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Functional characterization and substrate interactions of spore coat protein A laccase from *Bacillus velezensis* ST15 for broad-spectrum mycotoxin transformation

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Abstract

Persistent mycotoxin contamination across agricultural commodities threatens both consumers and livestock. In this study, a spore coat protein A (CotA) laccase was identified from *Bacillus velezensis* ST15, heterologously expressed in *Escherichia coli*, purified, and functionally characterized for the transformation of zearalenone (ZEN), aflatoxin B₁ (AFB₁) and alternariol monomethyl ether (AME). Transformation was observed for all four mycotoxins. This study provides, to our knowledge, the first experimental evidence that purified recombinant CotA laccase (rCotA) can transform AME. rCotA-mediated transformation was characterized under varied pH, thermal, and metal-ion conditions and monitored over time. The highest transformation efficiencies were achieved at pH 8.0 and 60–70 °C, whereas the effects of metal ions were substrate dependent. The major transformation products were tentatively annotated by ultra-performance liquid chromatography–high-resolution tandem mass spectrometry (UPLC–HRMS/MS) as hydroxylated or quinone derivatives, including 15-hydroxyzearalenone (15-OH-ZEN), 13-hydroxyzearalenone quinone (13-OH-ZEN-quinone), aflatoxin Q₁ (AFQ₁) or epi-aflatoxin Q₁ (epi-AFQ₁), 10-hydroxyalternariol (10-OH-AOH), and 10-hydroxyalternariol monomethyl ether (10-OH-AME). Molecular docking indicated that all four substrates could be favorably accommodated within the CotA binding pocket through

hydrogen-bonding and hydrophobic interactions. Transformation activity was further observed in naturally contaminated herbal matrices. Overall, the results broaden the established substrate repertoire of CotA laccases and highlight rCotA as a versatile candidate for transforming diverse mycotoxins in food and feed matrices.

Keywords: CotA laccase; *Bacillus velezensis*; mycotoxin biotransformation; alternariol monomethyl ether; molecular docking;

Introduction

With the expansion of global food production and storage, mycotoxin contamination has become a major threat to food safety and public health(Croatian Veterinary Institute et al. 2019; Deliklitas and Gokbulut 2025). Mycotoxins are toxic fungal metabolites that can accumulate in agricultural commodities before harvest and persist through processing and storage, with *Aspergillus*, *Penicillium*, and *Fusarium* among the principal producers(Ahmad et al. 2024; Shekhar et al. 2025). The combined toxicity, heat resistance, and environmental persistence of mycotoxins impose a lasting burden on food systems and public health(K et al. 2023). Representative contaminants include aflatoxins, zearalenone (ZEN), fumonisins, and ochratoxin A (OTA), whereas *Alternaria*-derived toxins are gaining prominence as emerging food-safety hazards(Sun et al. 2025). The well-established association between AFB₁ exposure and hepatocellular carcinoma supports its placement in Group 1 of the IARC carcinogenic

hazard classification(Ara et al. 2024; Niu et al. 2025). ZEN exhibits strong
estrogenic activity and can disrupt endocrine systems, leading to
reproductive disorders and an increased risk of hormone-related
diseases(Liu et al. 2025; Ruan et al. 2026; Zhang et al. 2026). Alternaria-derived toxins,
particularly alternariol (AOH) and alternariol monomethyl ether (AME),
have attracted increasing food-safety concern because of their reported
cytotoxic, genotoxic, and mutagenic effects. Nevertheless, their
enzymatic transformation remains far less explored than that of AFB₁ and
ZEN(Adhishree and Mukesh 2024; Vinogradova et al. 2026). These challenges
underscore the need for safe and effective strategies capable of targeting a
broad spectrum of mycotoxins.

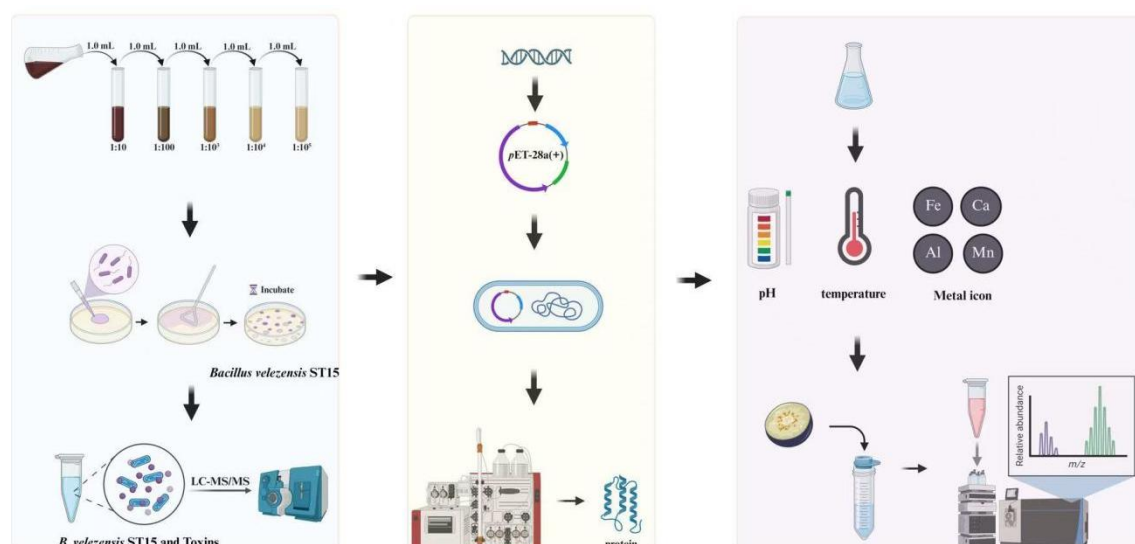
Conventional mitigation of mycotoxin contamination relies largely
on adsorption, irradiation, and treatment with chemical agents(Akhtar et al.
2025; Yao et al. 2026). However, these approaches usually involve high energy
consumption and may negatively affect food quality(Aasa et al. 2026; Ruiz 2025).
By contrast, biological approaches may provide greater selectivity and
environmental compatibility while better preserving food quality(Okoye et al.
2026; Stoev 2025; Sun et al. 2025; Tovar et al. 2025). Enzymatic transformation is
particularly promising because it can selectively modify mycotoxin
structures and potentially reduce their adverse biological effects(Jinpei et al.
2023; Zhong et al. 2025). Laccases are multicopper oxidases capable of acting
on chemically varied phenolic and aromatic substrates, and this catalytic

breadth supports their application to mycotoxin transformation(Jia et al. 2024). Laccase-mediated transformation has been reported for several major mycotoxins, including AFB₁, ZEN, T-2 toxin, fumonisin B₁ (FB₁), and ochratoxin A (OTA).(Mei et al. 2026; Xing et al. 2021a; Xu et al. 2025). Laccase can reduce the toxicity of these toxins by altering their chemical structures(Zaccaria et al. 2023). Laccase can render AFB₁ non-toxic by catalyzing the oxidation of key sites in the AFB₁ molecule(Bian et al. 2025; Cui et al. 2025). Furthermore, an 88% reduction in AFB₁ was reported after 24 h of treatment with Lac-W, an enzyme identified in *Weizmannia coagulans* strain 36D1(Wenbo et al. 2023). Another study found that supplementation with a redox mediator enabled Ery4 from *Pleurotus eryngii* to transform up to 90% of AFB₁(Loi et al. 2018). The oxidative capability of laccase enables ZEN to be transformed from a molecule with strong estrogenic activity into inactive degradation products(Wenbo et al. 2023). Current research indicates that laccases exhibit high efficacy in degrading AFB₁ and ZEN, however, studies on their activity toward AME are limited, representing a significant research gap(Loi et al. 2018; Priyanka et al. 2022).

Additionally, current laccase-based detoxification strategies still suffer from several critical limitations(Liu et al. 2024). First, their substrate spectrum is generally narrow, typically restricted to one or two mycotoxins, which limits their effectiveness in addressing co-contamination scenarios(Wang et al. 2024). Second, high degradation

efficiency often relies on the addition of redox mediators, leading to increased operational costs and potential safety concerns (Jia et al. 2024). Third, most reported laccases exhibit little or no activity toward emerging mycotoxins, particularly alternariol monomethyl ether (AME), highlighting a significant gap in current enzymatic detoxification strategies (Zhang et al. 2024).

To address these challenges, *B. velezensis* ST15 was identified as a mycotoxin-transforming strain, and genome mining guided the selection of CotA for recombinant production in *E. coli*. The resulting recombinant CotA laccase (rCotA) was evaluated for transformation activity toward ZEN, AFB₁, AOH, and AME. UPLC–HRMS/MS data were used for tentative product annotation, while putative enzyme–substrate interactions were modeled through molecular docking. The transformation activity of rCotA was further examined in naturally contaminated herbal matrices. Together, these analyses extend the recognized substrate repertoire of CotA laccases and support further investigation of CotA-based strategies for broad-spectrum mycotoxin management.



129

130 Materials and Methods

131 Reagents and culture media

132 The toxin panel comprised analytical standards of ZEN, AFB₁, FB₁,
 133 OTA, AOH, and AME (Pribolab, Shandong, China). Separate 1 mg/mL
 134 methanolic stocks were prepared for ZEN, AFB₁, OTA, AOH, and
 135 AME(Cui et al. 2025). Chromatographic analyses used HPLC-grade
 136 acetonitrile and methanol from Tianjin Siyou Fine Chemical Co., Ltd.
 137 (Tianjin, China). Other chemicals met analytical-grade specifications and
 138 were supplied by Guangzhou Chemical Reagent Factory (Guangzhou,
 139 China).

