	MF525127.1	*Brevundimonas* sp.	88	100	PP346128	1.23 ± 0.43
B13	OTL	MN880161.1	*Brevundimonas* sp.	97	100	PP346133	0.75 ± 0.01
B02	WTL	MK764985.1	*Staphylococcus succinus*	98	100	PP346123	0.3 ± 0
B22	ROL	MK847262.1	*Brevundimonas* sp.	90	98	PP346139	0.12 ± 0
B38	OTL	KF424684.1	*Kocuria palustris*	98	100	PP346148	0.07 ± 0

* **Isolation sources**: Fresh tea leaves (FTL); withered leaves (WTL); roller surfaces (RS); rotovaned leaves (RTL); oxidized leaves (OTL); fermentation beds (FB); and fired black tea (FBT); withering trays (WT) and rolling equipment (ROL).

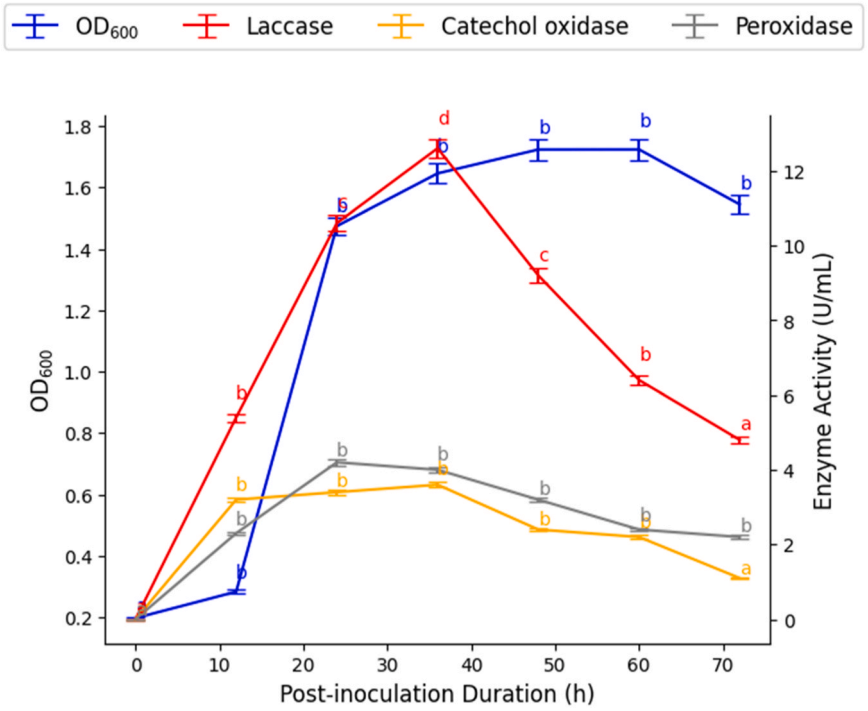

Fig. 1. The trend of extracellular enzymatic activities with bacterial growth in the nutrient broth media.

EGCG, mimicking black tea oxidative processing. HPLC-DAD chromatograms (Fig. 2) showed a concentration-dependent decrease in EGCG peak intensity with increasing enzyme volumes (31.25–500 μL), accompanied by new peaks for oxidation products.

Fig. 2 details the HPLC-DAD chromatogram (7) of the reaction mixture at 500 μL enzyme volume, showing prominent peaks for gallic acid (GA), gallocatechin gallate (GCG), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG), alongside eight overlaid chromatograms (1−8) corresponding to enzyme volumes 0 before incubation and 0–500 μL at the end of reactions. The dominant EGCG peak (retention

