Simultaneous transformation of ginsenoside Rb1 into rare ginsenoside F2 and Compound K by the extracellular enzyme from Aspergillus Niger Wu-16

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Research Article

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Abstract

Ginsenoside Compound K (CK) has high pharmacological activity for being widely used in the treatment and prevention of diseases. However, the content of CK in ginseng is very low or almost non-existent. In the present study, ginsenoside Rb1 was converted into ginsenoside F2 and CK by using the extracellular protein isolated from *Aspergillus niger* Wu-16. The results show that the crude enzymes produced by Wu-16 can catalyze the conversion of the substrate Rb1 to ginsenoside F2 and CK. The reaction time, pH, temperature, and ratio of enzyme to substrate for preparing ginsenoside F2 and CK were used as optimization factors. A reaction time of 48 h, pH=3, temperature of 55°C, and enzyme-to-substrate ratio of 0.8 were obtained. Furthermore, response surface optimization experiment was carried out to further obtain the maximum conversion rates of ginsenoside F2 and CK with 50.11% and 33.50% for 48 h, respectively. On this basis, the effect of metal ions on the enzymatic reaction showed that the conversion rate of ginsenoside Rb1 was increased remarkably by Co^{2+} with concentrations of 0.5 mM, and the highest conversion rate into rare ginsenoside CK reached 56.10%. Therefore, the advantage of enzymes catalysis from *A. Niger* Wu-16 could be a simple, convenient, and rapid method to produce rare saponin F2 and CK, this method provides a certain reference value for the production of rare ginsenosides.

1. Introduction

Ginsenoside is the main active component of ginseng, an important triterpenoid compound, which has good physiological and pharmacological activities, including anti-tumor, anti-inflammatory, and anti-aging biological functions (Hong et al., 2021; Duan et al., 2018a). According to the content, ginsenoside is divided into common and rare Ginsenosides. Common Ginsenosides (high content) account for more than 80% of the total Ginsenosides in ginseng root, such as Rb1 and Rb2 (Kochan et al., 2017; Wang et al., 2020a). The content of Rb1 is the highest in common Ginsenosides, while that of rare Ginsenosides such as F2 is low (Zang et al., 2017; Chen et al., 2020; Wang et al., 2011). Most of the minor ginsenosides generally exhibit good pharmacological activities. Rare ginsenoside F2 has a certain therapeutic effect on skin inflammation as a new treatment method, and F2 can inhibit human gastric cancer cells (Mao et al., 2016; Park et al., 2016). Ginsenoside CK shows a high absorption rate in blood and exerts pharmacological activity in the body (Akao T, 2021; Zhao et al., 2020). As a drug, it has anti-cancer, anti-diabetic, anti-allergic, and liver protection effects and has received widespread attention (Wong et al., 2015; Xiao et al., 2016). However, rare ginsenosides F2 and CK are difficult to produce because of their low content or because they are non-existent in ginseng or other plants (Yang et al., 2021). Ginsenoside Rb1 belongs to PPD-type ginsenosides and has the same core structure as ginsenoside F2 and CK. Therefore, the conversion of ginsenoside Rb1 into rare saponin F2 and CK is a significant and wise approach.

At present, the transformation of ginsenosides mainly includes physical, chemical, and biotransformation methods (Zheng et al., 2017). The basic principle of the conversion of common Ginsenosides to produce rare ginsenosides is that a series of deglycosylation reactions occur at specific positions of common Ginsenosides. Among these methods, the physical conversion of saponin has harsh conditions, high

energy consumption, and low yield, making it unsuitable for industrial production (Cui et al., 2019; Kochan et al., 2020; Ku, 2016). Chemical preparation methods have some disadvantages such as production of by-products, violent reaction process, difficulty in purification, and environmental pollution (Wang et al., 2020b; Zhang et al., 2020). In comparison with physical and chemical methods, biotransformation has quite good advantages. For example, biotransformation is a fast, convenient, efficient, and environmentally friendly method for obtaining rare Ginsenosides. Hence, biotransformation is a common method for ginsenoside transformation (Chen et al., 2021; Zhang et al., 2019). Many studies have focused on the production of rare ginsenosides by microbial enzyme (Eom et al., 2018; Fu, 2019). Moreover, the rare ginsenoside CK can be prepared from the common Ginsenosides Rb1 by microorganisms or enzymes produced from microorganisms. Lactic acid bacteria isolated from kimchi or enzyme preparation for culturing *Armillaria* can convert common Ginsenosides into highly active ginsenoside CK (Kim et al., 2018; Kim et al., 2012; Park et al., 2017). Quan et al. (Quan et al., 2011) discovered that ginsenoside Rb1 can be biotransformed by *leuconostoc mesenteroides* DC102 to obtain the CK, producing many intermediate products such as prosapogenins, gypenoside XVII, ginsenoside Rd, and ginsenoside F2 in this conversion reaction. Ginsenoside CK can be obtained from substrate ginsenoside Rb1 by the transformation of microorganism *Endophyte* JG09 from Platycodon grandiflorum, in which 168 h of culture, ginsenoside CK is rapidly produced (Cui et al., 2016). Although these studies provide a good solution on how to obtain rare ginsenoside CK, the biotransformation of ginsenoside Rb1 into F2 and CK encounters many problems, such as low conversion rate, long conversion time, production of many intermediate products, and difficult separation of product. In addition, the final F2 and ginsenoside CK is mainly used for disease treatment and drug development. Hence, the highly efficient bioconversion of ginsenoside F2 and CK is very important and innovative.

In the present study, the extracellular enzyme produced by the safe strain *A. niger* Wu-16 was extracted and used as catalyst to transform the main ginsenoside Rb1 to produce ginsenoside F2 and CK. The conditions of the enzymatic reaction, including temperature, pH, reaction time, and ratio of enzyme to substrate were optimized, and the influence of metal ions on the conversion reaction was explored. This research opens up a new path for the simultaneous and efficient production of ginsenoside F2 and CK and lays the foundation for the mass production of rare ginsenoside in the future.

2. Materials And Methods

2.1 Reagents

Ginsenoside standard products (purity greater than 98%), including Rb1, Rd, F2, and CK, were obtained from Chengdu Mansite Biotechnology Co., Ltd. Approximately 80% of ginsenoside Rb1 used as substrate was obtained from Zhejiang Jinainong Biotechnology Co., Ltd. Chromatographic-grade acetonitrile and methanol were used, and all other reagents were of analytical grade.

2.2 Methods

2.2.1 Screening and identification of strains

Strains were screened from ginseng rhizosphere soil, β-glucosidase-producing microorganisms were isolated using esculin-R2A agar, and then the red-brown to dark brown areas surrounded by β-glucosidase that hydrolyze surrounding materials were used to select the appropriate microorganism.

The strains were sent to Shanghai Shenggong Biotechnology Co., Ltd. for ITS rDNA gene sequencing. According to the comparison of the results, the NJ tree was constructed using MEGA4. The guided analysis was repeated for 1,000 times as a confirmation of each branch.

2.2.2 Activation of *A. niger* and its enzyme production conditions

The *A. niger* was inoculated from the slant medium into the PDB liquid medium, cultivated at 30°C and 150 rpm for 36 h to obtain the seed liquid, and inoculated into the enzyme-producing medium with 3% inoculum. The formula of the enzyme-producing medium was 2% bran, 0.2% $(NH4)_2SO_4$, and 0.5% KH_2PO_4. The samples was then cultured at 30°C and 150 rpm for 5 days.

2.2.3 Separation and concentration of enzymes

After stopping the fermentation, the culture broth was centrifuged at 10,000 rpm 4°C for 10 min, and 70% saturation $(NH_4)_2SO_4$ was added to the supernatant. After storing the mixture at 4°C for 6 h, it was centrifuged at 10,000 rpm for 10 min to obtain a protein precipitate and dissolved in deionized water. Then, the ammonium sulfate was removed and passed through a G25 desalting column. Enzyme powder was obtained by freeze-drying and subjected to SDS-PAGE experiment.