140 Three primer sets were custom synthesized by HuaDa
 141 Biotechnology Co., Ltd. (Guangzhou, China): primers for 16S ribosomal
 142 RNA (rRNA) gene analysis, *CotA* cloning, and plasmid verification.
 143 Commercial Luria–Bertani (LB) broth powder (Huankai, Guangzhou,
 144 China) was reconstituted in deionized water at 21 g/L. Materials from

Sangon Biotech (Shanghai, China) included competent *E. coli* DH5 α and BL21(DE3) cells. DNA polymerase and FastCut restriction endonucleases were from Takara Biomedical Technology (Beijing, China). The pET-28a(+) expression vector was provided by the MiaoLing Plasmid Platform (Wuhan, China).

Selection of bacterial isolates with ZEN-transforming activity

Red sour soup samples were collected in Kaili City, Guizhou Province, China (26.56° N, 107.98° E). Each 1-mL portion of each sample was combined with 9 mL of sterile saline, from which serial dilutions (10^{-2} – 10^{-5}) were prepared. Aliquots of the diluted suspensions were surface-inoculated onto MYP agar, and the plates were maintained at 37 °C for 24–48 h. Morphologically distinct colonies without an egg-yolk reaction halo were selected and purified by repeated streaking(Eva et al. 2022). Each purified isolate was incubated in broth supplemented with ZEN (1 μ g/mL) to determine its transformation capacity. Post-incubation ZEN levels were determined using the analytical protocol presented in Section 2.3(Tao et al. 2021). Isolates associated with a marked reduction in ZEN concentration were subjected to secondary screening under the same conditions. The isolate with the highest apparent transformation activity was selected. Genomic DNA was extracted, and the 16S rRNA gene was amplified using 27-F

(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492-R
(5'-ACGGCTACCTTGTTACGACT-3'). The amplicon was sequenced
and compared with National Center for Biotechnology Information
(NCBI) sequences by BLASTn. The isolate was assigned to *B. velezensis*
and designated ST15. The strain was cryopreserved at -80 °C. *Bacillus*
velezensis ST15 was deposited at the Guangdong Microbial Culture
Collection Center (GDMCC; 5th Floor, Building 59, No. 100 Xianlie
Middle Road, Yuexiu District, Guangzhou 510070, China) on 4 August
2026 under accession number GDMCC 68769.

$$\text{ZEN residual rate(\%)} = \frac{\text{Final ZEN concentration}}{\text{Initial ZEN concentration}} \times 100\%$$

Determination of toxin degradation by the ST15 strain

Quantification by UPLC-MS/MS

ZEN, AFB₁, AOH, and AME were determined by targeted UPLC-
MS/MS. An LC-20 chromatographic system (Shimadzu, Kyoto, Japan)
was interfaced with a 5500+ triple-quadrupole mass spectrometer (AB
SCIEX, Framingham, MA, USA). The instrument was equipped with an
electrospray ionization source and operated in multiple-reaction
monitoring (MRM) mode. Supplementary Table S1 summarizes the
declustering potential and collision energy settings applied to each
analyte (Prabhu et al. 2023).

For extract preparation, 100 µL of each fermentation sample was

combined with 900 μ L of acetonitrile acidified with 0.1% (v/v) formic acid. Following vortex mixing, particulate material was removed by centrifugation at $12,000 \times g$ for 10 min. The clarified extract was filtered through a 0.22- μ m nylon membrane and placed in an autosampler vial.

Analytes were resolved on an HSS T3 column (100 mm $\times$ 2.1 mm, 1.8 μ m; MicroPulite, China) thermostated at 40 $^{\circ}$ C. The aqueous eluent (A) contained 0.1% (v/v) formic acid, while acetonitrile was used as eluent B. The proportion of B was programmed as follows: 10% at 0–1 min, 10–90% at 1–5 min, 90% at 5–8 min, and 90–5% at 8–10 min. The flow rate and injection volume were 0.3 mL/min and 2 μ L, respectively.

UPLC-HRMS analysis

Transformation products of ZEN, AFB₁, AOH, and AME were characterized using an Orbitrap Exploris 120 high-resolution mass spectrometer equipped with an electrospray ionization source (Thermo Fisher Scientific, USA). The chromatographic program specified in Section 2.3.1 was applied without modification. Full-scan spectra were collected across m/z 70–1000 under both positive- and negative-ionization conditions.

The ion source was operated at sheath and auxiliary gas settings of 50 and 10 arbitrary units, respectively, and a capillary temperature of 320 $^{\circ}$ C. Electrospray potentials were +3.5 kV in positive-ion mode and –2.5 kV in negative-ion mode. Resolving powers of 30,000 and 15,000

were used for full-scan and tandem mass spectrometric acquisition, respectively. Stepped normalized collision energies were set to 30, 50, and 70.

Raw spectral data were processed using Xcalibur version 4.1 (Thermo Fisher Scientific, USA). Putative product structures were inferred from accurate-mass data, isotopic signatures, and diagnostic MS/MS fragment ions. Product structures were tentatively annotated from accurate mass, isotope patterns, and fragment ions; authentic standards were not available for confirmation.

Characterization of *B. velezensis* ST15

Determination of toxin degradation by the ST15 strain

Frozen stocks of ST15 were revived during an 18-h incubation at 37 °C in LB broth (5 mL), with agitation maintained at 180 rpm. Separate 1-mL aliquots of LB medium were supplemented with ZEN, AFB₁, FB₁, OTA, AOH, or AME to a final concentration of 1 µg/mL. Each preparation was then inoculated with the activated ST15 culture at 1% (v/v). Incubation was continued for 24 h at 37 °C with shaking at 180 rpm. Residual concentrations of the parent mycotoxins were determined using the UPLC–MS/MS procedure detailed in Section 2.3.1.

Whole-genome analysis and mining of candidate enzymes associated with ZEN transformation

Illumina paired-end reads were subjected to quality control and visualization using FastQC v0.12.1 to obtain clean reads(Science. et al. 2020). Genome assembly of the Illumina clean data was performed using SPAdes v3.12.0 with default parameters to generate assembled FASTA files(Anton et al. 2012). The assembly quality was assessed using QUAST v5.2.0, and visualized reports were exported in HTML format. Genome annotation was conducted using Prokka v1.14.6 (Seemann, 2014)(Ana et al. 2022). The MLST v2.23.0 tool was used to predict bacterial pubMLST typing profiles(A et al. 2017). Finally, the assembled genome was compared against the NCBI NT database using BLASTn to identify the most closely related bacterial species and infer functional characteristics. Candidate ZEN-degrading enzymes were identified through a systematic bioinformatic workflow using a curated ZEN-Degradase Database (ZDD v1.0), which was compiled from ZEN-degrading enzyme sequences collected globally from NCBI. Whole-genome sequences of ST15 were queried against the ZDD reference sequences via local BLASTp analysis.

Recombinant production and purification of CotA laccase

Genomic DNA from *B. velezensis* ST15 served as the template for amplification of the *cotA* coding region with PrimeSTAR Max DNA Polymerase (Takara, Japan). The primers were *cotA*-pET28a-F (5'-tggtggtgctcgagtgcggccgcTTACTGCTTATCCGTGACGTCCA-3') and

cotA-pET28a(+)-R

(5'-atgggtcgcggatccgaattcATGGCACTAGAAAAATTTGCAGATG-3').

The pET-28a(+) vector was linearized with *EcoR* I and *Not* I (Takara, Japan). Both the PCR product and digested vector were recovered using a DNA Gel Recovery Kit (Magen, Guangzhou, China). The purified fragments were assembled with the One Step Cloning Kit (Vazyme, Nanjing, China) to generate the pET-28a(+)-cotA expression construct. *E. coli* BL21(DE3) cells were subsequently transformed with the resulting plasmid.

Recombinant cells were grown in kanamycin-selective LB broth (50 µg/mL). At an OD₆₀₀ of 0.6, cultures received 0.1 mmol/L IPTG to induce rCotA expression, together with 1 mmol/L CuSO₄. Post-induction cultivation was conducted at 16 °C and 120 rpm for approximately 20 h. Cell biomass was recovered by centrifugation, suspended in phosphate-buffered saline, and disrupted ultrasonically. After centrifugation of the lysate (12,000 × g, 30 min, 4 °C), the supernatant containing soluble proteins was retained.

Recombinant CotA laccase (rCotA) was recovered from the soluble fraction using Ni-NTA affinity chromatography (Yeaden, Shanghai, China). Imidazole was removed by exchanging the eluate into 25 mmol/L Tris-HCl containing 150 mmol/L NaCl (pH 8.0) with a 3-kDa ultrafiltration device. Protein purity and apparent molecular mass were

evaluated by reducing SDS–PAGE with 10% resolving and 5% stacking gels.

Determination of mycotoxin degradation by rCotA protein

Detection and Identification of toxin Degradation Products by rCotA

Each 500-μL assay contained 25 mmol/L Tris–HCl (pH 8.0) and 150 mmol/L NaCl. One mycotoxin substrate and purified rCotA were added to final concentrations of 2 and 40 μg/mL, corresponding to 1 and 20 μg per assay, respectively. The reactions were allowed to proceed at 37 °C over a 24-h period. Aliquots obtained at 0 and 24 h were subjected to UPLC–MS/MS to determine the remaining parent mycotoxins. The 24-h aliquots were additionally examined by UPLC–HRMS/MS for tentative annotation of transformation products.