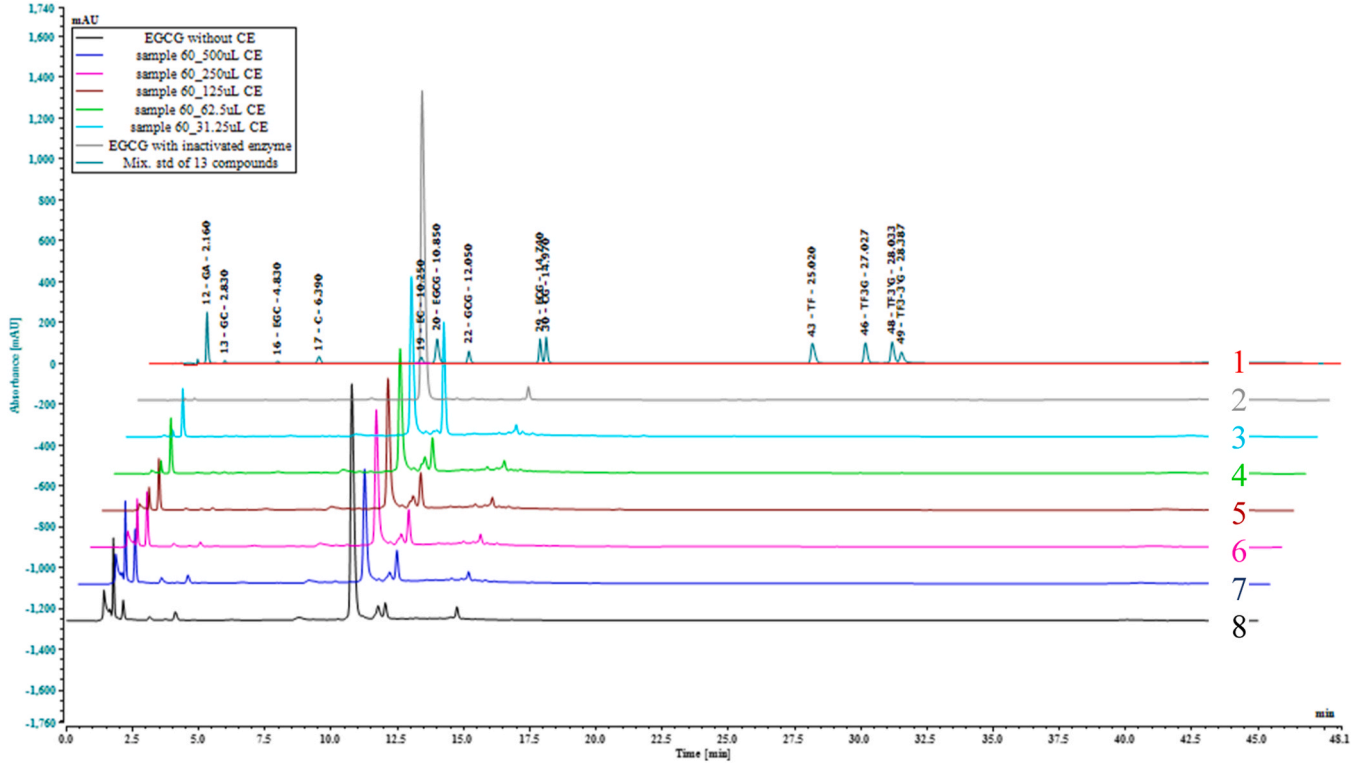

Fig. 2. HPLC-DAD chromatograms showing the effect of different crude enzyme volumes on EGCG degradation, along with the appearance of oxidation products in the sample vials. Detection at 280 nm. (1) Mixed standard of 13 compounds; (2) Control (EGCG) before incubation; (3) 31.25 μL; (4) 62.5 μL; (5) 125 μL; (6) 250 μL; (7) 500 μL enzyme treatment; and (8) Control (EGCG) with inactivated enzyme at the end of the reaction.

time 10.85 min) decreases in intensity from chromatogram A (0 μL, orange) to F (500 μL, blue), reflecting a reduction in EGCG concentration from 795.82 ± 55.71 mg/L (0 μL) to 333.33 ± 20.00 mg/L (500 μL), representing a ~58 % reduction (Table 3, Fig. 3A). This degradation is more pronounced than the previously reported 20–30 % EGCG loss typical in industrial tea fermentation (Aaqil et al., 2023). At the same time, the gallic acid peak (2.160 min) and EGC peak (4.830 min) increase, aligning with quantitative data showing gallic acid rising from 1.06 ± 0.02 mg/L to 29.81 ± 1.79 mg/L (Fig. 3A) and EGC from 1.14 ± 0.09 mg/L to 3.97 ± 0.24 mg/L. These results confirm the enzymatic breakdown of EGCG into monomeric catechins and polymeric pigments, consistent with traditional tea processing (Fang et al., 2019).