2.3 Enzyme-catalyzed ginsenoside Rb1 for the preparation of ginsenoside F2 and CK

First, 80% ginsenoside Rb1 10 mg was dissolved in 0.5 mL of acetic acid–sodium acetate buffer (pH = 5) and added with 0.27 mg enzyme. Then, the reaction was carried out at 45°C for 36 h. The precipitate contains ginsenoside F2 and CK and was added with 0.5 mL of methanol to be dissolved for HPLC detection.

2.4 Analysis method

HPLC was used to detect the content of conversion products from ginsenoside Rb1 in real time. Before that, a standard curve was fitted with standard ginsenoside F2 and CK to determine the concentration of ginsenoside F2 and CK in the sample, and four main factors such as temperature, pH, time, and enzyme-to-substrate ratio were considered for single-factor optimization experiment. Response surface optimization experiment was used to obtain more optimized and comprehensive results. In addition, the influence of metal ions on the enzymatic reaction was studied.

2.4.1 Ginsenoside F2 and CK standard curve

A total of 1.6 mg ginsenoside CK was dissolved in 2 mL chromatography-grade methanol to prepare a solution with a storage concentration of 0.8 mg•mL^{-1}. Approximately 12.5, 62.5, 125, and 500 μL and 1 mL of the stock solution were diluted with methanol to 1 mL to prepare concentrations of 0.01, 0.05, 0.1, 0.4, and 0.8 mg•mL^{-1}. The HPLC mobile phase is composed of A (water) and solvent B (acetonitrile). The solvent composition changes are as follows: 0–27 min, 35% B; 27–37.5 min, 35–65% B; 37.5–50 min, 65% B. Before injection, all samples need to be filtered with a 0.22 μm organic filter, the flow rate is 0.9 mL/min, the injection volume is 10 μL, and the measurement wavelength is 210 nm. The regression equation is based on the value between the concentration of ginsenoside CK (X, mg•mL^{-1}) and the peak area (Y). The regression equation is shown in formula (1):

$$Y = 3771090X + 12705 \qquad R^2 = 0.9987 \qquad (1)$$

The method for establishing the standard curve of ginsenoside F2 is the same as that of CK. Specifically, 1 mg•mL^{-1} V = 3 mL F2 standard wad prepared by diluting 1, 0.7, 0.4, 0.1, and 0.05 mL of the stock solution with methanol to 1 mL, yielding standard products with concentrations of 1, 0.7, 0.4, 0.1, and 0.05 mg•mL^{-1}, respectively. The regression equation was established from the value between the concentration of ginsenoside F2 and the peak area. The regression equation is established from the value between the concentration of ginsenoside F2 (X, mg•mL^{-1}) and the peak area (Y). The regression equation is shown in formula (2):

$$Y = 476045X - 26015 \qquad R^2 = 0.9983 \qquad (2)$$

The external standard method was used to quantitatively determine the content of ginsenoside F2 and CK after the enzymatic reaction, and a calibration curve was established. By comparing the retention time of ginsenoside F2 and CK in the sample with the standard, the content of ginsenoside F2 and CK in the sample was determined. All analyses were repeated twice, and the average value was used to build the model.

2.4.2 HPLC method

The HPLC method is the same as the 2.4.1 analysis of single factor and response surface method (RSM) experiments, and it was used to detect ginsenoside F2 and CK production to optimize enzyme catalysis conditions. The HPLC model was LC-16 produced by Shimadzu Corporation, and the column was C18-WR (4.6×250 mm, 5 μm).

2.5 Conversion reaction single factor experiment

The enzymatic reaction rate involves reaction temperature, time, acidity, and alkalinity of the reaction system, as well as the relationship between the amount of substrate and enzyme. These parameters have

also been reported in previous studies. Therefore, single-factor optimization experiments should be conducted.

Approximately 80% of ginsenoside Rb1 was dissolved in 0.5 mL of buffer and added with 0.27 mg enzyme, and the enzyme catalytic conditions include pH, temperature, reaction time, and the ratio of enzyme to substrate. Other factors in each single factor experiment are constant. The experiment was evaluated according to the conversion rate of the substrate, and all experiments were carried out in parallel in the three groups.

2.6 Response surface optimization experiment design

Based on the single-factor experiment optimization results, three main factors, namely, temperature, ratio of enzyme to substrate, and pH, were used to design response surface optimization experiments by using Box–Behnken design. Temperature (X1), ratio of enzyme to substrate (X2), and pH (X3) were used as the independent variable with three levels coded as + 1, -1, and 0 respectively. The three-factor, three-level experiment used the conversion rate of ginsenoside Rb1 as the response value to evaluate the reaction. The factors and their corresponding level combinations are shown in Table 1.

Table 1
Factors and levels

level	Factors		
	X1/°C	X2	X3
+ 1	60	1	4
0	52.5	0.8	3
-1	45	0.6	2

2.7 The influence of metal ions on enzyme catalysis

Metal ions can exhibit different important roles in the body. For example, Na^+, K^+, and Ca^{2+} exhibit the unique membrane transport function of ion pumps (Duan et al., 2018b). $MgCl_2$, $CaCl_2$, and $BaCl_2$ can increase the β-glucosidase activity of strains such as *Penicillium sclerotiae* (Kim et al., 2020). Based on common metal ions in the laboratory and previous literature studies, K^+, Na^+, Mn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, and Co^{2+} were used to study the effect on the enzymatic reaction by using the specific process is as follows:

0.5 mmol•L^{-1} of 0.5 mL of different metal ions was added to the reaction system and allowed to react under optimal catalytic conditions. The ginsenoside F2 and CK contents were detected by HPLC, and the conversion rate of ginsenoside Rb1 was calculated.

2.8 Statistical analysis

All experiments were carried out in triplicate, and the data were expressed as the mean ± standard deviation (SD) of three parallel measurements and obtained by using Origins 8.5. The value of $p < 0.05$ is considered to be statistically significant and credible.

3. Results And Discussion

3.1 Identification of the isolated strain

The 1,337 bp ITS sequence was uploaded to NCBI for comparison, and a phylogenetic tree was constructed as shown in Fig. 1. The strain belongs to the genus *Aspergillus*. It has the closest relationship with *A. niger* MT487766.1 and was named *A. niger* Wu-16.

Figure 1 should be here

3.2 Separation and concentration of enzymes from *A. niger*

The SDS-PAGE of extracellular enzymes is shown in Fig. 2, and the results show that the conversion of ginsenoside Rb1 to ginsenoside F2 and CK may be caused by various extracellular proteins secreted by *A. niger* Wu-16. This reaction is a process of the continuous removal of sugar groups, and β-glucosidase is the most common type of hydrolase. The molecular weights of the main extracellular proteins are 120, 60, 35, and 30 kDa, which are similar to those proteins with molecular weights of 43–138 kDa produced by *A. niger* in the literature (Jiang et al., 2021). The proteins can be used to produce high-value-added rare ginsenosides F2 and CK from low-cost Rb1.

Figure 2 should be here

3.3 Transformation of Rb1 by extracellular enzymes to produce ginsenoside F2 and CK

The extracellular enzyme of *A. niger* Wu-16 was applied to the transformation reaction of ginsenoside Rb1. As shown in Fig. 3, after 24 h, the substrate Rb1 was basically reacted, and three new peaks appeared compared with the blank control (Fig. 3a). By comparison with the peaks of Rb1, Rd, F2, and CK ginsenoside standards in Fig. 3d, the results showed that the extracellular enzyme hydrolyzed the substrate Rb1 to produce three new products as Rd at 13.5 min, F2 at 38.0 min, and CK at 46.2 min. As the reaction time increased, the output of ginsenoside CK increased, and the output of Rd and F2 first increased and then decreased during the conversion process (Figs. 3b and 3c). Therefore, ginsenoside CK is the final product, and Rd and F2 are the intermediate products, which consistent with the transformation pathway as Rb1→Rd→F2→CK reported in the literature(Fu et al., 2016; Yan Q, 2008).