Analysis of Enzymatic Properties of rCotA

Enzymatic properties, including optimal reaction temperature, optimal reaction pH, the influence of metal ions, and enzyme kinetics, were investigated in this study. The influence of pH on rCotA-mediated mycotoxin transformation was examined at selected values between 3.0 and 12.0, established by adjustment with HCl or NaOH. Temperature dependence was evaluated independently at selected points between 20 and 100 °C. Separate reaction mixtures were prepared with Ca²⁺, Cu²⁺,

Mg²⁺, Fe³⁺, Zn²⁺, K⁺, Li⁺, Mn²⁺, Co²⁺, Al³⁺, Ni²⁺, or EDTA. Each additive was tested individually at a final concentration of 2 mmol/L to determine its influence on rCotA-mediated mycotoxin transformation. For kinetic analysis, the enzymatic degradation reactions were carried out under defined conditions. The residual toxin concentrations were subsequently quantified, and degradation curves were constructed based on toxin removal over time. After the reaction, the toxin was extracted in the same way as above.

Molecular docking simulation of rCotA laccase and mycotoxins

In this study, molecular docking analyses between the selected ligands and targets were performed using the CB-Dock2 platform(Yang et al. 2022). CB-Dock2 is based on the CurPocket algorithm, which automatically identifies potential binding pockets within the protein structure and performs docking calculations using the AutoDock Vina scoring engine(Oleg and J 2010), detailed procedures have been described previously(Luo et al. 2024). Protein structures in PDB format were uploaded to the platform, which automatically identified and listed the five highest-scoring binding pockets. Ligand structures in SDF format were also uploaded, simplifying the docking process while improving accuracy. CB-Dock2 performed multiple docking simulations for each pocket and outputted the corresponding binding energies (kcal/mol) along with the

predicted three-dimensional conformations.

The highest-ranked binding pose, corresponding to the most favorable predicted energy, was retained for subsequent interaction analysis. Protein–ligand interactions were visualized and analyzed using PLIP (Protein-Ligand Interaction Profiler)(Schake et al. 2025) and PyMOL v2.6(A and L 2011) (Schrödinger, New York, NY, USA), generating three-dimensional interaction maps.

Evaluation of rCotA in naturally contaminated herbal matrices

The herbal material degradation system was established using 0.1 g of powdered mulberry fruit (*Morus alba*) or ziziphus seed (*Ziziphus jujuba* var. *spinosa*) in a total volume of 500 μ L ddH₂O, supplemented with 30 μ g of rCotA laccase. Reactions were performed at 70 °C, and samples were collected after 1 h to evaluate toxin degradation. After each incubation period, mycotoxins were extracted as described above.

Statistical analysis

Unless noted otherwise, values are reported as mean $\pm$ SD for three replicates ($n = 3$). Analyses were conducted in GraphPad Prism 10.0. Normality and homoscedasticity were examined using the Shapiro–Wilk and Levene tests, respectively. Data meeting both assumptions were analyzed by one-way ANOVA with Tukey’s multiple-comparison test. Statistical significance was set at $p < 0.05$, with * and ** denoting $p <$

0.05 and $p < 0.01$, respectively.

Results

Screening and characterization of *B. velezensis* ST15

A bacterial isolate designated ST15 was recovered from red sour soup and was assigned to *B. velezensis*. Colonies were white, flat, irregularly edged, and wrinkled (Fig. 1A). Gram-positive rods were observed by Gram staining (Fig. 1B). Under the tested culture conditions, OD₆₀₀ increased during the first 20 h and subsequently approached a plateau (Fig. 1C).

[Insert Fig. 1 near here]

[Insert Fig. 2 near here]

When a 1% (v/v) inoculum was incubated with individual mycotoxins at 1 µg/mL for 24 h, decreases of 93.65%, 75.39%, 35.48%, and 24.23% were measured for ZEN, AOH, AME, and OTA, respectively (Fig. 2). In contrast, neither AFB₁ nor FB₁ showed a statistically significant reduction.

Transformation of multiple mycotoxins by CotA laccase from *B. velezensis* ST15

The laccase family plays a significant role in the degradation of mycotoxins, research has discovered that the Lac-W from *Weizmannia*

coagulans 36D1 exhibits significant degradation effects on AFB₁, achieving a degradation rate of 88% within 24 hours(Wenbo et al. 2023). In the presence of a redox mediator, Ery4, a laccase from *Pleurotus eryngii*, was reported to achieve up to 90% removal of AFB₁(Loi et al. 2018). As multicopper oxidases, laccases can catalyze the oxidation of diverse phenolic substrates. The broad mycotoxin-transforming capacity of strain ST15 may therefore be partly attributable to CotA activity, although the involvement of other enzymes cannot be excluded.

A candidate *cotA* gene was identified in the ST15 genome by sequence comparison with reported ZEN-transforming enzymes. A fragment of approximately 1.5 kb was amplified and cloned into pET-28a(+) (Fig. 3A). Following production in *E. coli* BL21(DE3), rCotA was isolated by Ni–NTA affinity chromatography. On SDS-PAGE, purified rCotA migrated as a prominent band at approximately 70 kDa (Fig. 3B). After 20 µg of rCotA had been incubated with 1 µg of each toxin at 37 °C for 24 h, decreases of 98.58%, 90.32%, 96.56%, and 86.60% were measured for ZEN, AFB₁, AOH, and AME, respectively (Figs. 3C and 4). No significant transformation of FB₁ or tentoxin (TEN) was detected. The difference between the cellular and purified-enzyme results for AFB₁ indicates that culture-medium components or cellular metabolites may have influenced the reaction; this possibility was not directly tested(Fan et al. 2023).

[Insert Fig. 3 near here]

[Insert Fig. 4 near here]

Optimization of rCotA laccase reaction conditions

Transformation of ZEN, AFB₁, AOH, and AME by rCotA was observed in the absence of a redox mediator. Key parameters affecting rCotA-mediated mycotoxin transformation were evaluated. The highest transformation activity toward all four mycotoxins was observed at pH 8.0 (Fig. 5).

The degradation of four mycotoxins (ZEN, AFB₁, AOH, and AME) was strongly influenced by pH (Fig. 5). Under acidic conditions (pH 3–5), degradation was limited, particularly for AFB₁, AOH, and AME, while ZEN showed moderate degradation. Rates increased sharply near neutral pH, reaching high efficiencies at pH 7–8, with all toxins peaking at pH 8. Beyond this, degradation declined under strongly alkaline conditions (pH 9–12). These results indicate that the enzyme exhibits optimal activity under weakly alkaline conditions, highlighting pH 8 as the most favorable for simultaneous degradation of all four toxins.

[Insert Fig. 5 near here]

401 The temperature dependence of rCotA-mediated mycotoxin
402 transformation was subsequently characterized. Temperature significantly
403 influenced the degradation of ZEN, AFB₁, AOH, and AME (Fig. 6).
404 Transformation efficiency rose sharply over 20–60 °C, and all four parent
405 mycotoxins showed near-complete depletion within the 60–70 °C range.

406 Beyond this temperature range, the degradation efficiency gradually
407 decreased, although relatively high degradation rates were still observed
408 at 80–100 °C. Overall, the enzyme exhibited high catalytic activity within
409 a wide temperature range, with optimal degradation occurring at
410 approximately 60–70 °C.

411
412 *[Insert Fig. 6 near here]*
413

414 The results show that the degradation activity of rCotA toward four
415 mycotoxins (ZEN, AFB₁, AME, and AOH) was significantly influenced
416 by metal ions(Fig. 7). High concentrations of Cu²⁺ markedly inhibited
417 rCotA, reducing degradation of all substrates compared to the control.
418 EDTA also suppressed ZEN and AFB₁ degradation but enhanced AME
419 degradation. Ca²⁺ significantly inhibited AME degradation, while Fe³⁺
420 and Zn²⁺ slightly reduced degradation of some substrates (e.g., ZEN,
421 AFB₁). In contrast, K⁺, Li⁺, Mn²⁺, and Co²⁺ increased ZEN and AOH
422 degradation, indicating a promoting effect. These results demonstrate that

rCotA activity is metal-dependent and substrate-selective, suggesting that manipulation of specific ions can modulate its degradation efficiency for different mycotoxins. Fig 7 A–D correspond to ZEN, AFB₁, AME, and AOH, respectively.

[Insert Fig. 7 near here]

The kinetic analysis results (Fig.8) demonstrated that rCotA exhibited pronounced degradation activity toward ZEN AFB₁, AME, and AOH. For ZEN, AFB₁, and AME, degradation was initiated rapidly at the early stage of the reaction, with a substantial reduction observed within a short period. As the reaction progressed, the degradation efficiency further increased and gradually reached a plateau. In contrast, AOH showed a relatively slower degradation rate during the initial phase; however, its degradation extent increased steadily over time and eventually achieved a relatively high removal level. These results indicate that rCotA is capable of acting on multiple mycotoxins with diverse structural features, suggesting a certain degree of substrate adaptability.