Furthermore, Table 3 presents the concentrations of key oxidation products across enzyme volumes. The EGCG concentration before incubation (t = 0) was 816.24 mg/L, which is approximately 10 % below the theoretical value of 916.74 mg/L (from 1 mL of 20 mM EGCG diluted to 10 mL), likely due to impurities in the EGCG standard. After 2 h of incubation at 60 °C without enzyme (control, 0 μL), the concentration decreased to 795.82 ± 55.71 mg/L, attributable to minimal thermal degradation (Xu et al., 2019). Significant epimerization of EGCG resulted in substantial GCG formation, ranging from 310.42 ± 21.73–421.42 ± 29.50 mg/L, also attributable to the incubation temperature (Xu et al., 2019). Despite the limited impact of temperature alone, the observed 58 % reduction in EGCG highlights the pronounced effect of enzymatic activity (Fig. 3A).

Other catechins (Fig. 3B), including catechin (C, 3.55 ± 0.25–5.11 ± 0.31 mg/L), gallocatechin (GC, 4.75 ± 0.29–29.19 ± 2.04 mg/L), and epigallocatechin gallate (ECG, 89.32 ± 6.25–30.79 ± 1.85 mg/L), were detected, with significant changes ($p < 0.05$) indicating minor degradation or oxidative contributions. Theaflavin-3-gallate (TF3G) increased to 0.14 ± 0.01 mg/L at 31.25 μL, while theaflavin-3,3′-digallate (TF3,3′-diG) reached 4.474 ± 0.148 mg/L at 500 μL. Other theaflavins (TF, TF3'G) remained undetectable (0.00 mg/L).

The strong correlation between enzyme volume and oxidation products (Pearson's r = 0.92 for gallic acid, $p < 0.05$) suggests dose-dependent catalysis. The 60 °C incubation likely enhanced enzyme-substrate interactions, as *A. faecalis* PPOs exhibited thermostability in preliminary assays (data not shown), unlike typical plant PPOs (Li et al., 2013). The thermostability and pH stability of *A. faecalis* extracellular enzymes make them well-suited for tea processing conditions, which often involve elevated temperatures and varying pH levels. Notably, the laccase from *A. faecalis* XF1 exhibits optimal activity at 80 °C and remarkable stability in the 70–90 °C range, while the NYSO strain laccase retains full activity up to 70 °C with a 7-h half-life at 60 °C, confirming its suitability for the incubation conditions employed here (Mehandia et al., 2020; Abdelgalil et al., 2020). Additionally, the 60 °C incubation temperature is unlikely to affect polyphenol profiles significantly, as this temperature is commonly used in tea polyphenol extraction procedures without significant degradation of catechins or other phenolic compounds (Perva-Uzunalić et al., 2006; Vuong et al., 2011). However, oxidative agents, such as reactive oxygen species (ROS) or quinones generated during EGCG oxidation, may reduce enzyme activity by oxidizing critical amino acid residues or disrupting structural features like disulfide bridges, necessitating controlled conditions to maximize catalytic efficiency (Veetil et al., 2012). These controlled conditions, such as buffering to maintain pH stability, regulating oxygen levels, or adding antioxidants to scavenge ROS, ensure that enzymes like laccase maintain their activity, thereby optimizing catechin oxidation and tea pigment formation. These properties confirm that the 60 °C incubation temperature and the slightly acidic to neutral pH range typical of tea processing are compatible with both the enzymatic profile of *A. faecalis* and the stability of tea polyphenols, enabling effective catechin oxidation.

In addition to theaflavins (TFs), other oxidative coupling products, such as thearubegins (TRs) and theabrownins (TBs), were identified,

Table 3
Concentrations of oxidation products across enzyme volumes (mg/L, mean ± SD, n = 3) using HPLC quantification.