Figure 3 should be here

The effect of temperature on the conversion rate of ginsenoside Rb1 was investigated at the temperature range of 40–65°C, and the results are shown in Fig. 4. In the temperature range of 40–55°C, the conversion rate of ginsenoside Rb1 was greatly improved mainly because of the acceleration of the movement of molecules and increasing the possibility of enzyme contact with the substrate by increasing in temperature. Ginsenoside F2 is within the range of 40–60℃, and the yield is maintained above 50%. After 60 ℃, the yield decreased rapidly because of enzyme inactivation. At 50–55℃, the yield of ginsenoside CK was basically stable and relatively high, and the yield was 19.63% at 55 ℃, which is consistent with the optimum temperature (55°C) of ginsenoside hydrolyzing β-glucosidase in *Peritomies Bainier* sp. 229 (Yan et al., 2008). When the temperature was 55–65°C, the efficiency of catalyzing the substrate was remarkably reduced because of the rapid inactivation of one or several enzymes involved in the enzymatic reaction at high temperatures. To further explore the optimal temperature for the enzymatic reaction, we chose 45–60°C for further optimization experiments.

Figure 4 should be here

3.5 Effect of conversion time on conversion rate

The pH value, temperature, and enzyme/substrate ratio of the reaction were fixed values within the range of 24–108 h to study the effect of reaction time on enzymatic hydrolysis. The conversion rate of ginsenoside Rb1 was calculated, and the result is shown in Fig. 5. The yield of ginsenoside F2 was relatively stable, and the relative conversion rate exceeded 80%. The production of rare ginsenoside CK has been increasing. CK increased rapidly within 24–48 h, and its conversion rate reached a maximum of 32.09% because of the high substrate concentration in the early stage and the rapid conversion of the substrate into a product under high enzyme activity. After 48 h, the yield of CK slowly increased, mainly because the enzyme activity decreases with the increase of time at 55°C, leading to a decrease in catalytic efficiency. Therefore, according to actual production, 48 h is the most suitable reaction time, and the conversion efficiency is the highest.

Figure 5 should be here

3.6 Influence of pH on biotransformation to produce CK

The change of pH value will affect the binding and catalysis of protein catalyst and substrate molecule by changing the conformation of protein, the degree of dissociation of essential groups on enzyme active center, or the dissociation of substrate molecules. Considering that the catalyst for the conversion of Rb1 to F2 and CK is a mixture of multiple enzymes, theoretically, the most suitable pH value can make the catalyst exert the best catalytic activity, so that Rb1 can be efficiently converted into high value-added F2 and CK. Figure 6 shows that when the pH of the enzymatic reaction system was 3.0, the relative yield of CK reached 32.09%. At pH 3.0–6.0, the yield of CK shows a downward trend. At pH 4.0, F2 had the highest relative yield, similar to Jo's report (Jo et al., 2016), and β-D-glucosidase usually exerts its highest catalytic function under acidic conditions. Based on the present experiment, the final product can be selectively obtained by adjusting the pH of the reaction. At pH = 4, rare ginsenoside F2 accumulated in a large amount and decreased the pH value to obtain rare ginsenoside CK.

Figure 6 should be here

3.7 Optimization results of enzyme and substrate ratio

The influence of enzyme/substrate ratio on the hydrolysis of enzymatic reaction was studied, and the ratios were set to 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2. The results are shown in Fig. 7 as the ratio changes. When the ratio was in the range of 0.2–0.4, the yield of ginsenoside F2 increased rapidly, the ratio is 0.4, and the relative conversion rate reached 94.08%. As the ratio continued to increase, the yield of F2 basically remained stable. The yield of ginsenoside CK has been increasing as a whole. Specifically, when the ratio was in the range of 0.2–0.4, the yield of CK increased rapidly, but the relative conversion rate was low at 16.96%. As the ratio increased to 0.8, the output of CK nearly doubled to 31.66%, and when the ratio was 0.8, the yield of F2 was relatively high. Therefore, combined with the actual production cost, 0.8 is the best enzyme/substrate ratio for hydrolysis of ginsenoside Rb1 to ginsenoside CK.

Figure 7 should be here

3.8 Response surface design increases the conversion amount of prepared CK

The Design Expert.DX10 software was used to carry out response surface analysis, and 15 experiments with 3 central point repeats were conducted to determine the optimal conditions of the temperature, the ratio of enzyme to substrate, and enzymatic hydrolysis pH. The Box–Behnken design results are shown in Table 2. Tables 3 and 4 show the analysis of variance (ANOVA) results to evaluate the significance of the quadratic regression model equation and verify the feasibility of the equation. Significant differences were observed in the established quadratic polynomial models ($p < 0.001$) The coefficient of determination of ginsenoside F2 response value was as $R^2 = 0.9871$, and the F value was 42.58 based on Table 3. The coefficient of determination of ginsenoside CK response value was as $R^2 = 0.9861$, and the F value was 39.28 in Table 4, indicating that these two models are statistically significant and explaining the response of the relationship between the value and the independent variable. The results in the table show that the linear terms X1 and X3 of the model remarkably affect the production of ginsenoside F2 and CK. Among these statistically significant variables, pH (X3) is the most important ($p < 0.001$) factor affecting the hydrolysis process, followed by temperature (X1). By contrast, the ratio of enzyme to substrate (X2) only slightly affected the yield of ginsenoside F2 and CK. The relationship between the dual response value and each factor can also be seen intuitively from the response surface curves in Figs. 8 and 9. The regression model was used to study the transformation conditions of ginsenoside Rb1 to determine the optimal solution. The temperature was 55.03°C, the ratio of enzyme to substrate was 0.85, and the pH was 3.4. Under these conditions, the theoretical conversion rates of ginsenoside F2 and CK are 50.26% and 34.06%, respectively. Combined with the actual experimental results, the maximum conversion rates of ginsenoside F2 and CK were 50.11% and 33.50%, respectively, which are very consistent with the theoretical value, indicating that the process parameters have a certain practical value by the response surface method.

Table 2
The design and result of response surface optimization test

Run	Factor 1 A:temperature /°C	Factor 2 B:ratio of enzyme to substrate	Factor 3 C:pH value	Response 1 F2 yield /%	Response 2 CK yield/%
1	52.5	0.6	2	4.31	2.13
2	60	0.8	2	3.77	2.25
3	52.5	0.8	3	42.93	30.93
4	60	0.8	4	39.71	20.89
5	60	0.6	3	20.95	17.63
6	60	1	3	35.84	27.27
7	45	0.8	4	13.2	10.59
8	52.5	1	4	35.66	22.19
9	45	0.6	3	12.91	12.24
10	52.5	0.8	3	50.2	35.18
11	52.5	0.8	3	44.07	30.55
12	52.5	0.6	4	31.13	15.99
13	45	1	3	12.12	14.46
14	52.5	1	2	6.43	2.92
15	45	0.8	2	2.67	1.18

Table 3
Variance analysis results based on F2 yield

Source	Sum of Squares	df	Mean Square	F value	p-value Prob > F	
Model	3919.80	9	435.53	42.58	0.0003	Significant
A-temperature	440.60	1	440.60	43.08	0.0012	
B- ratio of enzyme to substrate	53.82	1	53.82	5.26	0.0703	
C-pH	1313.79	1	1313.79	128.45	< 0.0001	
AB	61.47	1	61.47	6.01	0.0578	
AC	161.42	1	161.42	15.78	0.0106	
BC	1.45	1	1.45	0.14	0.7218	
A^2	821.01	1	821.01	80.27	0.0003	
B^2	396.80	1	396.80	38.80	0.0016	
C^2	943.36	1	943.36	92.23	0.0002	
Residual	51.14	5	10.23			
Lack of Fit	20.56	3	6.85	0.45	0.7450	Not significant
Pure Error	30.58	2	15.29			
Cor Total	3970.94	14				

Table 4
Variance analysis results based on CK yield

Source	Sum of Squares	df	Mean Square	F value	p-value Prob > F	
Model	1784.35	9	198.26	39.28	0.0004	Significant
A-temperature	109.30	1	109.30	21.66	0.0056	
B-ratio of enzyme to substrate	44.42	1	44.42	8.80	0.0313	
C-pH	467.87	1	467.87	92.70	0.0002	
AB	13.76	1	13.76	2.73	0.1596	
AC	21.30	1	21.30	4.22	0.0951	
BC	7.32	1	7.32	1.45	0.2825	
A^2	248.27	1	248.27	49.19	0.0009	
B^2	138.29	1	138.29	27.40	0.0034	
C^2	863.49	1	863.49	171.08	< 0.0001	
Residual	25.24	5	5.05			
Lack of Fit	12.02	3	4.01	0.61	0.6712	Not significant
Pure Error	13.21	2	6.61			
Cor Total	1809.58	14				

Figure 8 should be here

Figure 9 should be here

3.9 Effects of different metal ions on enzymatic hydrolysis

The effect of metal ions on the conversion reaction of ginsenoside Rb1 is shown in Fig. 10. At 0.5 mM metal ion concentration, Ca^{2+} has the greatest effect on promoting the yield of ginsenoside F2, with a relative conversion rate of 78.03%. The metal ion with the greatest effect on the yield of ginsenoside CK was Co^{2+}, with a relative conversion rate of 56.10%. Other metal ions all have different degrees of promoting effect on the conversion reaction of ginsenoside Rb1, except for Fe^{2+}. Combining the yields of ginsenoside F2 and CK, it can be concluded that Co^{2+} has a significantly higher promoting effect on the enzymatic reaction than the other metal ions. It provides a reference value for the selection of metal ions during the preparation of immobilized enzyme materials.

