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Optimization of *Aspergillus Niger* fermentation for enzyme production and enzymatic hydrolysis of wheat bran for total ferulic acid via RSM

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Phenolic acids in wheat bran, particularly ferulic acid (FA), exhibit antimicrobial properties, but most FA is bound to cell wall polysaccharides via ester/ether linkages, severely limiting its bioavailability and extraction. Distinct from prior studies relying on commercial enzymes or single-component substrates, this study enhanced FA release through *Aspergillus niger* (*A. niger*) fermentation (no exogenous enzymes), utilizing a wheat bran-vinegar residue (9:1, w/w) substrate. Fermentation and destarched wheat bran (DSWB) enzymatic hydrolysis were optimized via Box-Behnken design and response surface methodology (BBD-RSM), yielding high enzyme activities: feruloyl esterase (16.63 ± 0.32 U/g), xylanase (3098.21 ± 47.27 U/g), and cellulase (21.12 ± 0.14 U/g). Hydrolysis released 5.41 ± 0.03 mg/g total FA (TFA) (including derivatives), which exerted significant antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, with MIC/MBC of 0.6/2.4 mg/mL (*E. coli*) and 0.3/1.2 mg/mL (*S. aureus*). This study offers valuable data for wheat bran-derived antimicrobials and enzyme-treated bran as feed antibiotic alternatives.

Keywords Ferulic acid, A. niger, Fermentation, Enzymatic hydrolysis, Wheat bran

As the international community is paying increasing attention to antimicrobial resistance (AMR) and drug residues arising from the widespread use of antibiotics, many countries and regional organizations—including the European Union (EU), the United States (U.S.), and China—have issued regulations to restrict or ban the use of antibiotics in livestock and poultry farming. Therefore, exploring safe, novel, and efficient alternatives to in-feed antibiotics in the "post-antibiotic era" has gradually become a research hotspot.

FA is a phenolic compound widely present in plants, which has garnered significant attention due to its potent antioxidant and anti-inflammatory properties, along with diverse applications in the fields of health and food[1–4]. Currently, research on FA has advanced to the levels of exploring its biological functions, innovation in delivery systems, optimization of production technologies, and specific applications in biomedicine and the food industry. The biological activity of FA constitutes the core of relevant research. Recent in vivo and clinical evidence highlights its preventive and therapeutic potential in the management of metabolic syndrome (MetS), which exerts such effects by improving glucose and lipid metabolism[5]. Furthermore, FA also possesses a range of additional properties, including antibacterial[6,7], anti-inflammatory[8,9], antioxidant[10], and other biological activities[11–14]. Owing to its non-residue in the body, non-pollution of the environment, and low toxicity or almost no toxicity, it is an ideal alternative to antibiotics[15].

FA can be extracted via diverse methods, which can be mainly categorized into traditional extraction methods and green extraction methods. Traditional extraction methods include liquid-liquid extraction (LLE)[16,17], solid-phase extraction (SPE)[18], etc. Their advantages lie in simple operation, but they suffer from drawbacks such as long extraction time, high energy consumption, large solvent usage, and relatively low extraction efficiency. In contrast, green extraction methods encompass ultrasound-assisted extraction (UAE)[19], microwave-assisted extraction (MAE)[20,21], enzyme-assisted extraction (EAE)[19,22], and supercritical fluid extraction (SFE)[23], etc.

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Their strengths include higher efficiency, greater environmental friendliness, and the ability to enhance the yield of bioactive compounds.

The extractable FA content in cereals reaches 0.5–3% (w/w), with the majority being in the trans isomer form and esterified with specific polysaccharides[24]. However, esterification hinders the access of depolymerizing enzymes to the cell wall, limiting the initial enzymatic degradation of the cell wall[25]. Therefore, to release this bound FA, specific enzymes are required to hydrolyze these ester linkages and polymer backbones[26,27]. Among these enzymes, feruloyl esterase (FAE) is the key one, as it can specifically hydrolyze the ester bonds between FA and polysaccharides[24,26]. Furthermore, glycoside hydrolases such as xylanase and cellulase act synergistically to degrade the cell wall structure, thereby enhancing the release efficiency of FA[27,28]. Studies have shown that, when G11 xylanase XynA and novel feruloyl esterase BpFae were used separately for hydrolyzing DSWB, the ferulic acid released was undetectable and 1.78%, respectively, whereas it was increased to 59.26% using the mixture of the two enzymes[29]. Enzymatic hydrolysis extraction, as a green and efficient method, has garnered increasing attention for releasing FA from agricultural by-products[30].

Microbial fermentation for enzyme production refers to a process where microorganisms grow under controlled conditions and produce enzymes[31]. Owing to its mild reaction conditions and high safety, this technology exhibits great application potential in multiple fields, including the food industry[32], textiles[33], etc. Meanwhile, microorganisms, as sources of enzymes, possess advantages such as short growth cycles, easy genetic manipulation, high enzyme production efficiency, and the ability to utilize cheap substrates[34,35]. By optimizing environmental and nutritional conditions, the fermentation process can significantly enhance enzyme yield and activity[36]. In light of these advantages, this study focused on optimizing the enzyme production process of *A. niger* fermentation through single-factor tests and the Box-Behnken experimental design. The aim is to address the issue of low enzymatic activity without the addition of commercial enzymes. Subsequently, further optimization of the enzymatic hydrolysis process for TFA release from bran was conducted via single-factor tests, Plackett-Burman regression analysis, Box-Behnken experiments, and response surface analysis. This approach aims to maximize the release of bound FA from bran and increase the added value of low-cost bran products.

Results

Correlation analysis between FAE, xylanase, and cellulase activities and TFA release

The amount of TFA released is positively correlated with the enzyme activities of FAE, xylanase, and cellulase, with correlation coefficients (R^2) of 0.7948, 0.7157, and 0.6643, respectively (Fig. 1). Therefore, the enzyme activities of FAE, xylanase, and cellulase are used as the first, second, and third indicators, respectively, for optimizing *A. niger* fermentation of wheat bran for enzyme production.

Screening of fermentation substrates and enzyme-producing strains

When the fermentation substrate was a mixture of vinegar residue and wheat bran at a ratio of 1:9, the FAE activities produced by *A. niger*, *N. sitophila*, and *T. reesei* all reached their highest levels (Fig. 2a, b and c). Notably, the FAE activity of *A. niger* was significantly higher than that of *N. sitophila*, and *T. reesei* (Fig. 2d).