Enzyme vol. (μL)	GA	GC	EGC	C	EC	EGCG	GCG	ECG	CG	TF3G	TF3-3′G
0 *	1.06 ± 0.02^{d}	7.12 ± 1.55^{b}	1.14 ± 0.09^{bc}	2.69 ± 0.25^{d}	20.67 ± 2.10^{bc}	816.24 ± 55.71^{a}	24.01 ± 6.12^{c}	79.32 ± 6.25^{a}	8.69 ± 2.71^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}
0	15.33 ± 1.07^{d}	9.19 ± 2.04^{a}	1.29 ± 0.37^{bc}	3.55 ± 0.25^{bc}	43.36 ± 3.04^{b}	795.82 ± 55.71^{a}	310.42 ± 21.73^{b}	89.32 ± 6.25^{a}	18.69 ± 2.71^{b}	0.00 ± 0.00^{b}	0.08 ± 0.01^{c}
31.25	24.69 ± 1.48^{bc}	4.91 ± 1.49^{a}	1.44 ± 0.39^{a}	5.11 ± 0.31^{a}	74.00 ± 4.44^{a}	531.72 ± 31.90^{b}	409.72 ± 24.58^{a}	80.00 ± 4.80^{a}	19.83 ± 6.59^{a}	0.14 ± 0.01^{a}	1.07 ± 0.12^{ab}
62.5	24.43 ± 1.71^{bc}	5.64 ± 0.39^{bc}	2.33 ± 0.23^{d}	3.14 ± 0.22^{cd}	14.82 ± 1.04^{c}	380.45 ± 26.63^{c}	398.82 ± 27.92^{a}	43.93 ± 3.08^{b}	17.56 ± 1.23^{b}	0.06 ± 0.00^{ab}	2.15 ± 0.15^{a}
125	24.20 ± 1.45^{c}	4.75 ± 0.29^{c}	2.28 ± 0.20^{d}	3.15 ± 0.19^{cd}	4.19 ± 0.25^{d}	413.46 ± 24.81^{c}	417.91 ± 25.07^{a}	44.18 ± 2.65^{b}	21.02 ± 1.26^{c}	0.00 ± 0.00^{b}	2.06 ± 0.12^{ab}
250	25.81 ± 1.81^{b}	6.12 ± 0.43^{bc}	2.82 ± 0.20^{d}	2.76 ± 0.19^{d}	11.55 ± 0.81^{c}	405.58 ± 28.39^{c}	421.41 ± 29.50^{a}	48.14 ± 3.37^{b}	20.44 ± 1.43^{c}	0.00 ± 0.00^{b}	3.52 ± 0.11^{b}
500	29.81 ± 1.79^{a}	6.36 ± 0.38^{b}	3.97 ± 0.24^{c}	3.08 ± 0.19^{cd}	7.58 ± 0.46^{cd}	333.33 ± 20.00^{d}	415.85 ± 24.95^{a}	30.80 ± 1.85^{c}	17.79 ± 1.07^{c}	0.00 ± 0.00^{b}	4.47 ± 0.15^{a}

Means denoted by different letters (a–f) within each column are significantly different (Tukey's test, $p < 0.05$). * denotes the control before incubation