Figure 10 should be here

4 Conclusions

In summary, mixed enzymes were isolated from the safe strain *A. niger* Wu-16 to prepare the production of rare saponin F2 and CK by catalyzing the substrate Rb1. The rare saponin F2 and CK could be increasingly accumulated when the substrate Rb1 is mixed with these extracellular enzymes. The results showed that this multi-enzyme could be used as biocatalyst to effectively convert the non-rare saponin Rb1 into the rare ginsenoside F2 and CK with Rd as intermediate product. To increase the production of rare saponin F2 and CK, we studied the effects of temperature, pH, reaction time, and enzyme substrate ratio on the conversion reaction. On this basis, the effect of metal ions on the conversion efficiency of the enzymes was explored. The promotion of the enzymatic reaction of Co^{2+} was remarkably higher than that of other metal ions, the enzyme conversion efficiency was the highest, and the conversion rate reached 56.10%. Interestingly, difference in pH affected the accumulation of the final product. Target product CK would accumulate in large quantities at pH = 3, while another high value-added rare ginsenoside F2 would accumulate in large quantities at pH = 4. The pH was adjusted to obtain the target product. This way of producing the rare saponin F2 and CK reported in this study has mild conditions, low cost, relative short reaction time, and high conversion efficiency in the catalysis of natural enzymes. The research results on the influence of reaction factors will help the commercial production of rare food-grade saponin such as F2 and CK. It provides a green and efficient method for the production of rare ginsenosides F2 and CK, laying a foundation for industrial production.

Declarations

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Author contributions All authors have contributed to the research concept and design. YF conceived and designed this research, specifically, conducting experiments, analyzing data and writing manuscripts. TZD participated in the screening of strains, and LXC participated in the resolution of problems during the experiment and the revision of the manuscript. FDD is mainly involved in project management, and WZS's main work is fund acquisition; project management; resources; supervision; writing-review and editing.

Data availability All relevant data generated during this study are included in the article.

Code availability Not applicable.

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate Not applicable.

Consent for publication All authors read the final manuscript and approved its submission to Biotechnology Letters.

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Figures

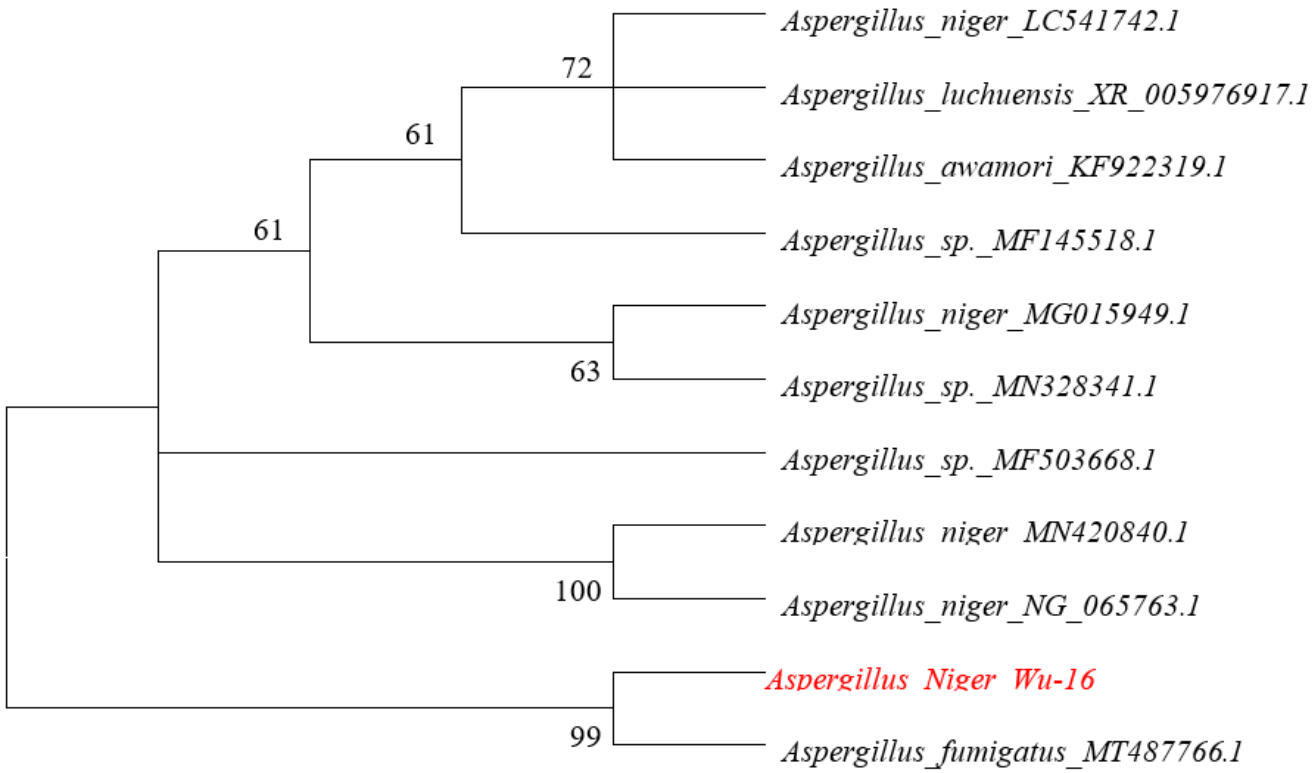

Figure 1

Phylogenetic tree based on ITS gene sequences showing the phylogenetic relationships between *Aspergillus niger* Wu-16 and related *Aspergillus* species