[Insert Fig. 8 near here]

Identification of mycotoxin degradation products

Products formed during rCotA-mediated mycotoxin transformation were characterized by UPLC–HRMS/MS. Accurate-mass and MS/MS fragmentation data supported the tentative annotation of two ZEN-derived products as 15-hydroxycarbazephenone (15-OH-ZEN) and 13-hydroxycarbazephenone quinone (13-OH-ZEN-quinone) (Fig. 9A–C). These assignments were consistent with those reported by Xing et al (Xing et al. 2021b). An AFB₁-derived product was tentatively annotated as AFQ₁/epi-AFQ₁ (Fig. 9D–E), consistent with earlier reports by Xing et al. and Guo et al (Guo et al. 2020; Xing et al. 2021). 10-OH-AOH was tentatively annotated as the predominant AOH oxidation product (Fig. 9F–G), as previously observed by Sun et al (Fan et al. 2023). In addition, AME was oxidized to 10-OH-AME (Fig. 9 H-I) was observed.

Notably, these oxidation products are generally reported to exhibit reduced toxicity compared to their parent compounds. In particular, AFQ₁ has been widely recognized as a less toxic metabolite of AFB₁ (Xing et al. 2021b), while hydroxylated derivatives such as 15-OH-ZEN, 10-OH-AOH, and 10-OH-AME are considered to possess diminished estrogenic or cytotoxic effects due to structural modifications of key functional groups (Xing et al. 2021b). These findings indicate that rCotA converts parent mycotoxins into structurally modified derivatives, whose toxicological profiles require further evaluation. However, product toxicity was not measured in the present study. The toxicological profiles of the tentatively

annotated products were inferred from published data and require validation in targeted in vitro and in vivo assays.

[Insert Fig. 9 near here]

Docking-derived models of CotA–mycotoxin interactions

Docking calculations performed with CB-Dock2 were used to explore the predicted accommodation of ZEN, AFB₁, AOH, and AME within the CotA binding pocket. Under an identical docking protocol, increasingly negative scores were interpreted as indicating more favorable predicted interactions. These computational scores were used only for relative comparison and not as experimental evidence of binding affinity or catalytic transformation.

As shown in Fig. 10, CotA exhibited good binding capacities toward ZEN, AFB₁, AOH, and AME, with docking scores of -8.3 , -7.9 , -7.4 , and -7.8 kcal/mol. These results indicate that all four mycotoxins can form relatively stable binding conformations within the active pocket of CotA. Among them, ZEN showed the strongest binding affinity, followed by AFB₁ and AME, while AOH displayed relatively weaker, yet still acceptable, binding

Detailed interaction analysis revealed distinct binding modes for each ligand. ZEN formed hydrogen bonds with Ser427, Gln145, and

Arg146, along with hydrophobic interactions involving Val426 and Trp463. AFB₁ interacted with Ser427, Thr480, Trp463, Arg146, Ala482, and Lys364, forming hydrophobic interactions, hydrogen bonds, and a salt bridge. AME showed a similar interaction pattern but involved more hydrogen bonds and fewer hydrophobic contacts, primarily interacting with Gln145, Ser427, Thr480, Trp463, Arg146, and Lys364. In contrast, AOH formed the highest number of hydrogen bonds with key residues such as Ser427, Thr480, Trp463, Arg146, and Lys364, accompanied by relatively limited hydrophobic interactions. These predictions identify residues for future mutational validation but do not establish a catalytic mechanism.

[Insert Fig. 10 near here]

Application of rCotA in naturally contaminated herbal matrices

Given the frequent co-occurrence of mycotoxins in cereals, rCotA-mediated transformation was further evaluated under mixed-substrate conditions. The results showed that rCotA exhibited effective degradation activity in the multi-mycotoxin degradation system (Fig 11). Within 1 h, the degradation of AME in the *Hovenia dulcis* samples was particularly pronounced. In contrast, the toxin levels

naturally present in the mulberry samples were relatively low; therefore, the degradation effect was less obvious. These results support further evaluation of rCotA as a biocatalyst for transforming structurally diverse mycotoxins in complex agricultural matrices.

[Insert Fig. 11 near here]

Conclusion

Genome mining of mycotoxin-transforming *B. velezensis* ST15 revealed a *cotA* gene, and the encoded laccase was subsequently produced in *E. coli* and functionally characterized. To the best of our knowledge, the transformation of AME by purified rCotA was experimentally demonstrated for the first time in this study, thereby expanding the known substrate range of CotA laccases toward *Alternaria* mycotoxins. rCotA transformed ZEN, AFB₁, AOH, and AME without an added redox mediator, demonstrating activity toward structurally diverse mycotoxins. In addition, CotA showed favorable thermal and alkaline stability, with optimal activity at pH 8.0 and 60–70 °C, consistent with practical processing conditions. The degradation products were tentatively identified as hydroxylated derivatives with reduced toxicity, suggesting its potential biosafety. Furthermore, CotA maintained effective detoxification performance in naturally contaminated cereal and herbal

matrices, demonstrating its application potential. Overall, this study highlights CotA as a promising broad-spectrum biocatalyst for the simultaneous detoxification of multiple mycotoxins and provides new insights into enzymatic strategies for controlling emerging *Alternaria* toxins. Industrial translation of rCotA will require catalytic optimization through enzyme engineering, scalable production, and rigorous safety assessment of its transformation products.

Discussion

Mycotoxins are secondary metabolites produced by various fungi in crops and food products, and their high toxicity, thermal stability, and environmental persistence have made them a long-standing concern in global food safety (Cheng et al. 2026; Torrijos et al. 2026). In practical production, multiple mycotoxins often coexist within the same commodity, which not only increases toxicological risks but also limits the effectiveness of single-target detoxification strategies (Réant et al. 2026). Biocatalytic systems capable of transforming several co-occurring mycotoxins within a single treatment are therefore of considerable practical value.

Strain ST15 was recovered from Hong Suan Tang, a traditional fermented food, and assigned to *B. velezensis* based on 16S rRNA gene sequence analysis. In vitro experiments demonstrated that ST15 effectively degraded ZEN, AOH, and AME, with particularly high efficiency against ZEN. However, although the ST15 strain exhibits

certain mycotoxin-degrading activity, its performance is constrained by metabolic byproducts and culture conditions, requiring relatively strict operational parameters and exhibiting slow degradation rates(Giuseppe et al. 2023; Greco et al. 2025). Moreover, as a living microorganism, ST15 cannot be directly applied in many food matrices. These constraints hinder the broader use of strain ST15 in food and feed systems.

Previous studies have reported that several laccases, including Lac2 from *Pleurotus pulmonarius*(Yanyi et al. 2021), Ery4 from *Pleurotus eryngii*(Loi et al. 2018), and BsCotA from *Bacillus subtilis*(Wang et al. 2019), are unable to directly degrade mycotoxins. In contrast, in the present study CotA completely degraded each toxin (ZEN, AFB₁, AME, and AOH) at an initial concentration of 1 µg/mL within 6 h at 70 °C and pH 8.0. Therefore, CotA exhibits superior toxin degradation efficiency compared with these fungal laccases. This superiority may be attributed to its unique molecular structure and catalytic mechanism. CotA is a thermostable and alkaliphilic laccase whose type 1 (T1) copper center copper center possesses a relatively high redox potential, facilitating electron transfer from the ZEN molecule to the catalytic center of the enzyme(Paulo et al. 2008). In addition, the hydrophobicity and spatial configuration of the CotA substrate-binding pocket may be more favorable for accommodating structurally complex phenolic compounds such as AFB₁ and ZEN(Zhou et al. 2025). In contrast, fungal laccases typically exhibit

optimal activity under mild conditions (25–40 °C, pH 4–6), and their conformational stability decreases under high-temperature and alkaline conditions, resulting in reduced catalytic efficiency(Wang et al. 2019).

Metal ion experiments further revealed that Mn^{2+} significantly enhanced the activity of CotA, whereas Cu^{2+} , Zn^{2+} , and Co^{2+} partially inhibited the enzyme. These results suggest that metal ions may regulate catalytic activity by influencing the electron transfer process at the enzyme's active site(Yongpeng et al. 2022). This observation is closely related to the catalytic mechanism of CotA. Mn^{2+} may act as an auxiliary factor in electron transfer, promoting electron transfer between the substrate and the T1 copper center, whereas the inhibitory effects of Cu^{2+} , Zn^{2+} , and Co^{2+} may result from competitive binding with the intrinsic copper ions in the active site or from interference with the protein conformation(Zhou et al. 2025). The response pattern of fungal laccases to metal ions differs markedly from that of CotA. For example, the laccase Ery4 from *P. eryngii* shows strict dependence on Cu^{2+} , whereas Mn^{2+} has little effect on its activity [37], further highlighting the fundamental differences in catalytic regulation between these two types of laccases(Wei et al. 2025).

The docking results suggest that CotA possesses a broad substrate-binding capacity toward structurally diverse mycotoxins, with binding affinity largely governed by the balance between hydrophobic interactions, hydrogen bonding, and electrostatic forces. The stable

binding conformations observed for ZEN, AFB₁, AOH, and AME indicate that these substrates can be effectively accommodated within the CotA active pocket, forming well-defined interaction networks with key residues. Such interactions not only ensure proper substrate positioning but also provide a structural basis for efficient electron transfer during the catalytic process.

