Heliyon 8 (2023) e12586

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Optimization of *Cordyceps sinensis* fermentation *Marsdenia tenacissima* process and the differences of metabolites before and after fermentation

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ARTICLE INFO

Keywords:
Medicinal fungi
Response surface method (RSM)
UHPLC-Q-TOF-MS
Traditional Chinese medicine
Two-way liquid fermentation

ABSTRACT

In this paper, we explored the interaction of factors which influenced the *Cordyceps sinensis* fermentation *Marsdenia tenacissima* (Roxb.) Wight et Arn, a *Dai* (a national minority of China) medicine, and the optimal fermentation conditions. The differences of *C. sinensis* metabolites in normal state (CN) and products of two-way liquid fermentation of *C. sinensis* and *Marsdenia tenacissima* (CM) and *Marsdenia tenacissima* (MT). The interactive effect of factors was analyzed and the best conditions are obtained through the box-behnken design (BBD) in response surface methodology (RSM). All metabolites were determined by ultra high performance liquid chromatography quadrupole time of flight mass spectrometer (UHPLC-Q-TOF-MS), analyzed and identified by metabonomics technology. Results showed that the optimum fermentation conditions were the concentration of raw medicinal materials is 160 g/L, the fermentation time is 6 days, the inoculation volume is 9.5%, the rotating speed is 170 rpm. 197 metabolites were identified in both positive ion and negative ion. 119 metabolites were significantly different between CN and CM. 43 metabolites were significantly different between CM and MT. Differential metabolic pathways were enriched. In conclusion, this paper optimizes the bidirectional fermentation process of *M. tenacissima* and *C. sinensis* through response surface methodology, and analyzes the changes of components from the level of metabonomics, so as to provide reference for exploring medicinal fungi fermentation of traditional Chinese medicine.

1. Introduction

Cordyceps sinensis, a valuable traditional Chinese medicine, a fungus of the genus Cordyceps of ergot family [1]. It is an ascomycete parasitic on the larvae of bats and moths. According to traditional Chinese medicine theory, its gas is slightly fishy, its taste is slightly bitter, its nature is sweet and flat, and it belongs to the lung and kidney meridians. It can be used to treat some diseases caused by lung and kidney deficiency, such as cough, asthma, hemoptysis and sweating caused by lung deficiency [2]. It can also be used to treat a series of symptoms caused by kidney deficiency, such as sored waist and knees, pain in back and loin, dizziness and tinnitus, hair loss and premature senility [3]. Its extract has anti-tumor [4], anti-HIV and anti-oxidant effects [5], as well as anti-aging [6] and whitening effects. *C. sinensis* powder is a dry powder obtained from the mycelium isolated from *C. sinensis* through fermentation. It can promote metabolism and enhance the immune capacity of the body. At present, the main ingredient of Jin-shui-bao capsule (a drug sold in the Chinese market) is Fermented *C. sinensis* powder. Fermented *C. sinensis* powder has similar efficacy to *C. sinensis* [7, 8].

Marsdenia tenacissima (Roxb.) Wight et Arn, also known as "Tong-guan-teng" or "Wu-gu-teng" in traditional Chinese medicine, it is the stems and roots of *M. tenacissima*, a plant of Asclepiadaceae family. It tastes bitter and cold, and belongs to the lung meridian. It has the effects of relieving cough and asthma, eliminating phlegm, promoting lactation, clearing away heat and toxic material [9]. About 196 chemical constituents have been identified from different parts of the plant, including steroids, triterpenes and organic acids. Steroids are the main characteristics and bioactive components of this plant [10]. At present, the single raw material of Xiao-ai-ping injection, a commercially available anti-cancer drug, is *M. tenacissima* [11]. Its extract also has certain anti-tumor pharmacological effects [12]. It was found that *M. tenacissima* extract can inhibit the proliferation, migration and invasion of lung cancer cells [13].

Medicinal fungi refer to the fungi used as drugs to treat diseases. China is also the first country to use medicinal fungi to prevent and treat diseases. The medicinal fungi recorded in "Shennong Bencao Jing", one

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https://doi.org/10.1016/j.heliyon.2022.e12586
Received 12 July 2022; Received in revised form 28 October 2022; Accepted 15 December 2022

of the well-known ancient classic Chinese herbal medicine books dated back to 220–280 AD include *Poria cocos, Ganoderma lucidum, C. sinensis, Auricularia*, etc. In their metabolic activities of growth and development, they can produce enzymes, proteins, fatty acids, amino acids, peptides, polysaccharides (carbohydrates), alkaloids, sterols, terpenes, glycosides, vitamins and other substances with pharmacological activities or inhibitory or therapeutic effects on human diseases in mycelium, sclerotia or fruiting body [14]. Clinically, they can directly use mycelium, sclerotia or fruiting body, or use the effective substances isolated from the fungi.

The two-way liquid fermentation technology is adopted, and its products include mycelium and fermentation liquid. Typical examples are "*Cordyceps*". Some only use mycelium, and some concentrate and dry the fermentation liquid and apply it together or separately with mycelium. Some directly apply fungi powder extract, and some extract polysaccharide and other effective components before they are used for medicine [15]. Through the fermentation of medicinal fungi and traditional Chinese medicine, on the one hand, the extract of traditional Chinese medicine is used as the base material to produce medicinal fungus chemical components for the fermentation and culture of medicinal fungi [16]. At the same time, It may enhance the metabolic activities of medicinal fungi and enhance their pharmacological effects, fermentation shortens the medicinal fungi propagation cycle, reduces the production cost and improves the production efficiency. On the other hand, it is conducive to increase the precipitation of active components in traditional Chinese medicine rattan and produce new chemical components and new efficacy [17]. As a new processing method of traditional Chinese medicine, it broadens the application scope of traditional Chinese Medicine [18].