For xylanase, *A. niger* fermentation yielded the highest enzyme activity, showing no significant difference from that of *A. niger* fermented with pure wheat bran but significantly higher than that of *N. sitophila*, and *T. reesei* (Fig. 2e, f, g and h).

Regarding cellulase, all three strains (*A. niger*, *N. sitophila*, *T. reesei*) produced higher enzyme activities when fermented with the 1:9 mixed substrate compared to pure wheat bran (Fig. 2i, j and k). Among them, the cellulase activity of *T. reesei* was significantly higher than that of *A. niger* and *N. sitophila* (Fig. 2l).

Since FAE and xylanase activities were much more strongly correlated with TFA release than cellulase activity, the 1:9 vinegar residue-wheat bran mixture (hereafter referred to as the mixed substrate) was selected as the fermentation substrate, and *A. niger* was chosen as the enzyme-producing strain.

Single-factor effects on enzyme production

The activities of FAE, xylanase, and cellulase produced by *A. niger* during wheat bran fermentation increased initially and then decreased with prolonged fermentation time. All three enzymes reached their maximum

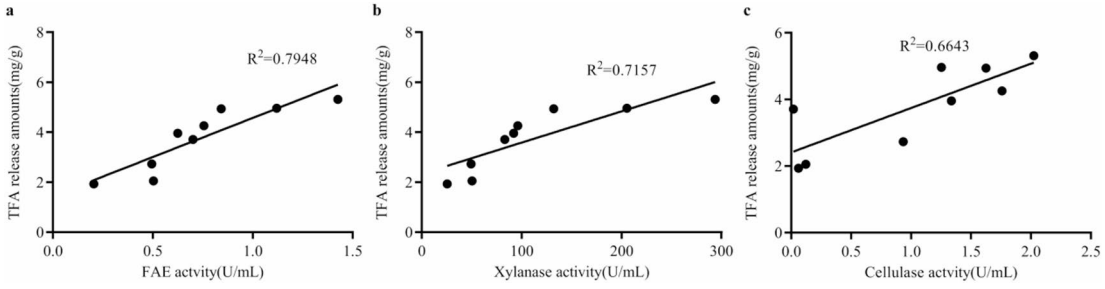

Fig. 1. Correlation analysis between enzyme activities and TFA release. (**a**) Correlation between FAE activity and TFA release; (**b**) Correlation between xylanase activity and TFA release; (**c**) Correlation between cellulase activity and TFA release.

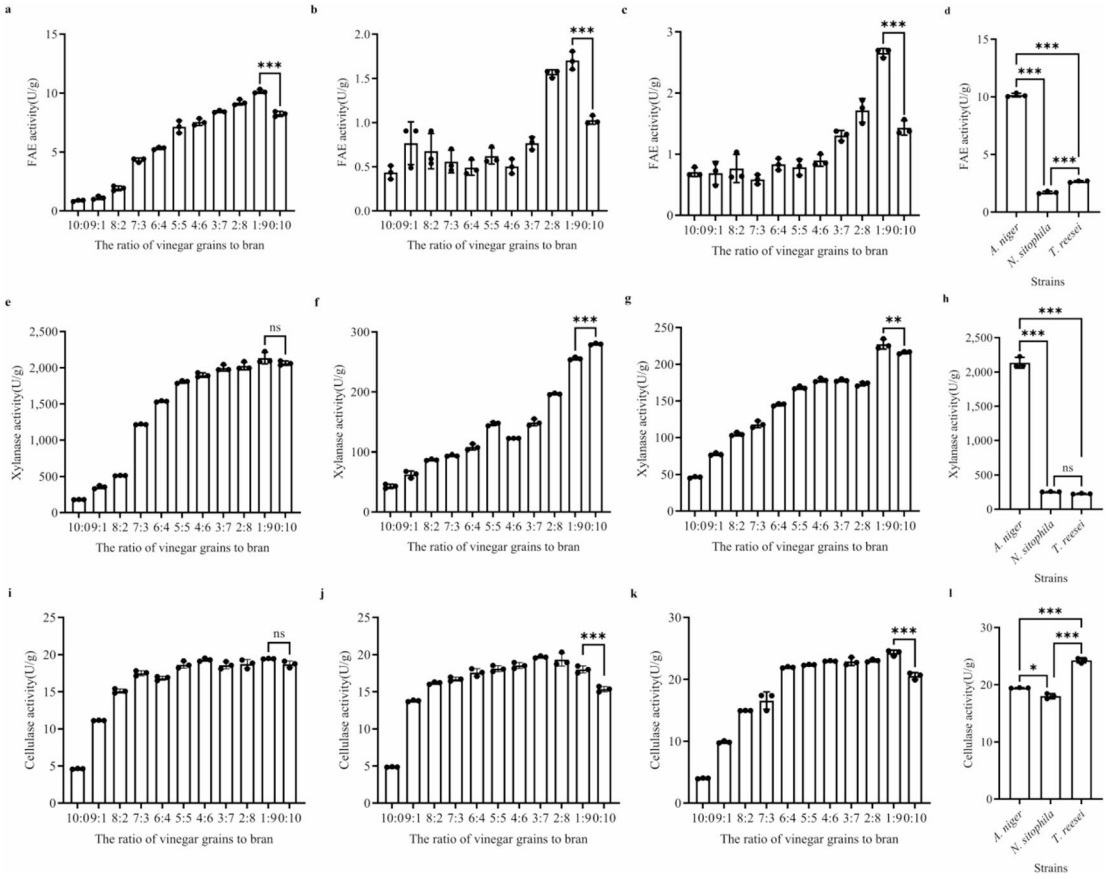

Fig. 2. Effects of substrates and strains on enzyme activities. (**a**–**c**) FAE activities of *A. niger*, *N. sitophila*, and *T. reesei* following fermentation with different substrates, respectively; d: Comparison of FAE activities among *A. niger*, *N. sitophila*, and *T. reesei* following fermentation at a vinegar residue: wheat bran ratio of 1:9; (**e**–**g**) Xylanase activities of *A. niger*, *N. sitophila*, and *T. reesei* following fermentation with different substrates, respectively; (**h**) Comparison of xylanase activities among *A. niger*, *N. sitophila*, and *T. reesei* following fermentation at a vinegar residue: wheat bran ratio of 1:9; (**i**–**k**) Cellulase activities of *A. niger*, *N. sitophila*, and *T. reesei* following fermentation with different substrates, respectively; (**l**) Comparison of cellulase activities among *A. niger*, *N. sitophila*, and *T. reesei* following fermentation at a vinegar residue: wheat bran ratio of 1:9. Data are presented as mean ± SEM from three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns, not significant.

activities on the 3rd day of fermentation (Fig. 3a). With respect to inoculum size, FAE and xylanase activities peaked at a 10% inoculum, while cellulase activity also remained relatively high under this condition (Fig. 3b).