Notably, the efficient degradation of multiple mycotoxins by CotA is reflected not only in its high reaction rate but also in the markedly reduced toxicity of the resulting products. Some other microbial enzymes may produce intermediate compounds with relatively higher toxicity during mycotoxin degradation. For instance, in certain fungal laccase systems, the oxidation of ZEN can generate quinone-type intermediates, which may retain estrogenic activity or exhibit cytotoxicity(Okoye et al. 2026). Moreover, studies have reported that microbial degradation of AFB₁ can produce highly reactive intermediates such as AFB₁-8,9-epoxide, which are considered key species responsible for the mutagenic and carcinogenic effects of AFB₁(León and Aguayo 2025). By comparison, the main products of CotA-catalyzed ZEN degradation are 15-OH-ZEN and 13-OH-ZEN-quinone, whose estrogenic activity is reduced by more than 100-fold relative to the parent toxin(Jelle et al. 2011; Xing et al. 2021). In the AFB₁ degradation system, a newly detected product was tentatively identified as AFQ₁ or its epimer epi-AFQ₁, exhibiting significantly reduced

620 toxicity(Liu et al. 2025; Subagia et al. 2024). For *Alternaria* toxins, the primary
621 degradation products were 10-hydroxy-alternariol (10-OH-AOH) for
622 AOH and 10-hydroxy-alternariol monomethyl ether (10-OH-AME) for
623 AME(den Hollander et al. 2025; Henriqueta et al. 2023; Huikang et al. 2023). These
624 products are typically generated through hydroxylation or quinone
625 formation reactions and generally show substantially lower toxicity and
626 biological activity compared with their parent toxins. Therefore, CotA not
627 only demonstrates high catalytic efficiency but also markedly reduces the
628 toxicity of degradation products, highlighting its potential for mycotoxin
629 detoxification in food applications. Docking simulations predicted
630 favorable accommodation of the four mycotoxins within the CotA
631 binding pocket through hydrogen-bonding and hydrophobic contacts with
632 surrounding residues, potentially supporting catalytic oxidation.

633 Interestingly, while ST15 showed limited degradation of AFB₁ at the
634 cellular level, purified CotA effectively catalyzed its breakdown. This
635 discrepancy may result from inhibitory effects of cellular metabolites or
636 components in the growth medium, which could interfere with the
637 interaction between the toxin and the enzyme active site(Gao et al. 2024; Wu et
638 al. 2024). Similar phenomena have been reported in other microbial
639 degradation systems, highlighting the importance of further investigating
640 the influence of cellular environments on CotA activity to fully
641 understand the overall degradation mechanism(Sun et al. 2023; Yan et al. 2023).

Finally, in cereal powder matrices, CotA effectively degraded multiple mycotoxins present in mulberry and Ziziphus jujuba samples, maintaining high degradation efficiency even under conditions of multi-toxin coexistence. These findings indicate that CotA exhibits robust catalytic performance in complex food matrices, supporting its feasibility for practical applications in medicinal herbs, grains, and feed products.

Statements and Declarations

CRedit authorship contribution statement

Xin Wang: Writing – original draft, Validation, Software, Investigation, Data curation. Xinyu Zhao: Supervision Methodology, Investigation, Funding acquisition. Qingping Wu: Supervision Methodology, Investigation, Funding acquisition. Jinqi Xiao: Writing – review and editing, Validation. Yongcheng Lu: Writing – review and editing, Validation. Meiqing Deng: Investigation. Weipeng Guo: Supervision, Funding acquisition. Xianhu Wei: Supervision, Funding acquisition. Youxiong Zhang: Supervision, Funding acquisition. Qihui Gu: Supervision, Funding acquisition. Yuwei Wu: Supervision, Funding acquisition. Ling Chen: Writing – review & editing, Supervision, Funding acquisition, Formal analysis.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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873 **Figure legends**

874 **Fig. 1** Morphological and growth characteristics of *Bacillus velezensis* ST15. a Colony morphology. b
875 Gram-stained cells. c Growth profile

876 **Fig. 2** Changes in individual mycotoxin concentrations after incubation with *Bacillus velezensis* ST15 at
877 37 °C for 24 h. Values are presented as mean $\pm$ SD (n = 3)

878 **Fig. 3** Recombinant production and transformation activity of spore coat protein A laccase. a
879 Amplification of the approximately 1.5-kb *cotA* coding sequence from *Bacillus velezensis* ST15. b SDS–
880 PAGE analysis of purified His-tagged recombinant CotA laccase (rCotA). c Parent-mycotoxin depletion
881 after 24 h of incubation with rCotA

882 **Fig. 4** UPLC–MS/MS chromatograms for zearalenone (a), aflatoxin B₁ (b), alternariol (c), and alternariol
883 monomethyl ether (d) before and after treatment with rCotA

884 **Fig. 5** Effect of reaction pH on rCotA-mediated transformation of the four mycotoxins. Values are
885 presented as mean $\pm$ SD (n = 3)

886 **Fig. 6** Effect of reaction temperature on rCotA-mediated transformation of the four mycotoxins. Values
887 are presented as mean $\pm$ SD (n = 3)

888 **Fig. 7** Influence of metal ions and EDTA on rCotA-mediated transformation of zearalenone (a), aflatoxin
889 B₁ (b), alternariol monomethyl ether (c), and alternariol (d). Values are presented as mean $\pm$ SD (n = 3)

890 **Fig. 8** Time-course profiles of rCotA-mediated transformation of zearalenone (a), aflatoxin B₁ (b),
891 alternariol monomethyl ether (c), and alternariol (d). Values are presented as mean $\pm$ SD (n = 3)

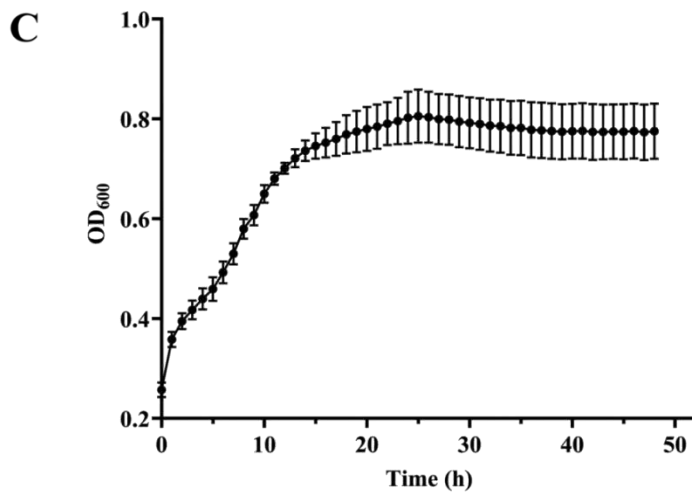
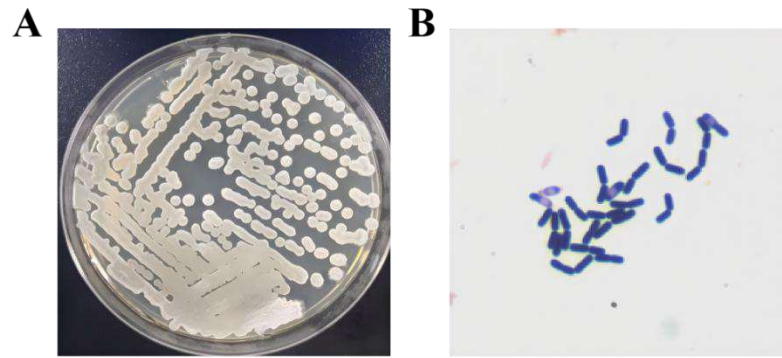
892 **Fig. 9** UPLC–HRMS/MS analysis of products formed during rCotA-mediated mycotoxin transformation.
893 Spectra are shown for zearalenone, 15-hydroxyzearalenone, and 13-hydroxyzearalenone quinone (a–c);

894 aflatoxin B₁ and aflatoxin Q₁/epi-aflatoxin Q₁ (d, e); alternariol and 10-hydroxyalternariol (f, g); and
895 alternariol monomethyl ether and 10-hydroxyalternariol monomethyl ether (h, i). Product assignments
896 are tentative because authentic standards were unavailable

897 **Fig. 10** Docking-derived poses of zearalenone (a), aflatoxin B₁ (b), alternariol (c), and alternariol
898 monomethyl ether (d) in CotA. Blue solid, grey dashed, and yellow dashed lines indicate hydrogen bonds,
899 hydrophobic contacts, and salt bridges, respectively