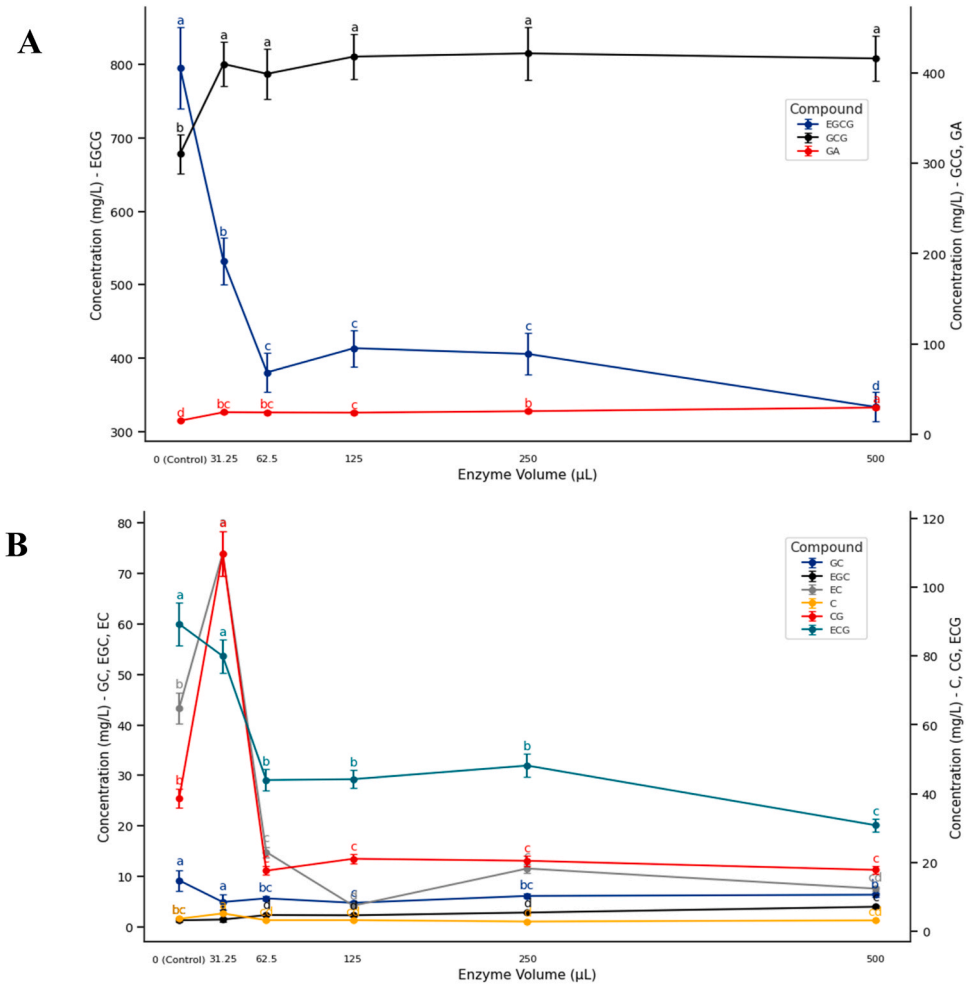

Fig. 3. EGCG oxidation by *Alcaligenes faecalis* crude enzyme filtrate at 0, 31.25, 62.5, 125, 250, 500 µL, major products GA/GCG (**A**), and other catechins (GC, EGC, C, EC, ECG, CG) (**B**), SD error bars and letters denoting Tukey's HSD pairwise comparison results ($p < 0.05$).

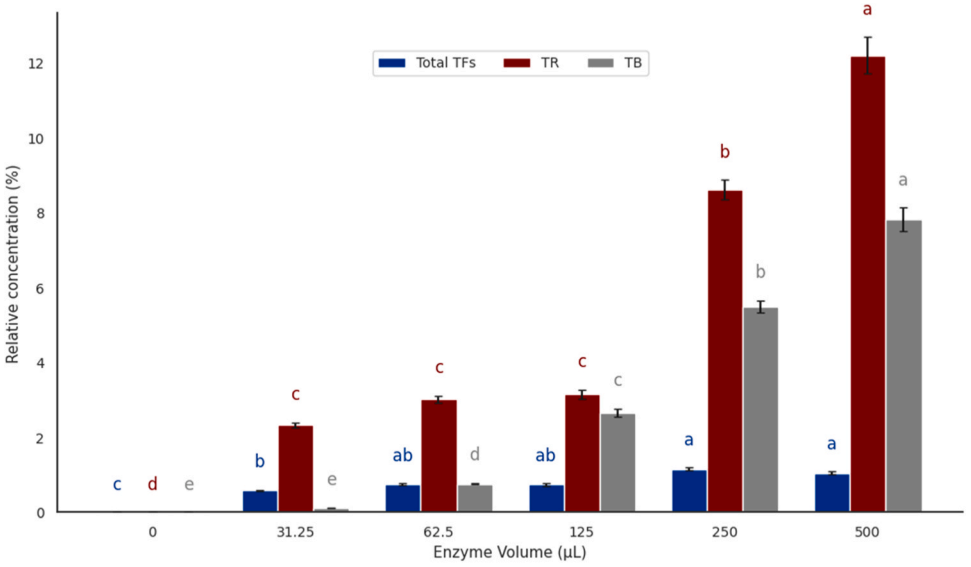

Fig. 4. Relative concentrations (%) of total theaflavins (TFs), thearubegins (TRs), and theabrownins (TBs) formed at different enzyme volumes. Bars represent mean ± standard deviation (n = 3). Different letters above the bars indicate statistically significant differences ($p < 0.05$) according to Tukey's HSD test.