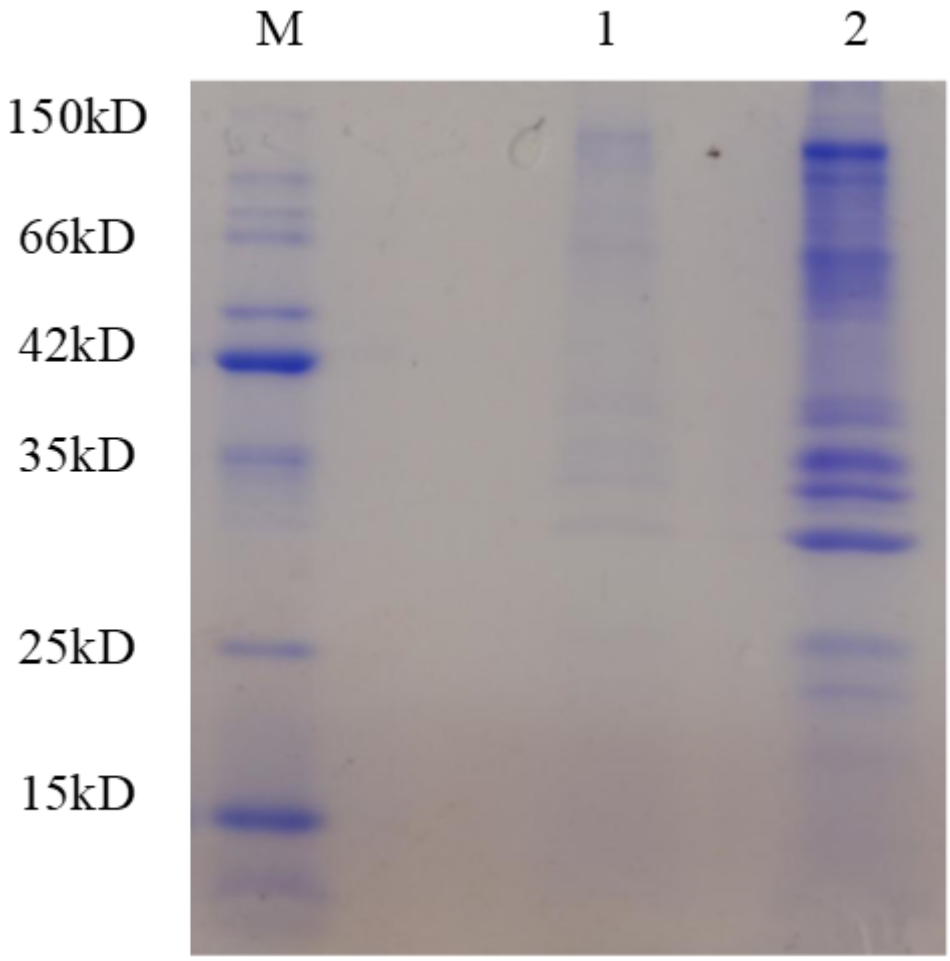

Figure 2

From the SDS-PAGE before and after concentrate of the enzyme produced by *Aspergillus niger* Wu-16. Lane M: Protein molecular mass marker, Lane 1: Before concentrate, Lane 2: After concentrate

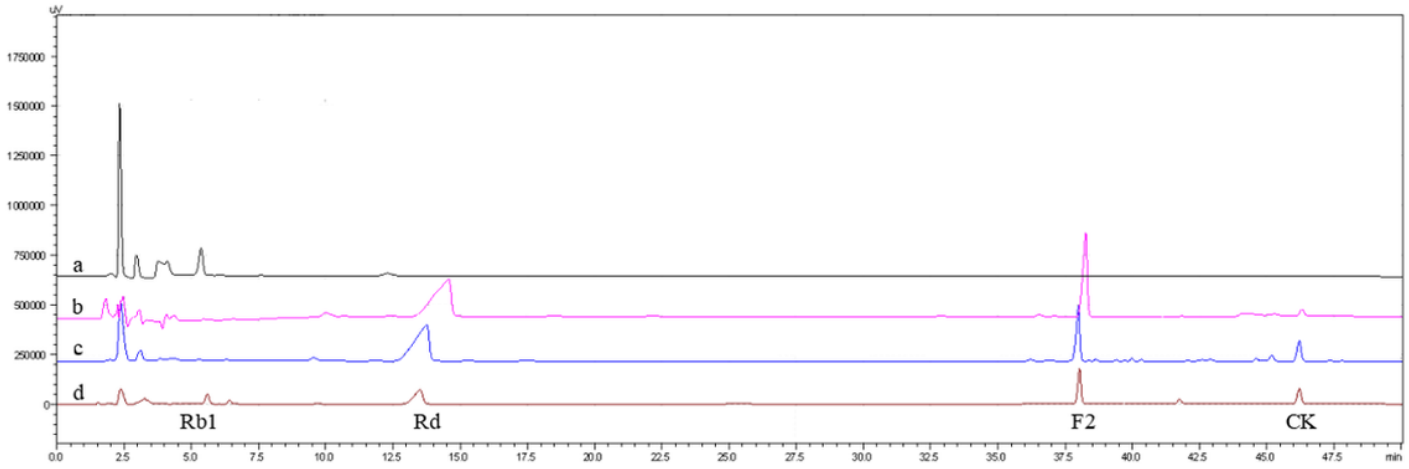

Figure 3

HPLC chart of the ginsenoside Rb1 substrate (a) and its enzymatic reaction for transform products with 24 h (b) and 48 h (c), and the standard products (d)

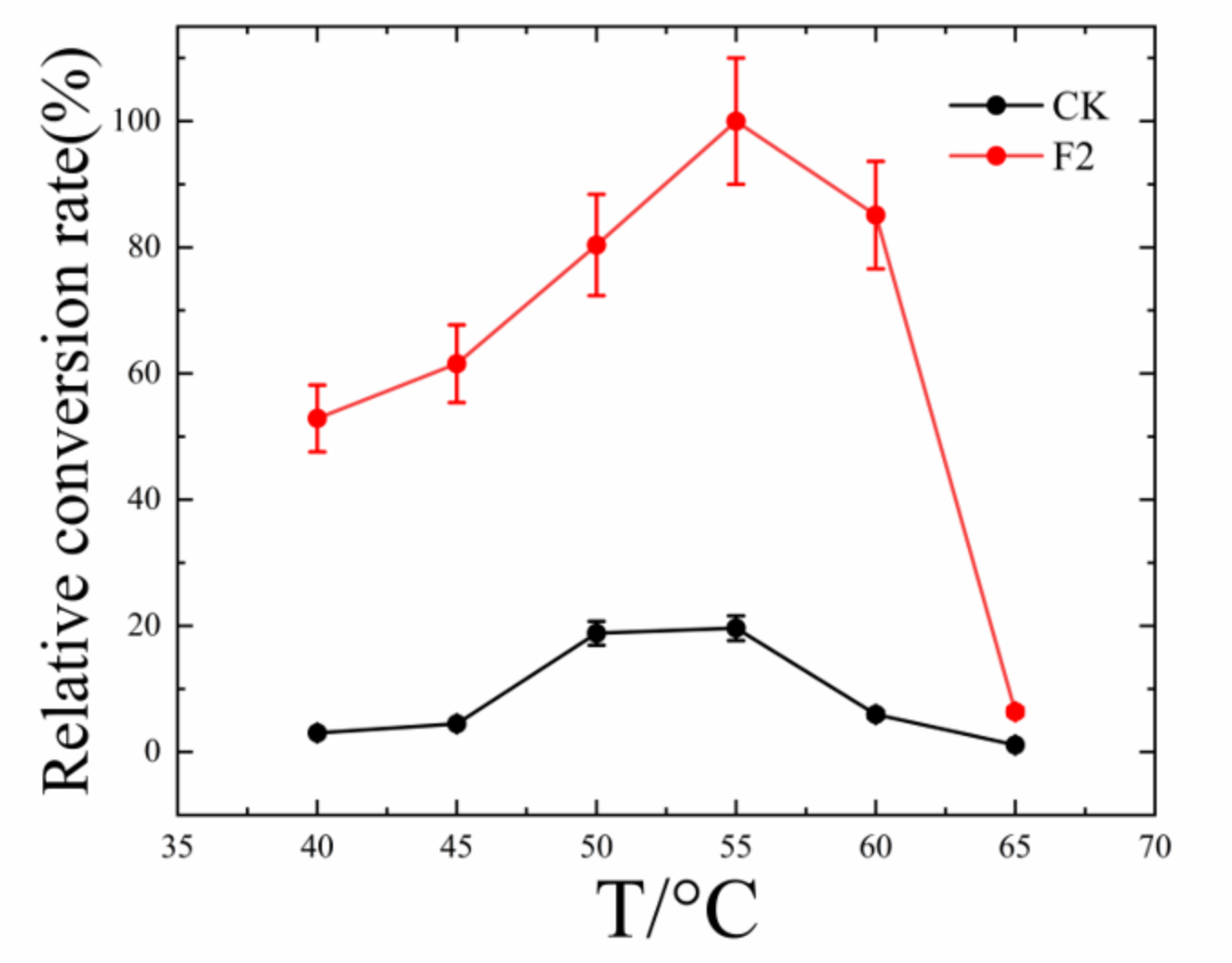

Figure 4

Effect of temperature on enzymatic hydrolysis as determined by adding 10 mg substrate, 0.27 mg enzyme, and 0.5 mL of pH=5.0 glacial acetic acid–anhydrous sodium acetate buffer and reacting the, at different temperatures (40–65°C) for 36 h