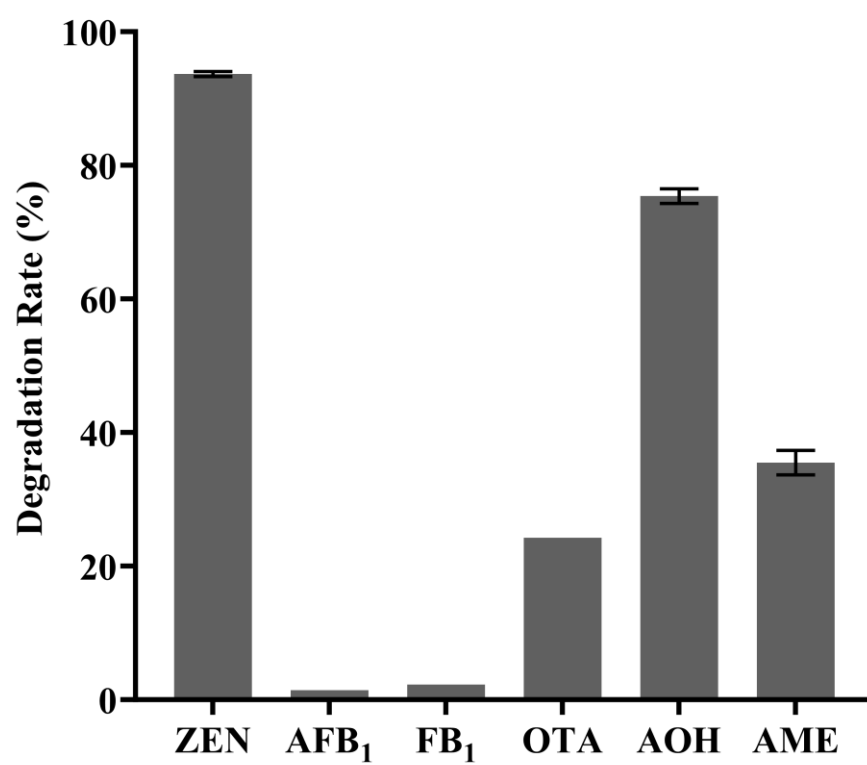
900 **Fig. 11** Changes in naturally occurring mycotoxin concentrations after rCotA treatment of three
901 plant-derived samples. Values are presented as mean $\pm$ SD (n = 3)