indicating the formation of complex tea pigments. Fig. 4 demonstrates a significant increase in pigment accumulation, with total TFs rising from 1.11 % in the control to 4.16 % at the highest enzyme concentration. TR and TB levels also exhibited substantial increases. The TF yield (≤4.16 %) aligns with traditional black tea fermentation, which typically yields 1–6 % TFs, depending on cultivar and processing conditions (Aaqil et al., 2023; Sang et al., 2011). However, controlled micro-scale experiments do not replicate these yields in large-scale production, where lower TF percentages are common. To address this, the synergistic action of endogenous plant polyphenol oxidases (PPOs) and peroxidases, which drive oxidative coupling during leaf fermentation, could enhance yields. Recent studies suggest that co-incubating microbial enzymes with partially purified plant-derived oxidases can boost pigment formation and catechin turnover (Wang et al., 2024). Thus, combining *A. faecalis* enzymes could optimize TF production in future large-scale strategies.

While *A. faecalis* enzymes show promise for microbial-assisted tea oxidation, safety considerations are critical due to the bacterium's classification as an opportunistic pathogen. This Gram-negative, aerobic bacterium, commonly found in soil, water, and the human intestinal tract as a saprophyte, is rarely isolated from healthy individuals (Batt, 2014). However, it has been linked to rare nosocomial infections, particularly in immunocompromised patients or those with indwelling medical devices, with concerns heightened by the emergence of multidrug-resistant strains (Huang, 2020). To mitigate risks, this study used crude enzyme filtrates (acellular extracts) harvested at 36 h, eliminating viable cells and minimizing contamination risks. This approach aligns with biotechnological practices where *A. faecalis* cell-free extracts are safely used in processes like phenol degradation, biosurfactant production, and mycotoxin detoxification (Ray and Pattnaik, 2024). For large-scale tea flavor enhancement, prioritizing purified or immobilized enzyme preparations will ensure compliance with food safety standards while avoiding the use of live *A. faecalis* cells, thus maintaining consumer safety.

3.4. Potential mechanism of EGCG oxidation by crude enzyme of *A. faecalis*

Epigallocatechin gallate (EGCG) is a major catechin found in green tea, characterized by a complex polyphenolic structure comprising three interconnected rings. The A-ring, which is a dihydroxy-substituted benzene ring with hydroxyl groups at the 5 and 7 positions, contributes to the molecule's antioxidant properties. The C-ring is a heterocyclic pyran ring, linking the A- and B-rings. The B-ring, which is a trihydroxy-substituted benzene ring with hydroxyl groups at the 3′, 4′, and 5′ positions, enhances the molecule's redox reactivity. Additionally, EGCG possesses a galloyl group (derived from gallic acid) esterified to the C-ring at the 3-position, further amplifying its biological activities. The arrangement of these functional groups enables EGCG to undergo redox reactions, metal chelation, and intermolecular interactions crucial to its bioactivity. Fig. 5 shows the molecular structure of EGCG, highlighting its four-ring system organized around the central C-ring (Wei et al., 2016).

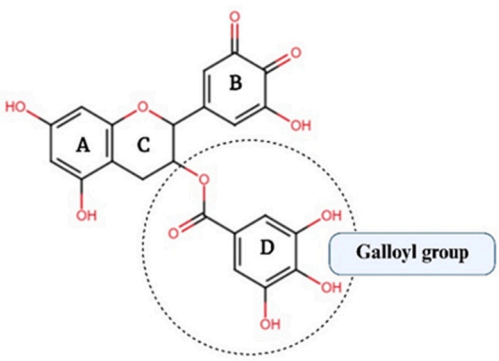

Fig. 5. Molecular structure of EGCG, made up of three rings A, B, and D joined by the C-pyran ring. Based on (Wei et al., 2016).