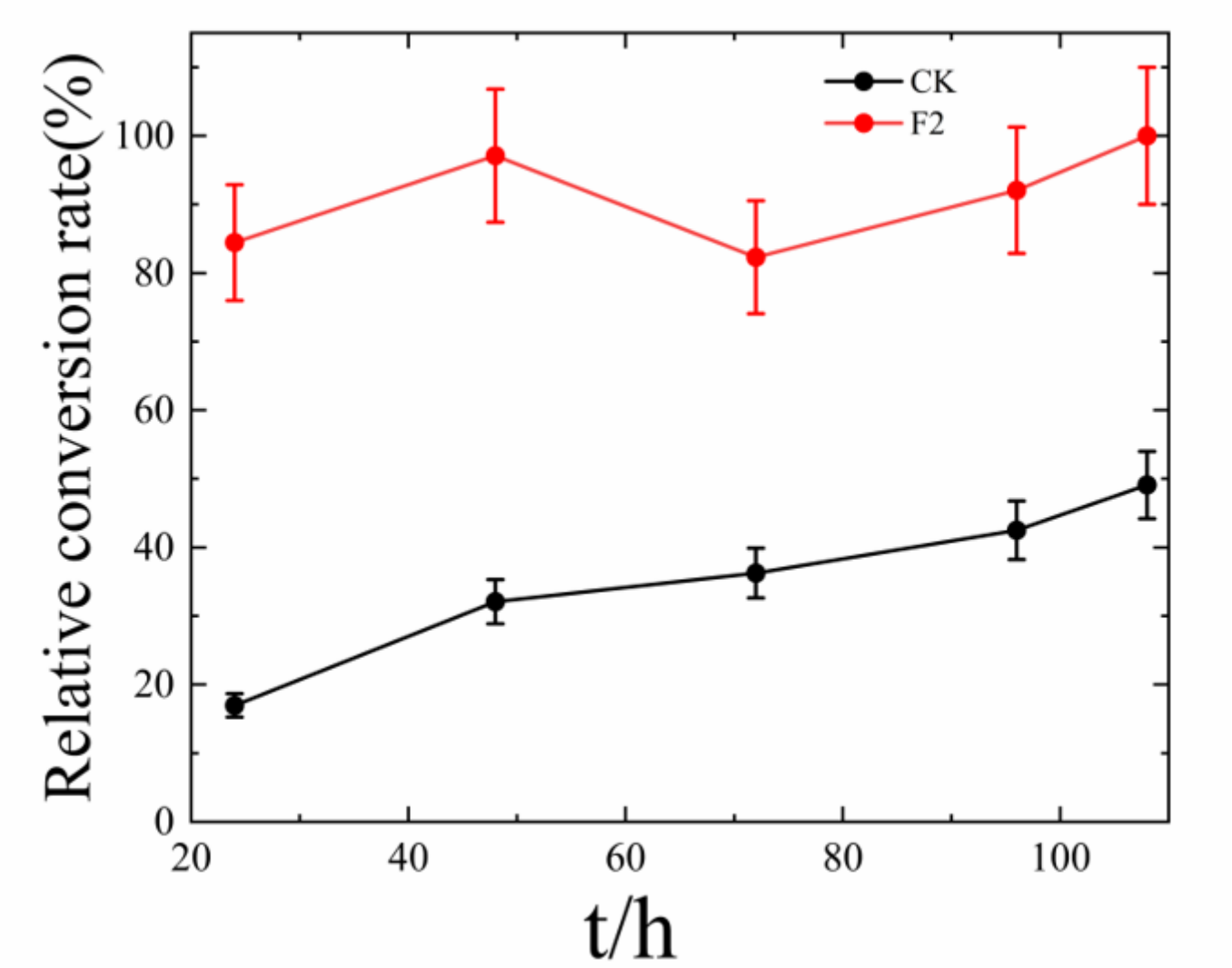

Figure 5

Effect of time on enzymatic hydrolysis. By adding 10 mg substrate, 0.27 mg enzyme, and 0.5 mL of pH=5.0 glacial acetic acid–anhydrous sodium acetate buffer, reaction was carried out at 55°C for 24–108 h

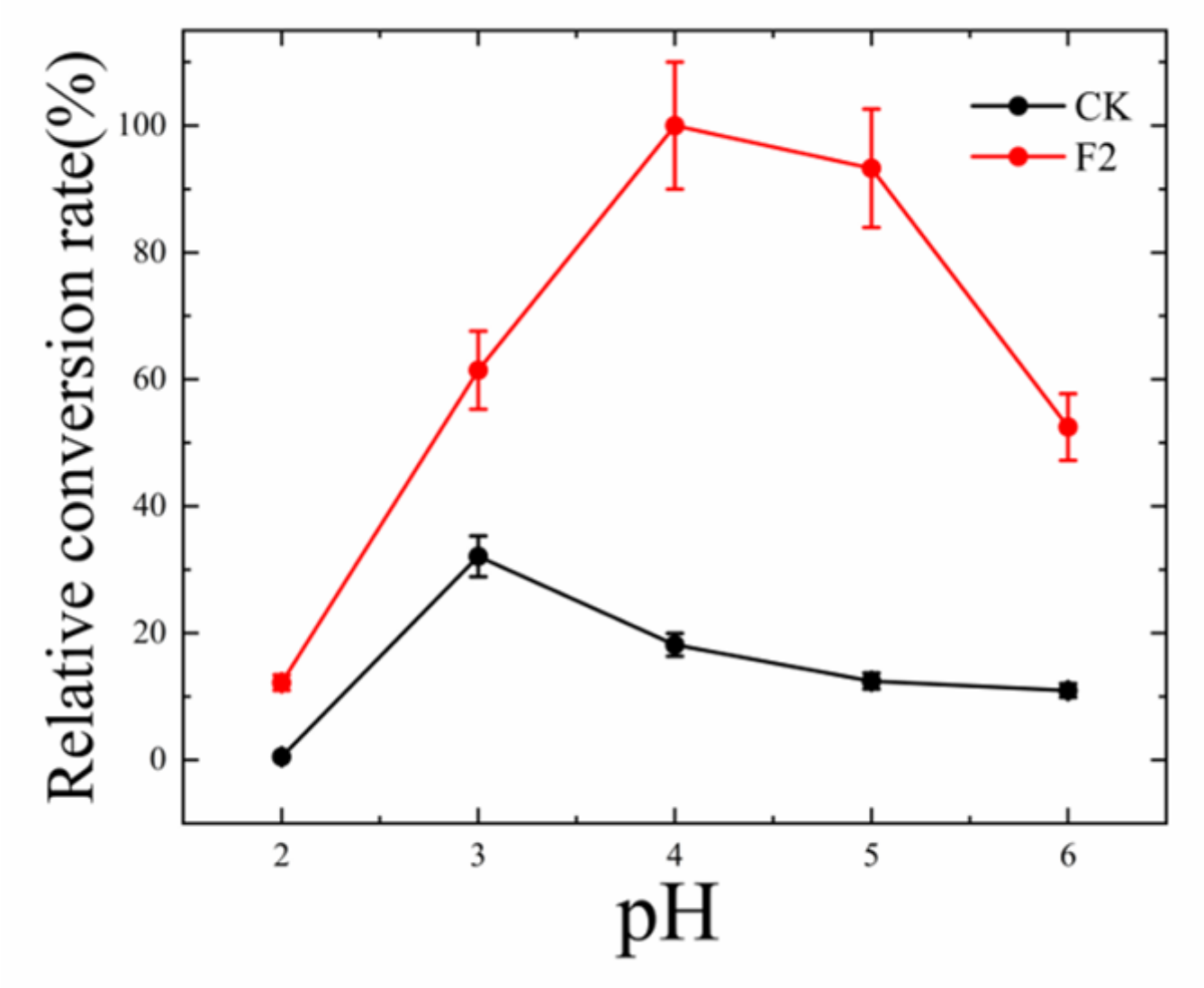

Figure 6

Influence of pH value on enzymatic hydrolysis. The reaction was performed at 45°C for 36 h by adding 10 mg substrate, 0.2 mg enzyme, 0.5 mL buffer of different pH values (glycine–hydrochloric acid buffer (pH 2-–3), and glacial acetic acid–anhydrous sodium acetate buffer (pH 4.0–6.0)