Regarding moisture content: FAE activities were 9.978 U/g, 10.008 U/g, and 10.137 U/g at 55%, 60%, and 65%, respectively, with no significant differences among groups; xylanase activities were 2620.488 U/g, 2654.180 U/g, and 2520.145 U/g under the same moisture conditions, also showing no significant differences; cellulase activities at 60% and 65% moisture (19.812 U/g and 21.158 U/g, respectively) were similarly non-significant (Fig. 3c). Based on these results, moisture content was excluded as a variable in subsequent Box-Behnken design and response surface methodology experiments.

For nitrogen sources: When NH_4Cl, peptone, or yeast extract was used as the nitrogen source, FAE activities (11.380 U/g, 11.418 U/g, 12.019 U/g) and xylanase activities (2476.200 U/g, 2601.445 U/g, 2530.400 U/g) showed no significant differences. However, cellulase activity with yeast extract was significantly lower than that with NH_4Cl. Furthermore, all three enzyme activities in the yeast extract group were significantly higher than those in the control group (Fig. 3d).

With respect to yeast extract concentration: FAE activity peaked at 1.28%, but xylanase activity at this concentration was significantly lower than that at 1.60%, 1.92%, and 2.24%; cellulase activity showed no significant differences across all tested concentrations (Fig. 3e).

For carbon sources: Xylose yielded the highest FAE activity. Xylanase activity showed no significant differences between sucrose and xylose groups, and neither differed from the control. Cellulase activity was significantly lower than that in the glucose group but comparable to the control (Fig. 3f). Regarding xylose concentration: FAE and xylanase activities showed no significant differences between 0.96% and 1.28%, while cellulase activity peaked at 1.28% (Fig. 3g).

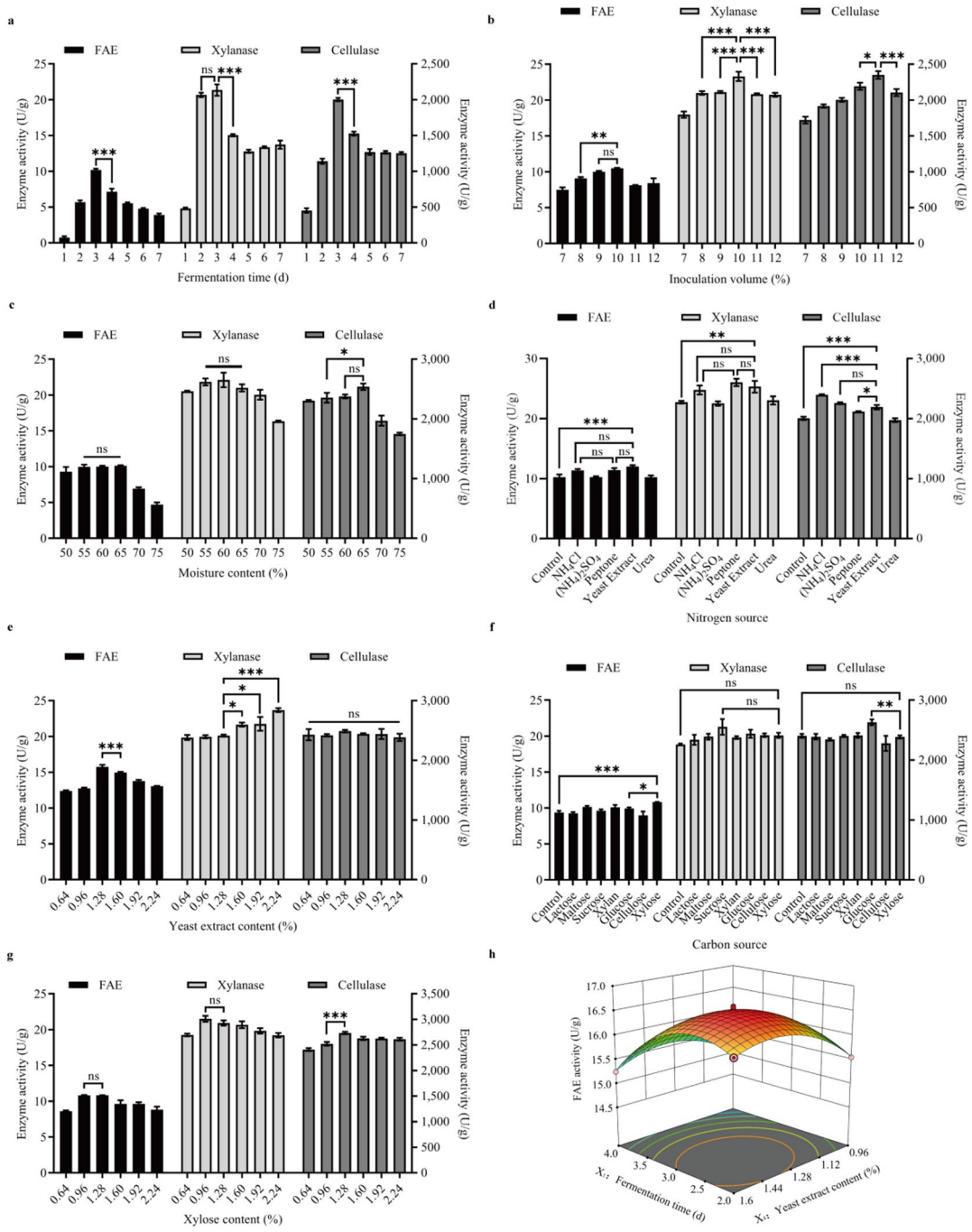

Fig. 3. Results of single-factor experiments for enzyme production by *A. niger* fermentation. (**a**) Effects of fermentation time on enzyme production; (**b**) Effects of *A. niger* seed culture inoculum size on enzyme production; (**c**) Effects of moisture content on enzyme production; (**d**) Effects of different nitrogen sources on enzyme production; (**e**) Effects of yeast extract concentration on enzyme production; (**f**) Effects of different carbon sources on enzyme production; (**g**) Effects of xylose concentration on enzyme production; (**h**) Response surface plot showing the interaction between fermentation time and yeast extract concentration on FAE activity produced by *A. niger*. Data are presented as mean ± SEM from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

Combined with the above results and correlation analysis between enzyme activities and TFA release, the optimal parameters for *A. niger* fermentation-based enzyme production were determined as follows: fermentation time of 3 days, inoculum size of 10% (*A. niger* seed culture), moisture content of 60%, yeast extract as nitrogen source (1.28%), and xylose as carbon source (1.28%).