Crude enzyme filtrates from *A. faecalis* catalysed EGCG oxidation, producing gallic acid, as well as some catechins, including EGC, and theaflavins, suggesting its involvement in multiple enzymatic pathways (Fig. 5). Enzymatic treatment led to the generation of catechin derivatives and theaflavins, strongly suggesting the involvement of PPO and peroxidase-like activities (Teng et al., 2017). The reaction efficiency increased significantly at 60 °C, likely due to enhanced enzyme-substrate interaction and optimal catalytic kinetics. In contrast, control reactions with heat-inactivated filtrate showed negligible EGCG degradation, underscoring the critical dependence on active enzymatic machinery. These findings highlight the potential of microbial enzymes in driving controlled polyphenol bioconversions under mild, industry-relevant conditions.

Mechanistically, the EGCG oxidation likely proceeds through multiple pathways: ester hydrolysis, where the galloyl group is cleaved to release gallic acid; C-ring opening, which converts EGCG into epigallocatechin (EGC) and simpler catechins (Zhu et al., 2020); and oxidative coupling, involving ortho-quinone intermediates that lead to the formation of dimeric compounds such as theaflavins (Nuraini et al., 2025). These transformations align with previously described microbial or enzymatic polyphenol bioconversions and support the hypothesis that *A. faecalis* significantly contributes to catechin degradation and tea pigment biosynthesis. These key oxidative pathways, such as oxidation, ester hydrolysis, C-ring cleavage, and dimer formation, are illustrated in Fig. 6, which highlights the structural modifications resulting in gallic acid, EGC, and theaflavins. However, crude enzyme extracts inherently contain a mixture of catalytic activities, including non-specific hydrolases and oxidases, which may contribute to EGCG breakdown independently of the intended PPO or laccase pathways. This enzymatic promiscuity complicates mechanistic attribution and underscores the need for purification to isolate specific activities (Westphal and van Berkel, 2021; Zhuang et al., 2024). Without purification, it remains challenging to distinguish targeted biotransformation from background enzymatic noise, particularly in polyphenol-rich systems where EGCG is highly reactive.

Overall, the study demonstrates that black tea processing harbours a diverse bacterial community, with certain isolates, particularly *A. faecalis,* exhibiting promising enzymatic activity relevant to tea biochemistry. These findings pave the way for exploring microbial inoculation or targeted biotransformation strategies to modulate black tea quality, pigment formation, and catechin profiles under mild, industry-relevant conditions.

4. Conclusions

This study has demonstrated that black tea processing supports a diverse and enzymatically active bacterial community, with *Alcaligenes faecalis* emerging as a key species linked to elevated extracellular PPO activity. Of the 43 bacterial isolates examined, 27 exhibited PPO activity, with the highest levels observed in isolates from the later stages of processing, such as oxidised and fired leaves. The growth dynamics and enzymatic profile of *A. faecalis* revealed that laccase and catechol oxidase activities peaked during the late exponential phase (36 h), whereas peroxidase activity reached its maximum earlier, indicating distinct temporal patterns of enzymatic regulation. Crude enzyme filtrates harvested at the optimal growth phase effectively oxidised EGCG, leading to the formation of gallic acid, simpler catechin derivatives, and theaflavins. Mechanistic analysis suggested that enzymatic oxidation proceeds via ester hydrolysis, C-ring cleavage, and oxidative coupling reactions, mirroring key biochemical transformations characteristic of traditional black tea fermentation. These findings highlight the potential of *A. faecalis*-derived enzymes as biocatalysts for enhancing tea