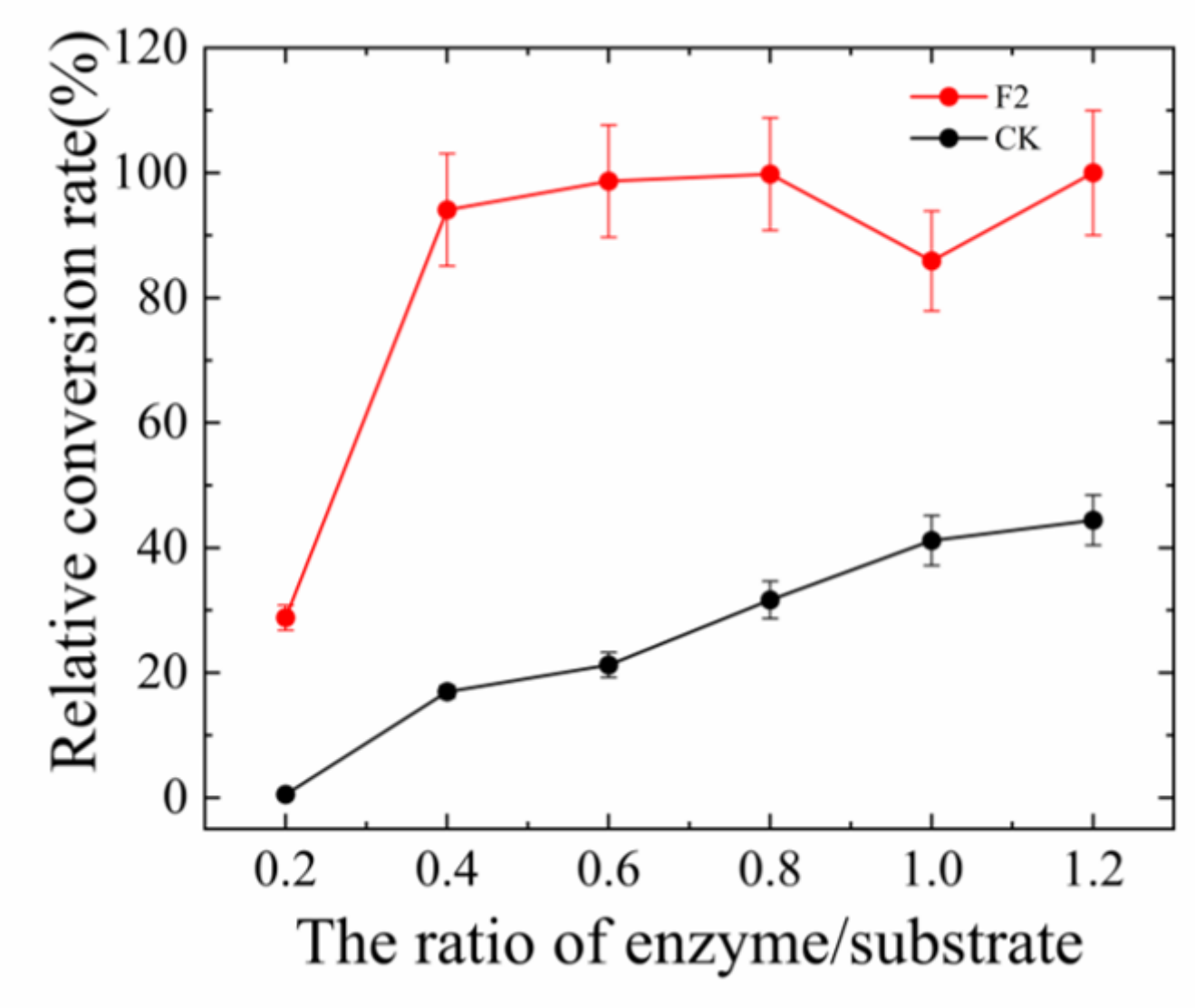

Figure 7

Influence of the ratio of enzyme to substrate on enzymatic hydrolysis. By adding 10 mg substrate, 0.5 mL glacial acetic acid–anhydrous sodium acetate buffer with pH=5.0, and lyophilized enzymes of different quality (1–12 mg), the reaction was performed for 36 h at 45°C

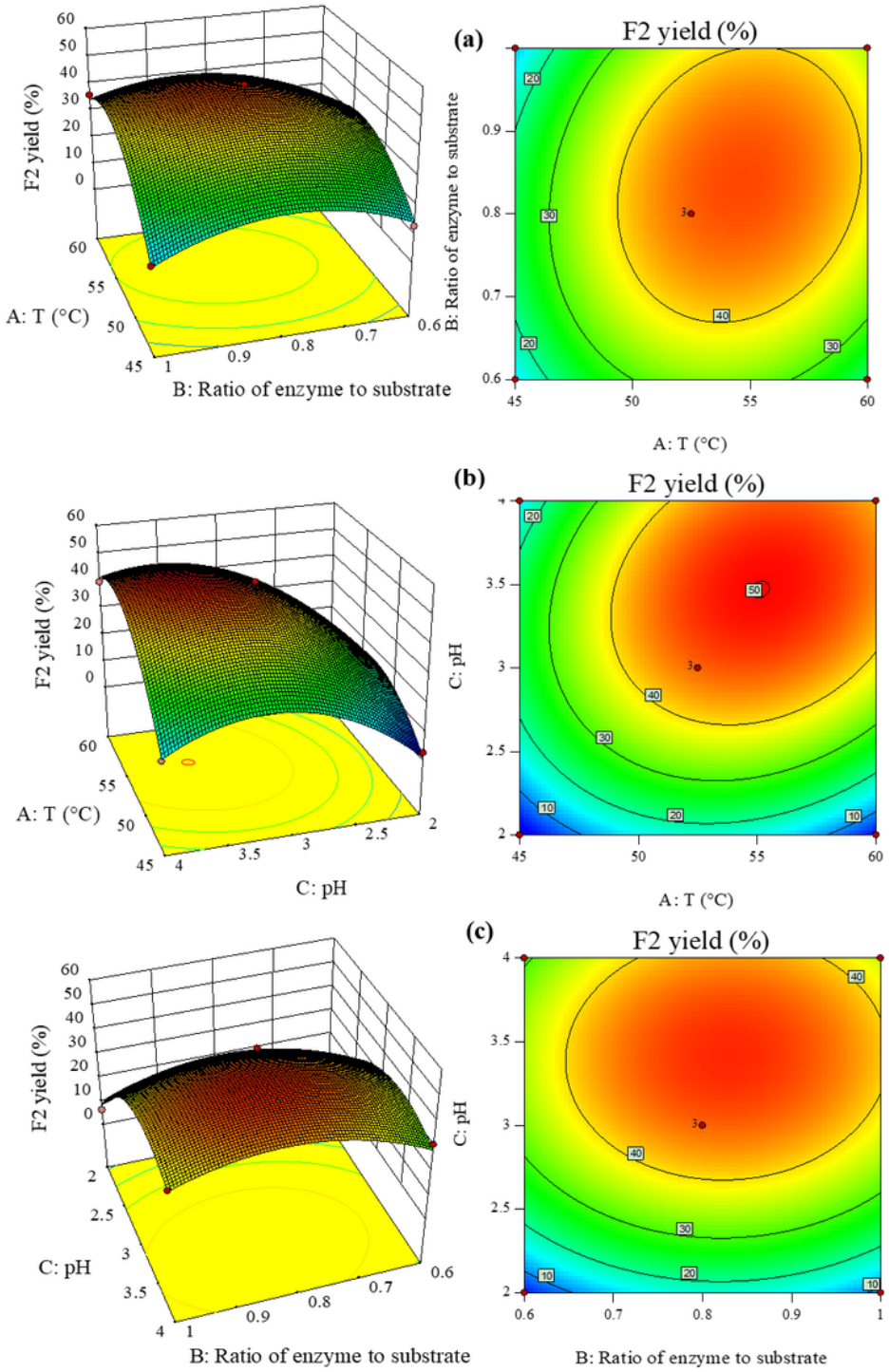

Figure 8

Three-dimensional surface and interaction effect and contour map of the three factors of enzymatic hydrolysis temperature, substrate to enzyme ratio, and pH on the yield of ginsenoside F2