Enzyme production Box-Behnken experiment

Based on the results of the Box-Behnken experiment (Supplemental Table S6), the data were subjected to multiple regression fitting using Design-Expert 10.0 software, yielding a quadratic multiple regression Eq. (1) for FAE activity (Y) with respect to the coded independent variables: fermentation time (X_1), inoculum size (X_2), xylose concentration (X_3), and yeast extract concentration (X_4).

$$\begin{aligned} Y = & -43.35349 + 3.11494X_1 + 9.36480X_2 + 2.75347X_3 + 11.89327X_4 \\ & + 0.029312X_1X_2 + 0.111957X_1X_3 - 0.305338X_1X_4 + 0.081424X_2X_3 \\ & + 0.040712X_2X_4 - 0.063612X_3X_4 - 0.587553X_1^2 - 0.488216X_2^2 \\ & -1.55532X_3^2 - 4.14751X_4^2 \end{aligned} \quad (1)$$

Analysis of variance (ANOVA) for the regression model revealed a P-value < 0.001 (Supplemental Table S7), indicating that the overall model exerted a highly significant effect on the experimental results and was credible. The lack-of-fit term showed a P-value of 0.8203 ($P > 0.05$), which was not significant, confirming the model's appropriateness. With a coefficient of determination (R^2) of 0.9894 and an adjusted R^2 (R^2_{Adj}) of 0.9788, the model exhibited good fitness, with a strong correlation between predicted and actual values and minimal error, thus demonstrating high reliability. A coefficient of variation (CV) of 0.43% indicated good experimental repeatability. Therefore, the model was reliable and could accurately predict the response value (FAE activity) within the designed range.

The interaction between factors X_1 and X_4 significantly affected the response value (Supplemental Table S7). Using Design-Expert 10.0 software, a response surface plot was generated to visualize the interaction effect of X_1 and X_4 on FAE production. Under the conditions of 10% inoculum size and 1.28% xylose concentration, FAE activity increased gradually with extended fermentation time and increased yeast extract concentration, reaching a peak before decreasing gradually (Fig. 3h).

Enzyme hydrolysis single-factor test

The release of TFA from DSWB via enzymatic hydrolysis with the mixed enzyme increased rapidly within the first 10 min, followed by a gradual rise over time, and finally reached a peak before stabilizing at 120 min (Fig. 4a).

TFA release reached its maximum when the enzyme mixture pH was 4.5, solid-liquid ratio was 1:30, and enzymatic hydrolysis temperature was 50 °C (Fig. 4b, c and d). Supplementation of Ca^{2+} to the mixed enzyme further enhanced TFA release, with the highest release observed at a Ca^{2+} concentration of 5.0 mmol/L (Fig. 4e and f).

Thus, the optimal parameters for enzymatic hydrolysis of DSWB to release TFA were determined as follows: enzymatic hydrolysis time of 120 min, enzyme mixture pH of 4.5, solid-liquid ratio of 1:30, hydrolysis temperature of 50 °C, and Ca^{2+} supplementation at 5.0 mmol/L.

Enzymatic Box-Behnken test

Plackett-Burman design-based screening showed that enzymatic hydrolysis temperature, pH, and solid-liquid ratio significantly affected TFA release ($P < 0.05$), confirming these three factors as the key determinants of TFA release from DSWB via enzymatic hydrolysis with *A. niger* fermentation broth. Thus, these factors were selected for subsequent optimization using Box-Behnken response surface design. The experimental results (Supplemental Table S8) were subjected to multiple regression fitting using Design-Expert 10.0 software, yielding a quadratic regression Eq. (2) for TFA release (Y) with respect to the coded independent variables: enzymatic hydrolysis temperature (X_1), pH (X_2), and solid-liquid ratio (X_3).

$$\begin{aligned} Y = & 5.3940 - 0.2300X_1 + 0.0276X_2 + 0.0524X_3 \\ & + 0.0332X_1X_2 + 0.0743X_1X_3 - 0.0180X_2X_3 \\ & - 0.5073X_1^2 - 0.3620X_2^2 - 0.4180X_3^2 \end{aligned} \quad (2)$$

ANOVA for the regression model revealed a P-value < 0.001 (Supplemental Table S9), indicating the model exerted a highly significant effect on the experimental results and was credible. The lack-of-fit term had a P-value of 0.8659 ($P > 0.05$), which was non-significant, confirming the appropriateness of the model. With a coefficient of determination (R^2) of 0.9989 and an adjusted R^2 (R^2_{Adj}) of 0.9974, the model exhibited excellent fitness, with a strong correlation between predicted and experimental values, minimal error, and high reliability. A CV of 0.47% further demonstrated good experimental repeatability and accurate results. Overall, the model was reliable and could accurately predict TFA release within the designed parameter range (Tables 1, 2, 3, 4, 5, 6, 7, 8 and 9).

Significance testing of the regression coefficients showed the three factors exerted effects on Y in the order $X_2 > X_3 > X_1$. The significant two-factor interactions affecting Y were $X_1 \times_2$ and $X_1 \times_3$, with the X_1 and X_3 interaction having a highly significant effect (Supplemental Table S9). To visualize these interactions, response surface plots were generated via Design-Expert 10.0 software to illustrate the effects of $X_1 \times_2$ and $X_1 \times_3$ on TFA release.

When the solid-liquid ratio was fixed at 1:30, TFA release increased gradually with rising enzymatic hydrolysis temperature and pH, reaching a maximum before decreasing gradually (Fig. 4g). When pH was fixed at 4.5, TFA release changed with variations in enzymatic hydrolysis temperature and solid-liquid ratio (Fig. 4h), exhibiting the same trend as observed in Fig. 4g.