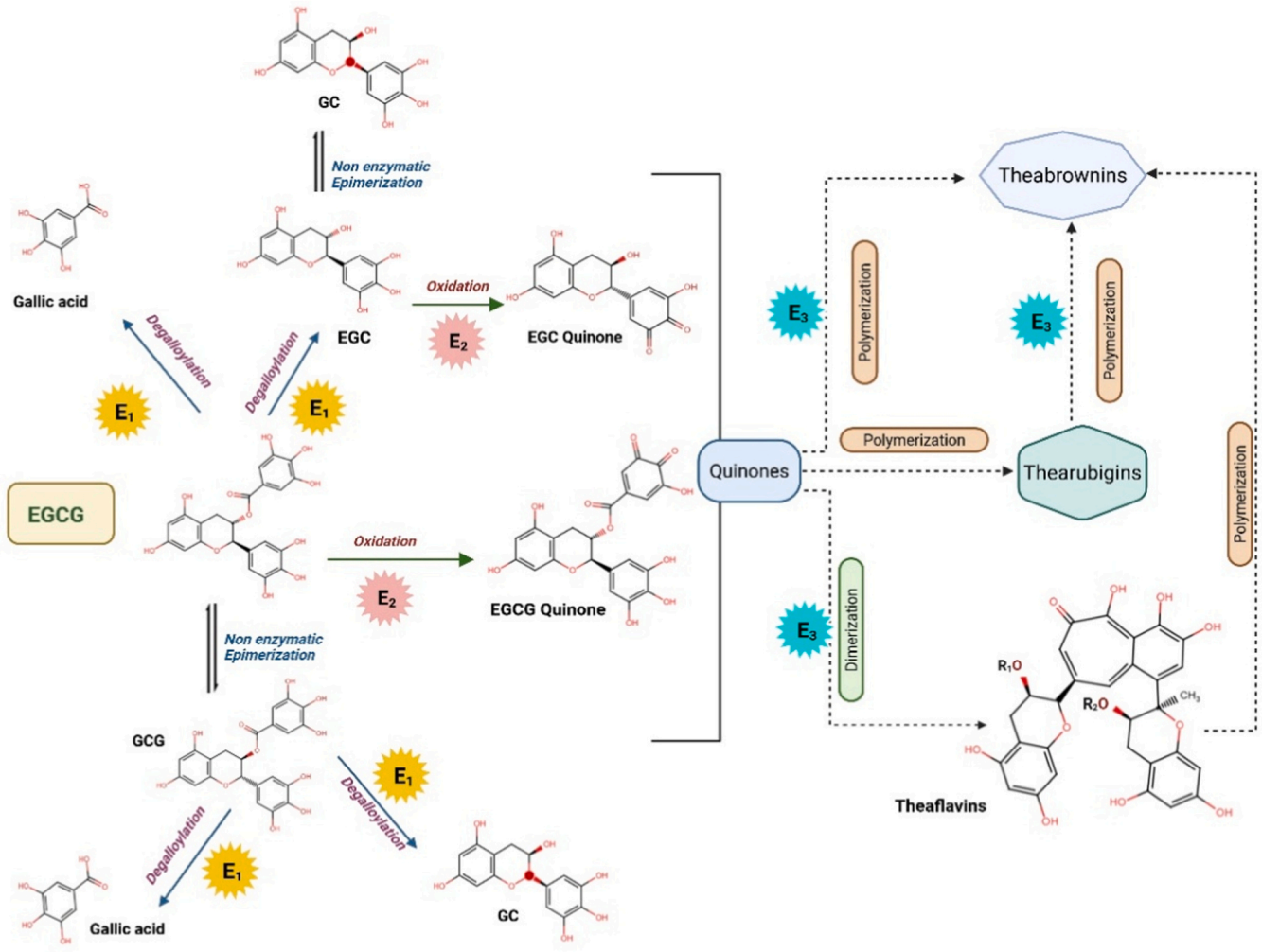

Fig. 6. Proposed pathway of EGCG biotransformation to different tea polyphenols (E1- Alpha glucosidase or Esterases; E2-Catechol oxidase/O_2 or Peroxidase/R_2O_2 or Laccase; E3-Peroxidase/R_2O_2 or Laccase). R_2O_2 is a peroxide, such as H_2O_2.

fermentation, offering a more controlled and consistent alternative to relying solely on endogenous leaf enzymes. Scaling up microbial biotransformation processes and validating their stability and efficacy in industrial settings will be essential to support the integration of biotechnology-driven innovations in tea processing, quality enhancement, and production efficiency.

CRediT authorship contribution statement

Prishanthini Muthulingam: Writing – original draft, Visualization, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ali Rashidinejad:** Writing – review & editing, Validation, Supervision, Investigation, Formal analysis, Conceptualization. **David Popovich:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **P.A. Nimal Punyasiri:** Writing – review & editing, Supervision, Conceptualization. **Chandrika M. Nanayakkara:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Carl H. Mesarich:** Writing – review & editing, Validation, Supervision.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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Data availability statement

The data presented in this study are available upon request from the corresponding author.

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