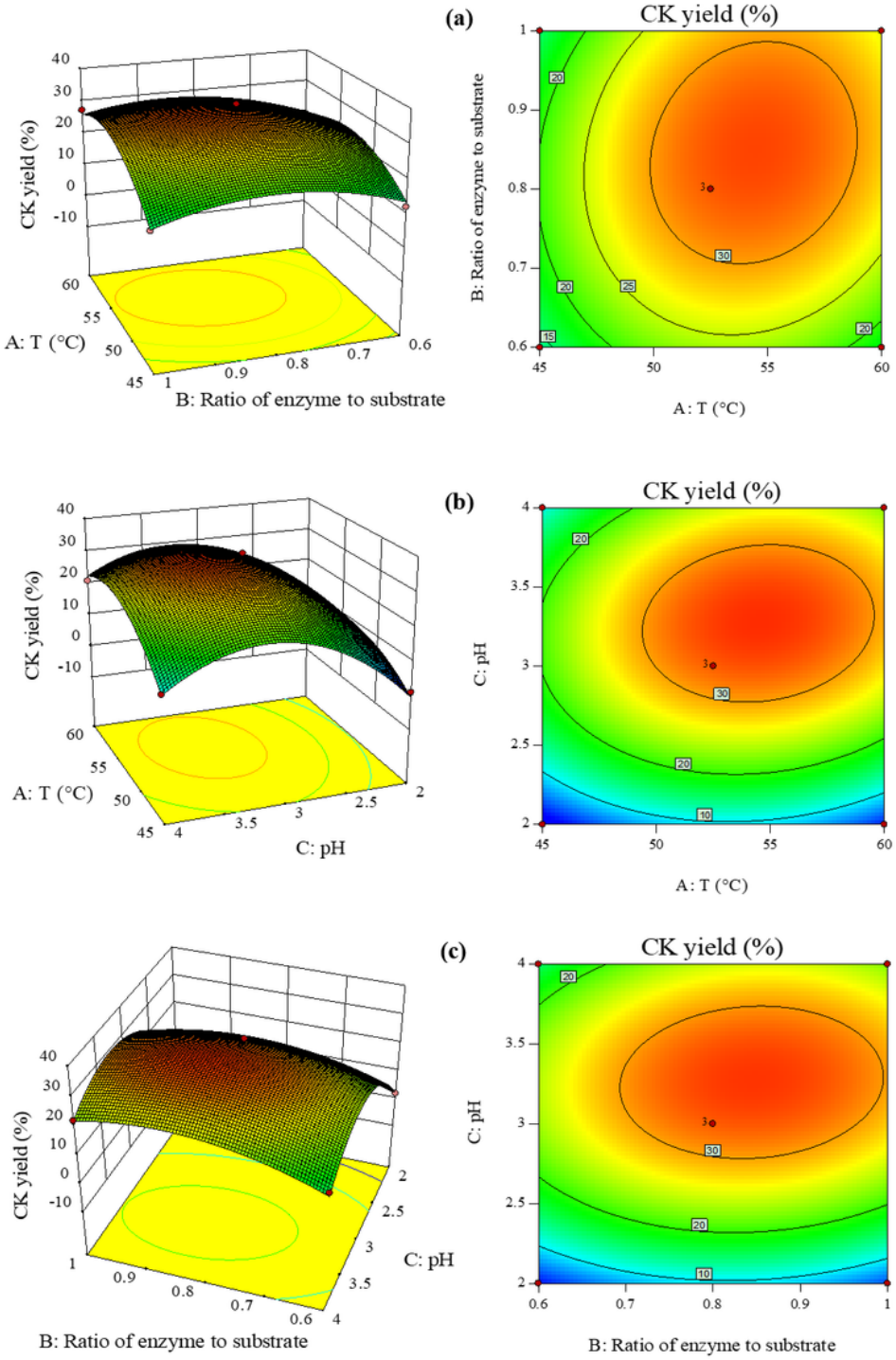

Figure 9

Three-dimensional surface and interaction effect and contour map of the three factors of enzymatic hydrolysis temperature, substrate to enzyme ratio, and pH on the yield of ginsenoside CK

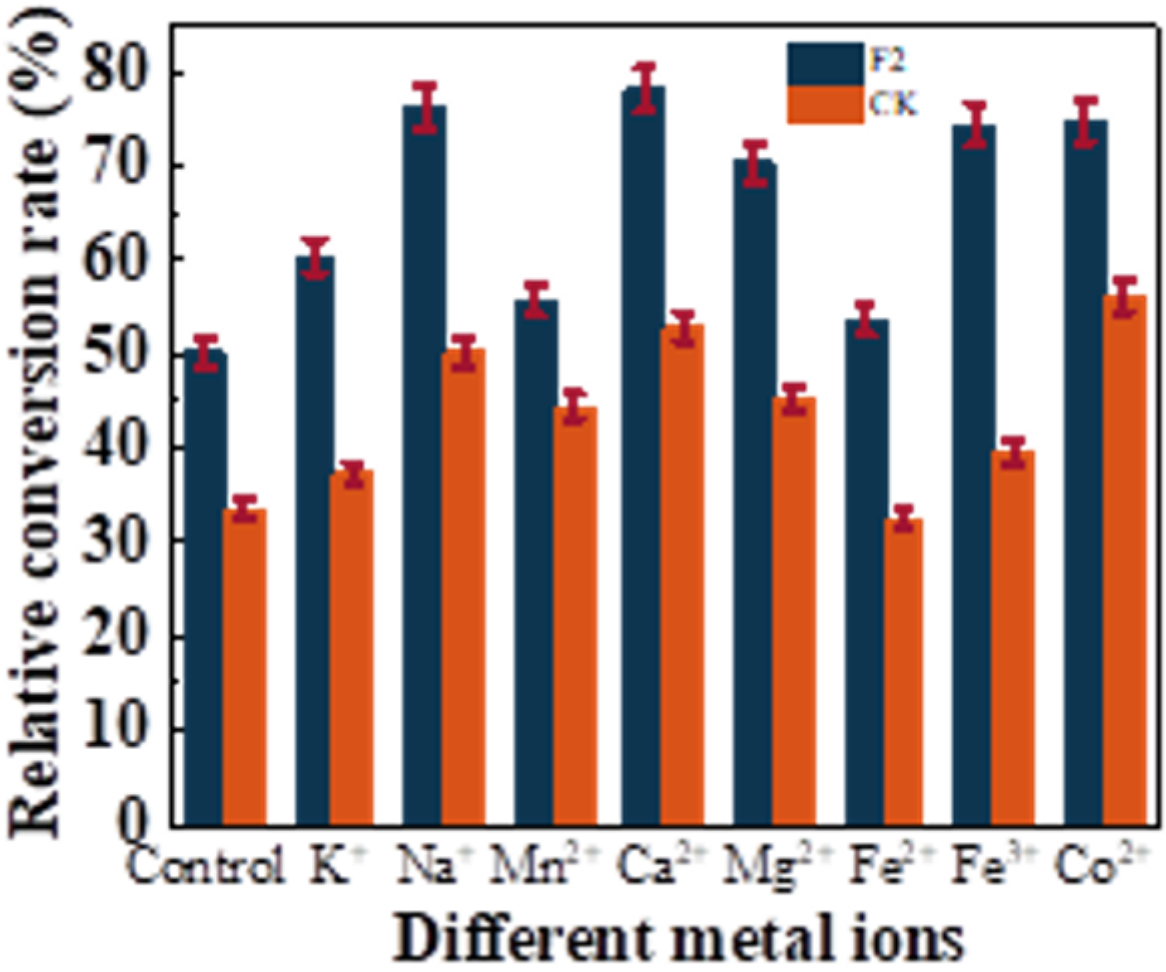

Figure 10

Effects of different metal ions on the ginsenoside F2 and CK-producing activity of extracellular enzymes from *A. niger* Wu-16 using ginsenoside Rb1

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