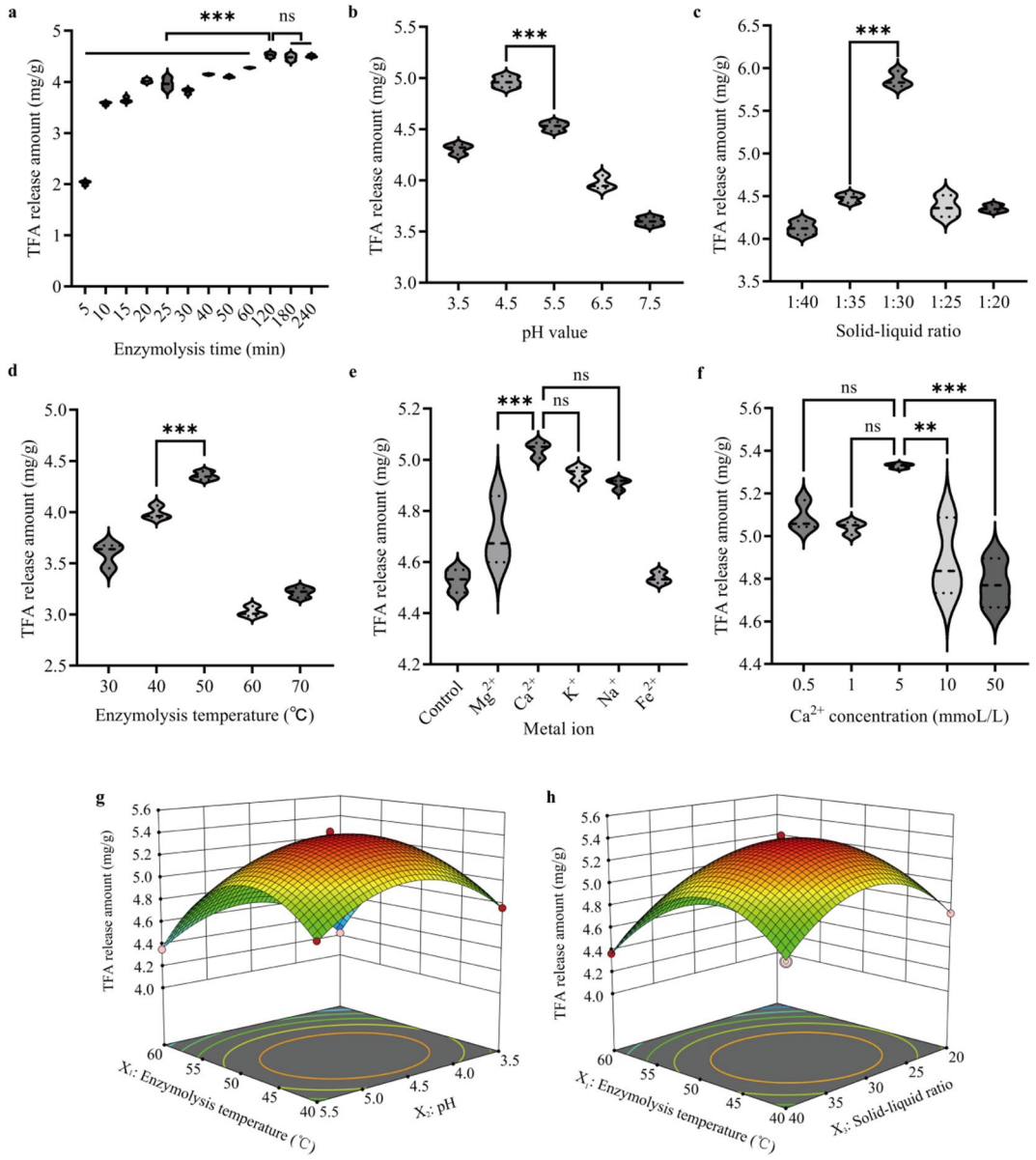

Fig. 4. Results of single-factor experiments for TFA release from DSWB via mixed enzyme hydrolysis. (**a**) Effects of hydrolysis time on TFA release; (**b**) Effects of pH on TFA release; (**c**) Effects of solid-to-liquid ratio on TFA release; (**d**) Effects of hydrolysis temperature on TFA release; (**e**) Effects of different metal ions on TFA release; (**f**) Effects of Ca^{2+} concentration on TFA release; (**g**) Response surface plot showing the interaction between hydrolysis temperature and pH on TFA release; (**h**) Response surface plot showing the interaction between hydrolysis temperature and solid-to-liquid ratio on TFA release. Data are presented as mean ± SEM from three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns, not significant.

Levels	Fermentation time/h	Inoculation amount/%	Moisture content/%	Nitrogen source	Nitrogen source content /%	Carbon source	Carbon source content /%
1	24	8	50	Urea	0.64	Glucose	0.64
2	48	9	55	$(NH_4)_2SO_4$	0.96	Maltose	0.96
3	72	10	60	Peptone	1.28	Xylan	1.28
4	96	11	65	NH_4Cl	1.60	Cellulose	1.60
5	120	12	70	Yeast Extract	1.92	Sucrose	1.92
6	144	—	75	—	2.24	Xylose	2.24
7	168	—	80	—	—	Lactose	—

Table 1. Single factor experiment on enzyme production from wheat Bran by *A. niger* fermentation.

Factors	Level		
	-1	0	1
Fermentation time X_1/d	2	3	4
Inoculation amount X_2/%	9	10	11
Carbon source concentration X_3/%	0.96	1.28	1.6
Nitrogen source concentration X_4/%	0.96	1.28	1.6

Table 2. Box-Behnken test design for enzyme production from wheat Bran by *A. niger* fermentation.

Levels	Enzymolysis time/min	pH value	Solid-liquid ratio	Enzymolysis temperature/℃	metal ion	Concentration of metal ion /mmol/L
1	5	3.5	1:40	30	Mg^{2+}	0.5
2	10	4.5	1:35	40	Ca^{2+}	1.0
3	15	5.5	1:30	50	K^{+}	5.0
4	20	6.5	1:25	60	Na^{+}	10.0
5	25	7.5	1:20	70	Fe^{2+}	50.0
6	30	–	–	–	–	–
7	40	–	–	–	–	–
8	50	–	–	–	–	–
9	60	–	–	–	–	–
10	120	–	–	–	–	–
11	180	–	–	–	–	–
12	240	–	–	–	–	–

Table 3. Single factor experiment on TFA production from enzymolysis of wheat bran.