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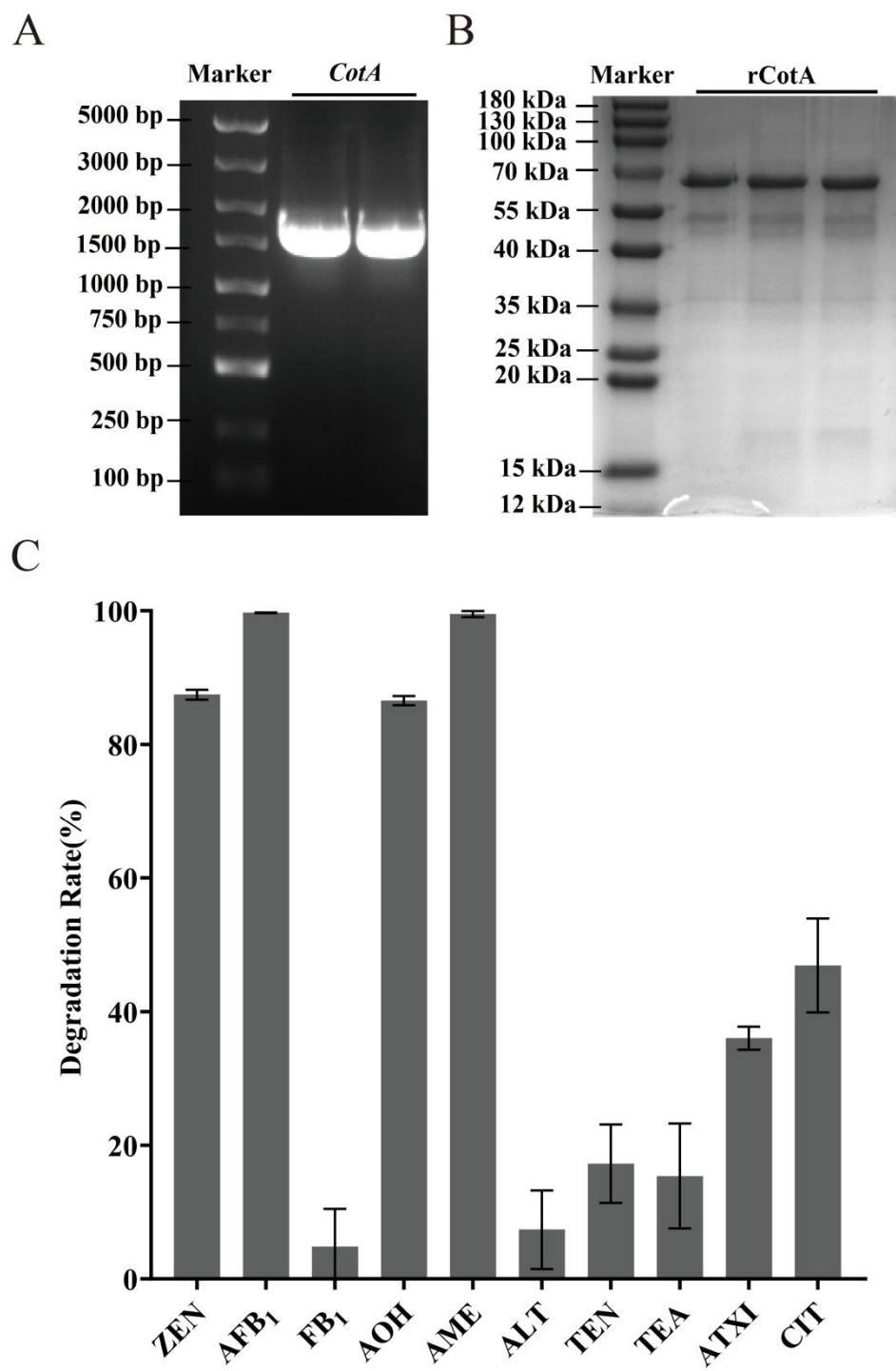
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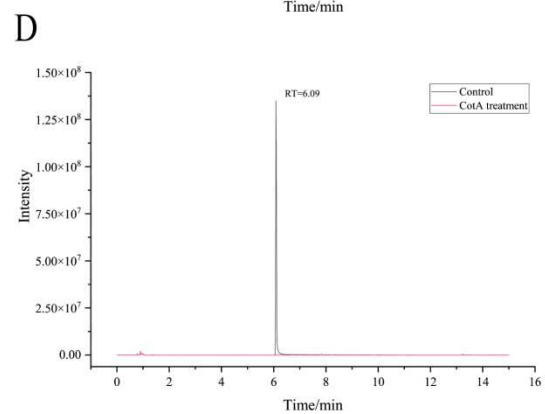
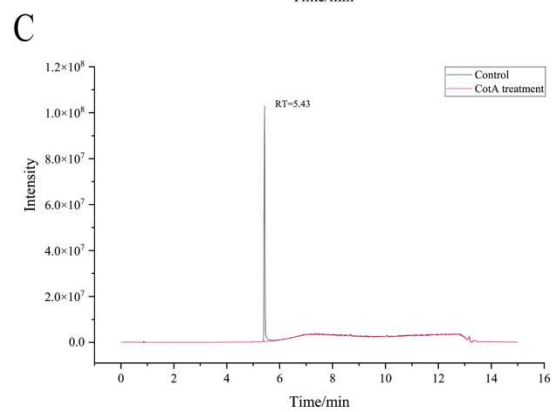
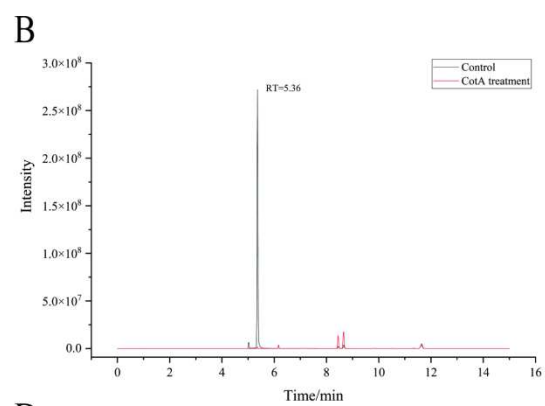
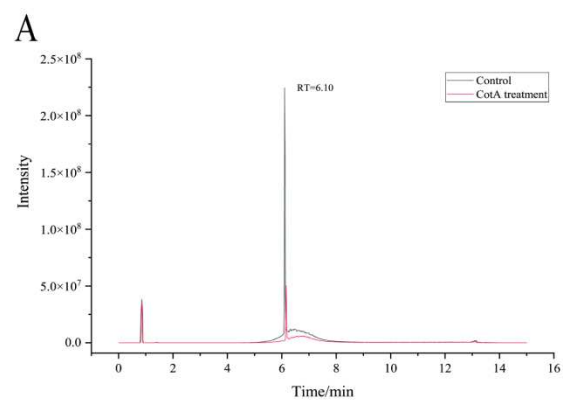
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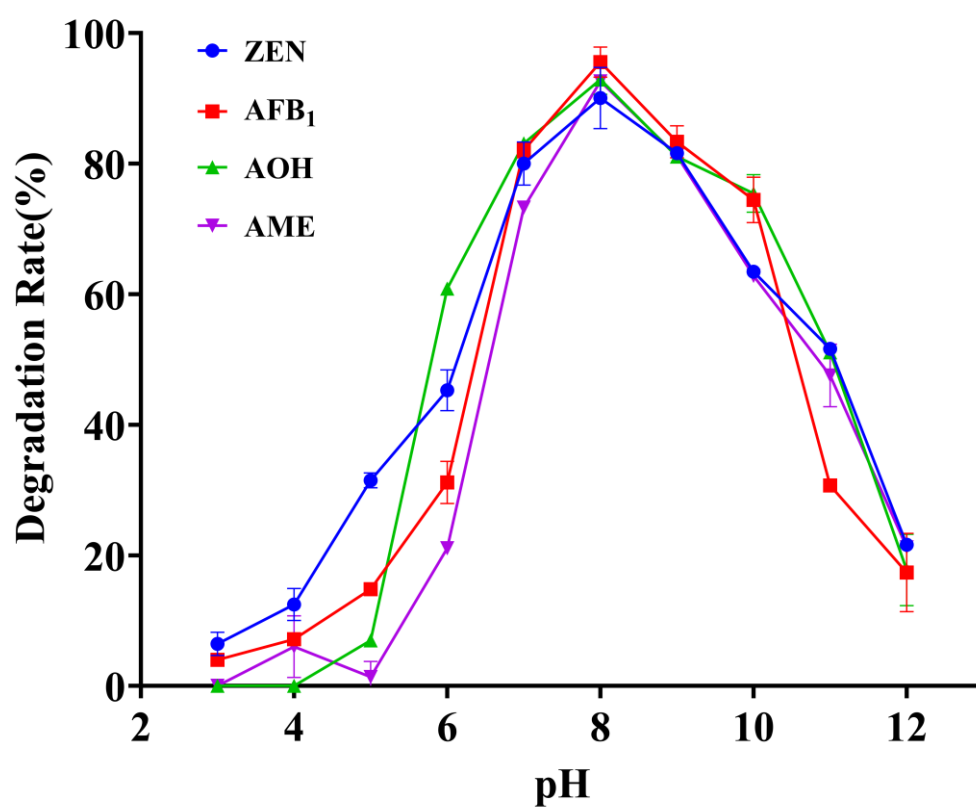
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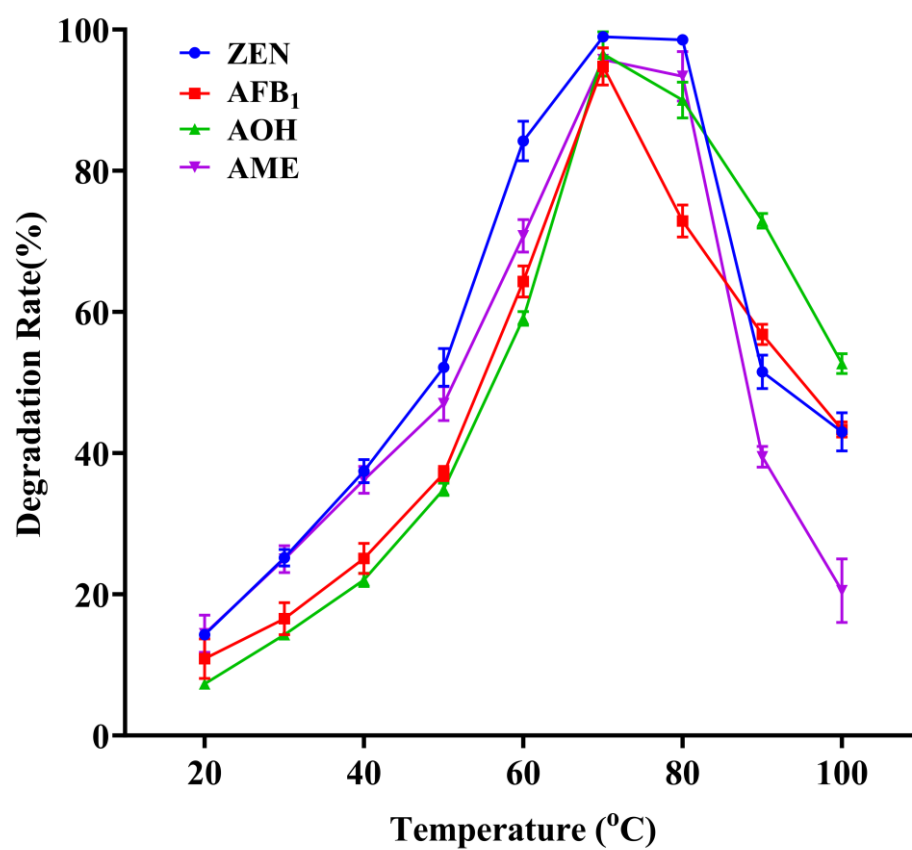
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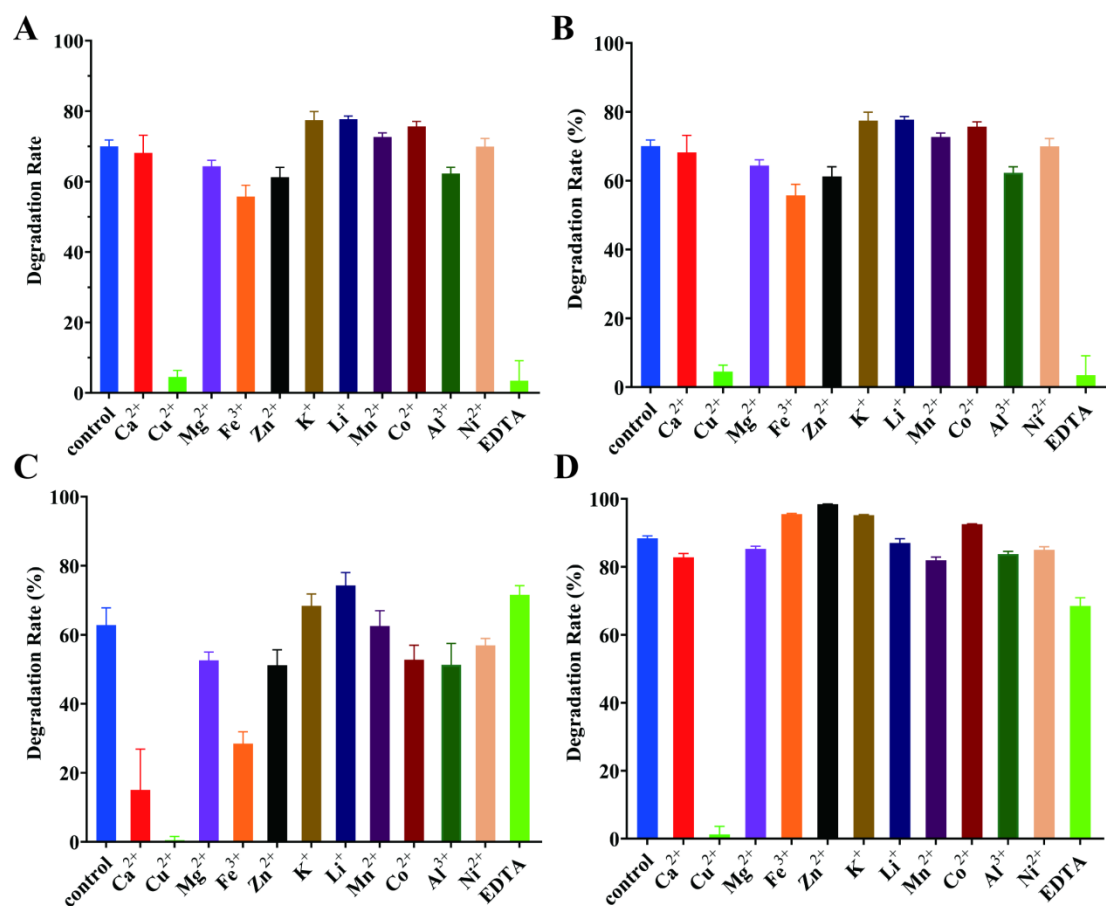
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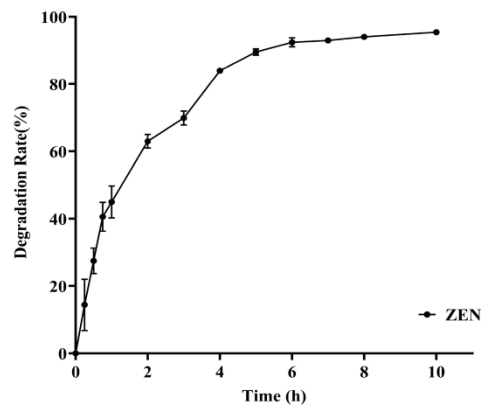
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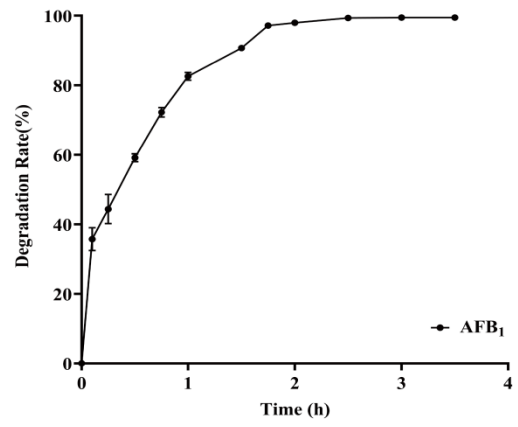
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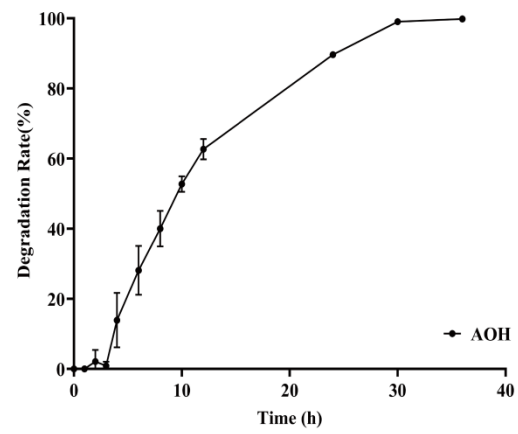
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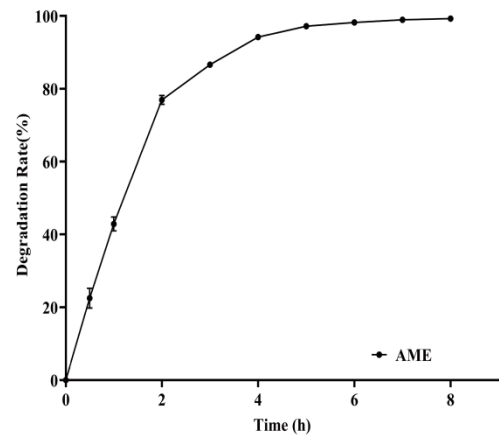
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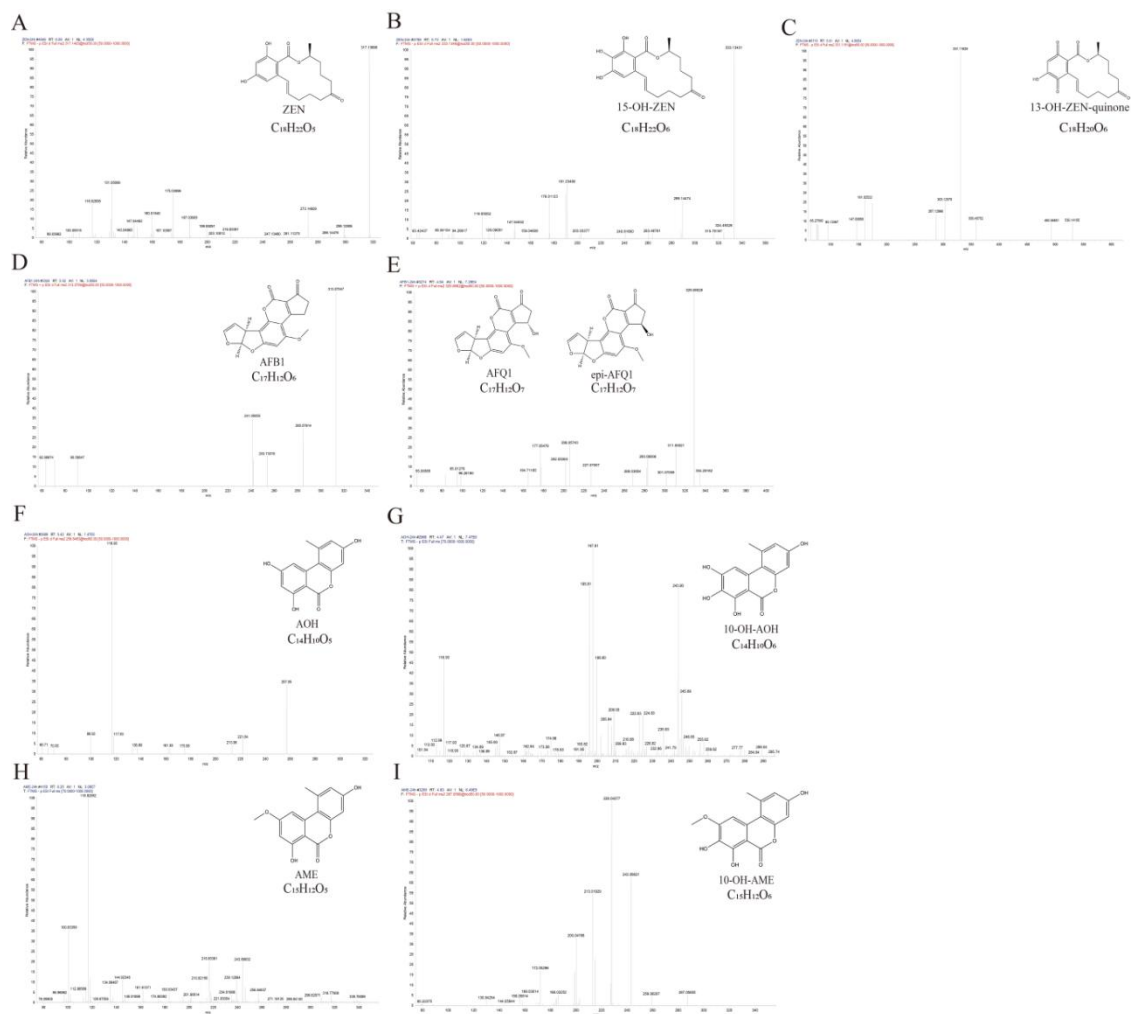