Factors	Level		
	-1	0	1
Enzymolysis time A/min	60	120	180
Enzymolysis temperature B/℃	40	50	60
$CaCl_2$ concentration C/mmol/L	1.0	5.0	10.0
pH value D	3.5	4.5	5.5
Solid-liquid ratio E	1:35	1:30	1:25

Table 4. Plackett-Burman test design for TFA production from enzymolysis of wheat bran.

Factors	Level		
	-1	0	1
Enzymolysis temperature X_1/℃	40	50	60
pH value X_2	3.5	4.5	5.5
Solid-liquid ratio X_3	1:35	1:30	1:25

Table 5. Box-Behnken test design for TFA production from enzymolysis of wheat bran.

Antibacterial activity of TFA

The TFA obtained in this study exhibited significant concentration-dependent inhibitory activity against *E. coli*. When *E. coli* (initial concentration: 1×10^6 CFU/mL) was treated with 0.6 mg/mL TFA for 24 h, the cell concentration decreased to 8×10^4 CFU/mL (significantly lower than the initial level). At 2.4 mg/mL TFA, the *E. coli* concentration was essentially 0 CFU/mL, representing a 99.9% reduction relative to the initial concentration (Fig. 5a). Correspondingly, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of TFA against *E. coli* were determined as 0.6 mg/mL and 2.4 mg/mL, respectively.

Similarly, TFA significantly inhibited the growth of *S. aureus* with a trend mirroring that observed for *E. coli*. For *S. aureus* (initial concentration: 1×10^6 CFU/mL), treatment with 0.3 mg/mL TFA for 24 h reduced the cell concentration to ~6×10^5 CFU/mL, while 1.2 mg/mL TFA resulted in an essentially 0 CFU/mL cell concentration (99.9% reduction; Fig. 5a). Thus, the MIC and MBC of TFA against *S. aureus* were 0.3 mg/mL and 1.2 mg/mL, respectively.

Test groups	Fermentation time X_1/d	Inoculation amount X_2/%	Xylose concentration X_3/%	Yeast concentration X_4/%	FAE enzyme activity U/g
T1	4	10	1.28	1.6	15.2506
T2	4	11	1.28	1.28	15.0161
T3	3	10	1.6	1.6	16.1365
T4	3	10	1.6	0.96	15.6675
T5	3	10	1.28	1.28	16.5795
T6	3	10	1.28	1.28	16.5665
T7	3	9	1.6	1.28	16.0063
T8	3	11	1.28	0.96	15.2767
T9	4	10	0.96	1.28	15.42
T10	3	10	0.96	1.6	16.3059
T11	4	9	1.28	1.28	15.2637
T12	3	10	1.28	1.28	16.5274
T13	4	10	1.28	0.96	14.9901
T14	2	10	1.28	1.6	16.2017
T15	3	10	1.28	1.28	16.3841
T16	2	10	0.96	1.28	16.2277
T17	3	9	0.96	1.28	16.0975
T18	3	11	1.28	1.6	15.6806
T19	3	10	1.28	1.28	16.5925
T20	2	10	1.6	1.28	16.0453
T21	4	10	1.6	1.28	15.3809
T22	2	10	1.28	0.96	15.5503
T23	2	11	1.28	1.28	15.6545
T24	3	10	0.96	0.96	15.8108
T25	3	11	1.6	1.28	15.6806
T26	2	9	1.28	1.28	16.0193
T27	3	11	0.96	1.28	15.6675
T28	3	9	1.28	0.96	15.5503
T29	3	9	1.28	1.6	15.902

Table 6. Box-Behnken test design and response value for enzyme production from wheat Bran by *A. niger* fermentation.

Parameter	Quadratic sum (SS)	Free degree (DF)	MS	F Value	Significance
model Model	6.20	14	0.4428	93.53	***
X1	1.60	1	1.60	337.25	***
X2	0.2892	1	0.2892	61.09	***
X3	0.0312	1	0.0312	6.60	*
X4	0.5771	1	0.5771	121.89	***
X1 × 2	0.0034	1	0.0034	0.7259	
X1 × 3	0.0051	1	0.0051	1.08	
X1 × 4	0.0382	1	0.0382	8.07	*
X2 × 3	0.0027	1	0.0027	0.5736	
X2 × 4	0.0007	1	0.0007	0.1434	
X3 × 4	0.0002	1	0.0002	0.0358	
X12	2.24	1	2.24	472.95	***
X22	1.55	1	1.55	326.55	***
X32	0.1645	1	0.1645	34.75	***
X42	1.17	1	1.17	247.11	***
Residual	0.0663	14	0.0047		
Lack of Fit term	0.0373	10	0.0037	0.5146	
Pure Error	0.0290	4	0.0072		
Cor Total	6.27	28			

Table 7. ANOVA for the regression model of enzyme production by *A. niger* fermentation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Test groups	Enzymolysis temperature X_1/℃	pH value X_2	Solid-liquid ratio X_3	TFA release amounts mg/g
T1	50	4.5	30	5.357
T2	40	5.5	30	4.758
T3	50	4.5	30	5.389
T4	40	4.5	40	4.666
T5	50	3.5	20	4.514
T6	40	3.5	30	4.765
T7	50	3.5	40	4.663
T8	60	5.5	30	4.351
T9	50	4.5	30	5.382
T10	60	4.5	40	4.368
T11	50	5.5	20	4.601
T12	50	5.5	40	4.678
T13	50	4.5	30	5.423
T14	40	4.5	20	4.718
T15	50	4.5	30	5.419
T16	60	3.5	30	4.225
T17	60	4.5	20	4.123

Table 8. Box-Behnken test design and response value for TFA production from enzymolysis of wheat bran.

Parameter	Quadratic sum (SS)	Free degree (DF)	MS	F value	Significance
model Model	3.12	9	0.3468	685.61	***
X1	0.4232	1	0.4232	836.66	***
X2	0.0061	1	0.0061	12.07	*
X3	0.0219	1	0.0219	43.39	***
X1 × 2	0.0044	1	0.0044	8.74	*
X1 × 3	0.0221	1	0.0221	43.60	***
X2 × 3	0.0013	1	0.0013	2.56	
X12	1.08	1	1.08	2141.82	***
X22	0.5518	1	0.5518	1090.83	***
X32	0.7357	1	0.7357	1454.43	***
Residual	0.0035	7	0.0005		
Lack of Fit term	0.0005	3	0.0002	0.2382	
Pure Error	0.0030	4	0.0008		
Cor Total	3.12	16			

Table 9. ANOVA for the regression model of TFA production from enzymolysis of wheat bran. *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

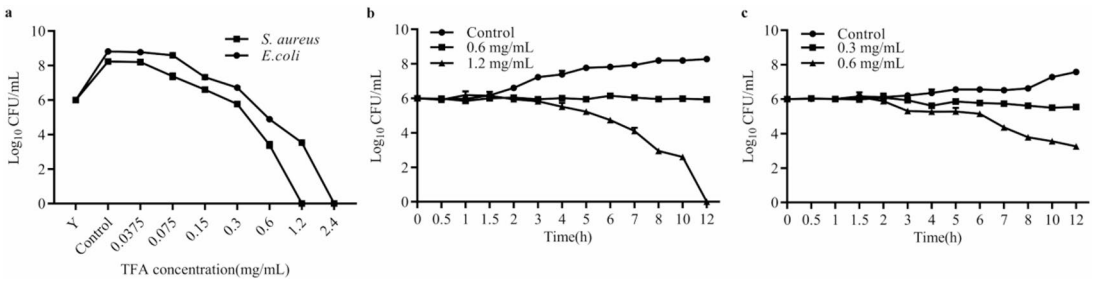

Fig. 5. Antibacterial activity of TFA. (**a**) MICs and MBCs of TFA against *E. coli* and *S. aureus*; (**b**) Time-kill curve of TFA against *E. coli*; (**c**) Time-kill curve of TFA against *S. aureus*.Data are presented as mean ± SEM from three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

The time-kill curve of TFA against *E. coli* (Fig. 5b) showed that all groups remained in the adaptation phase within the first 1.5 h, with negligible changes in cell concentration. After 1.5 h, *E. coli* entered the exponential growth phase, and the control group exhibited a gradual increase in cell concentration. In contrast, the group treated with 0.6 mg/mL TFA (MIC) showed almost no change in cell concentration over 12 h. When treated with 1.2 mg/mL TFA, the *E. coli* concentration decreased rapidly after 3 h and reached 0 by 12 h. These results confirm that TFA at its MIC inhibits *E. coli* growth, with antibacterial activity exhibiting both time- and concentration-dependence.

Consistently, the time-kill curve against *S. aureus* (Fig. 5c) showed that the bacterium remained in the adaptation phase within 2 h, with minimal changes in cell concentration. After 2 h, *S. aureus* entered the growth phase, and the control group's cell concentration increased gradually. Treatment with 0.3 mg/mL TFA (MIC) resulted in almost no change in *S. aureus* concentration over 12 h, while 0.6 mg/mL TFA led to a gradual decrease in cell concentration after 2 h. These findings indicate that TFA similarly exerts time- and concentration-dependent antibacterial activity against *S. aureus* at its MIC.

Discussion

With the global escalation of concerns regarding antimicrobial resistance and drug residues stemming from the extensive use of antibiotics, numerous countries—including the EU, the United States, and China—have enacted regulations to restrict or prohibit antibiotic use in livestock farming[37–39]. Consequently, the development of safe, novel, and effective antibiotic alternatives for animal feed has emerged as a pivotal research focus in the "post-antibiotic era." Wheat bran, a ubiquitous feed ingredient, contains FA, a compound with broad-spectrum antibacterial activity that effectively inhibits pathogenic bacteria such as *E. coli* and *S. aureus*[7]. Owing to its non-residue property, minimal environmental impact, and low toxicity, FA represents an ideal antibiotic alternative[40,41].

This study aimed to maximize TFA release from DSWB via enzymatic hydrolysis using a mixed enzyme solution derived from fermentation by a high enzyme-producing *A. niger* strain. RSM was employed to optimize both the enzyme production (fermentation) and enzymatic hydrolysis processes. Fermentation strain and substrate are critical determinants of enzyme activity: the selected *A. niger* strain exhibited favorable traits including high enzyme productivity, good stability, safety, reliability, and ease of cultivation; meanwhile, fermentation substrates must provide comprehensive nutritional support for microbial growth, proliferation, and enzyme synthesis—collectively emphasizing their pivotal roles in enzyme production. Based on correlation analysis between the activities of FAE, xylanase, cellulase, and TFA release, *A. niger* was selected as the fermentation strain, and a 1:9 mixture of vinegar residue and wheat bran was identified as the optimal fermentation substrate.

To optimize the fermentation process, a sequential approach combining single-factor experiments, Box-Behnken design, and RSM was adopted. The operationally feasible optimal parameters were determined as follows: moisture content 60%, fermentation time 64 h, inoculum size 9.80% (seed suspension concentration: 1.3×10^8 CFU/mL), xylose concentration 1.21%, and yeast powder concentration 1.38%. Under these conditions, FAE activity reached 16.63 ± 0.32 U/g, which was highly consistent with the predicted value (16.65 U/g). This marked a significant improvement compared to a previous report of 7.68 mU/g FAE activity from *A. niger*-mediated solid-state fermentation of wheat bran[42].

Subsequently, the fermented mixed enzyme solution was used for DSWB hydrolysis. The parameters governing TFA release during enzymatic hydrolysis were optimized via a stepwise strategy involving single-factor experiments, Plackett-Burman regression analysis, Box-Behnken design, and RSM. The optimal hydrolysis conditions were: hydrolysis time 120 min, temperature 48 °C, pH 4.5, solid-to-liquid ratio 1:30.5, and $CaCl_2$ concentration 5.0 mmol/L. Under these conditions, TFA release reached 5.41 ± 0.03 mg/g, which was not statistically significant ($p > 0.05$) from the predicted value (5.42 mg/g). This represented a marked enhancement compared to a prior study where lactic acid bacteria fermentation of bran achieved a maximum FA release of 1.39 mg/g[43]. The superior TFA release in the current study is likely attributed to the synergistic actions of multiple enzymes: FAE and xylanase collaborate to degrade ferulated plant cell wall polysaccharides[44], while FAE and cellulase jointly enhance the hydrolysis of lignocellulosic substrates[45]. The combined activity of these three enzymes enables more efficient plant cell wall degradation, thereby promoting the release of esterified FA—consistent with previous findings documenting enzyme synergy in FA liberation[44,46].