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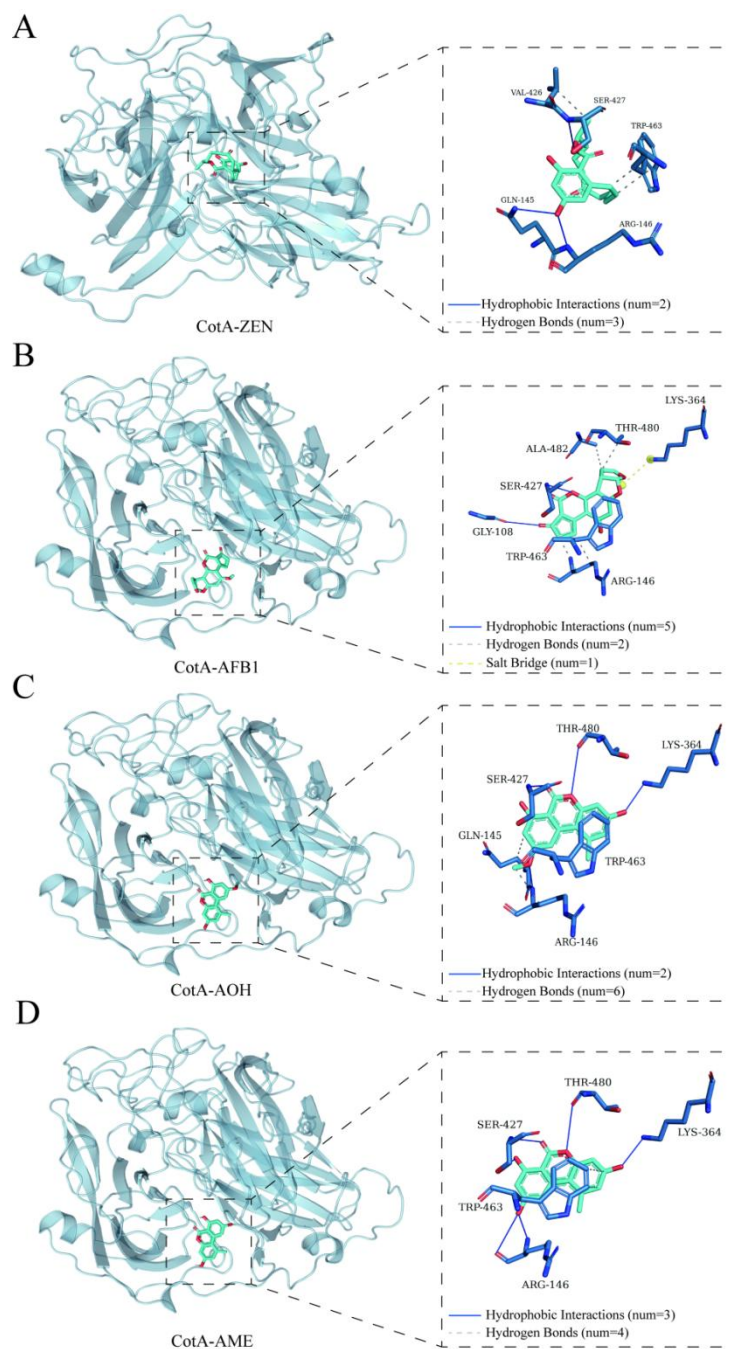
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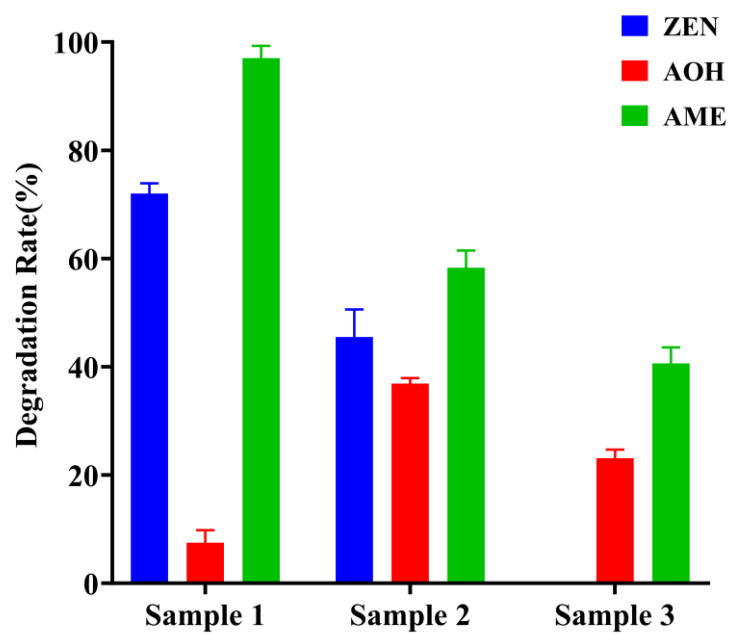
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