Furthermore, antimicrobial activity assays of the crude TFA extract revealed significant antibacterial efficacy. The MIC and MBC against *E. coli* were 0.6 mg/mL and 2.4 mg/mL, respectively, while those against *S. aureus* were 0.3 mg/mL and 1.2 mg/mL. Notably, the antibacterial effects exhibited both time- and concentration-dependence, confirming the strong antibacterial activity of the TFA extract.

The novelty of this study resides in the use of an *A. niger*-fermented mixed enzyme solution for DSWB enzymatic hydrolysis, coupled with systematic optimization of both fermentation and hydrolysis parameters via RSM. While the current results confirm that *A. niger* fermentation of wheat bran yields a multi-enzyme system (including FAE) with high biological activity, and that this crude enzyme solution effectively enhances TFA release from DSWB, several limitations warrant future exploration. Despite the demonstrated in vitro antibacterial activity of the extracted TFA against *E. coli* and *S. aureus*, the in vivo efficacy and safety of TFA remain to be verified. Additionally, the potential of TFA to exert other biological functions merits further investigation.

Materials and methods

Strains

A. niger, *N. sitophila*, and *T. reesei* were preserved by the Animal Biochemistry Laboratory of Shanxi Agricultural University.

Correlation analysis between FAE, xylanase, and cellulase activities and TFA release

Equal masses of wheat bran were supplemented with mixtures of FAE, xylanase, and cellulase that varied in enzyme activity levels. After measuring TFA release from the wheat bran, linear regression analyses were performed—with the activities of FAE, xylanase, and cellulase as the independent variables (x-axis) and TFA release as the dependent variable (y-axis).

Screening of fermentation substrates and enzyme-producing strains

With the activities of FAE, xylanase, and cellulase as the evaluation indicators, mixtures of wheat bran and vinegar residue at different proportions were fermented by *A. niger*, *N. sitophila*, and *T. reesei*, respectively, to screen for optimal enzyme-producing substrates and fermentation strains.

Single-factor test for enzyme production

A single-factor experiment was conducted using the control variable method to investigate seven factors potentially influencing enzyme production during fermentation: fermentation time, inoculum size, moisture content, nitrogen source, nitrogen source concentration, carbon source, and carbon source concentration (Supplemental Table S1). FAE activity was used as the primary evaluation indicator, xylanase activity as the secondary indicator, and cellulase activity as the tertiary indicator.

Box-Behnken design for enzyme production

Using FAE activity as the response variable, a four-factor, three-level Box-Behnken design was implemented using Design-Expert 10.0 software, with the factors being fermentation time (X_1), inoculum size (X_2), xylose concentration (X_3), and yeast extract concentration (X_4). The levels of each factor were as follows: fermentation time (days): 2, 3, and 4; inoculum size (%): 9, 10, and 11; nitrogen source (yeast extract) concentration (%): 0.96, 1.28, and 1.6; and carbon source (xylose) concentration (%): 0.96, 1.28, and 1.6 (Supplemental Table S2).

Single-factor test for enzyme hydrolysis

The parameter combination optimized in Sect. 4.4 was adopted for *A. niger* fermentation for enzyme production, and the resulting crude enzyme mixture was used for the enzymatic hydrolysis of DSWB to release TFA.

With TFA release as the evaluation indicator, a single-factor experiment was conducted using the control variable method to investigate six factors potentially affecting TFA release from DSWB via enzymatic hydrolysis: enzymatic hydrolysis time, pH, solid-liquid ratio, hydrolysis temperature, metal ion type, and metal ion concentration (Supplemental Table S3).

Box-Behnken design for enzyme hydrolysis

Design-Expert 10.0 software was used to perform Plackett-Burman regression analysis on five factors potentially affecting TFA release from enzymatically hydrolyzed DSWB: hydrolysis time, hydrolysis temperature, $CaCl_2$ concentration, pH, and solid-liquid ratio (Supplemental Table S4).

Subsequently, a three-factor, three-level Box-Behnken experiment was designed using the three key factors identified from the Plackett-Burman screening: hydrolysis temperature, pH, and solid-liquid ratio, which served as independent variables, with TFA release as the response variable. The levels of each factor were as follows: hydrolysis temperature (℃): 40, 50, and 60; pH: 3.5, 4.5, and 5.5; and solid-liquid ratio: 1:35, 1:30, and 1:25 (Supplemental Table S5).

Antimicrobial tests

E. coli and *S. aureus* were activated, and a viable cell suspension with a concentration of 1×10^9 CFU/mL was prepared using the serial dilution method. The TFA crude solid extract obtained in this study was dissolved in 5 mL of prewarmed (50 °C) nutrient broth (NB). Subsequently, 5 μL of the 1×10^9 CFU/mL bacterial suspension was added, resulting in final TFA concentrations of 0.0375, 0.075, 0.15, 0.3, 0.6, 1.2, and 2.4 mg/mL, with a final bacterial concentration of 1×10^6 CFU/mL. The control group contained no TFA crude extract. All mixtures were incubated in a shaking incubator at 37 °C with constant agitation at 180 rpm for 24 h. The MICs and MBCs of the TFA crude extract against *E. coli* and *S. aureus* were determined via serial dilution.

Subsequent time-kill kinetic analyses were performed based on the determined MIC values. The TFA crude solid extract was dissolved in 5 mL of prewarmed (50 °C) NB, and 5 μL of the 1×10^9 CFU/mL bacterial suspension was added to achieve final TFA concentrations of 1×MIC and 2×MIC, with a final bacterial concentration of 1×10^6 CFU/mL. The control group contained no TFA crude extract. The tubes were incubated in a shaking incubator at 37 °C with constant agitation at 180 rpm. Samples were collected at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, and 12 h for bacterial counting via serial dilution, with three replicates per time point.

Statistical analysis

Statistical analysis was performed via ANOVA. The data represent three independent experiments and are presented as the means ± SEMs. A p value of < 0.05 was considered to indicate statistical significance.

Data availability

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

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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