Response surface methodology (RSM) is an experimental statistical method rising in the West in the early 1990s. It is a statistical method to fit the functional relationship between factors and response values in a small area with a simple primary or quadratic polynomial model [19]. It is mainly used to explore the optimal fermentation conditions to obtain the most fermentation products. In the study of succinic acid produced by *Actinobacillus succinogenes* fermentation, response surface methodology was used to optimize the production process [20].

In recent years, metabonomics is a newly developed discipline after genomics and proteomics. It is an important part of systems biology. After that, it has developed rapidly and penetrated into many fields such as traditional Chinese medicine and food field [21]. This technique can be used to identify different kinds of traditional Chinese medicine and analyze their property differences [22, 23]. It can also be used to analyze the changes of components before and after fermentation [24, 25]

At present, the relevant research on the fermentation of *C. sinensis* mainly focuses on the yield, structure and activity of extracellular polysaccharides of its fermentation products [26]. There are few studies on the process optimization of traditional Chinese medicine fermentation by *C. sinensis* and the related metabolites. In this paper, we explored the factors affecting the fermentation of *C. sinensis* and the optimal fermentation conditions through single factor experiment and response surface experiment, and then the changes of metabolites *C. sinensis* before and after fermentation are analyzed by metabonomics method. Finally, enriched differential metabolic pathways.

Table 1. Levels of influencing factors.

Factors	Levels		
	-1	0	1
A [Drug concentration (g/L)]	80	140	200
B [fermentation time (d)]	2	5	8
C [Inoculation volume(%)]	6	8	10
D [Speed(rpm)]	120	150	180

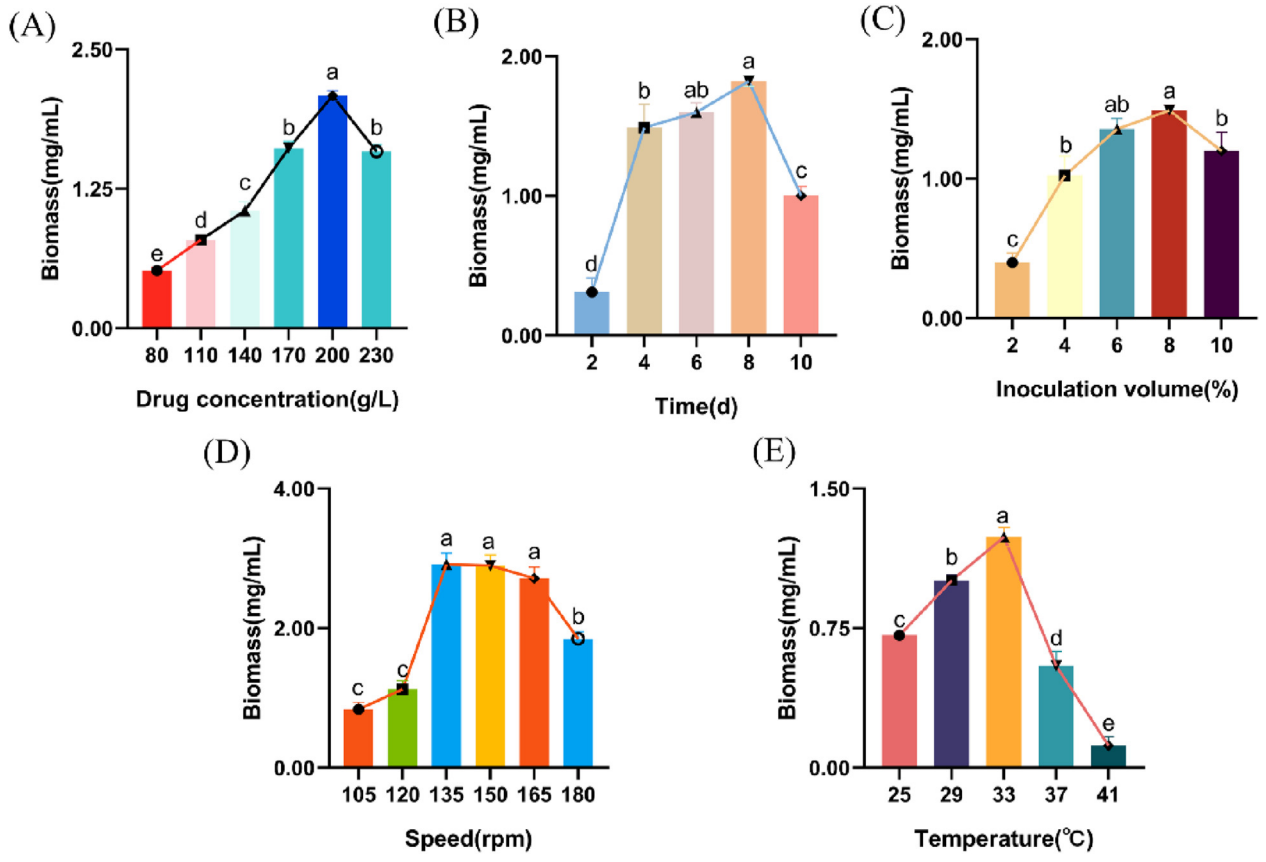

Figure 1. Effect of single factor on biomass (A) Effect of drug concentration on biomass (B) Effect of time on biomass (C) Effect of inoculation on biomass (D) Effect of speed on biomass (E) Effect of temperature on biomass abcde indicates the difference at each point ($p < 0.05$), and the same letter indicates no significant difference.

2. Material and methods

2.1. Experimental strain

It is deposited in laboratory 404, school of pharmacy, Minzu University of China, Beijing, China.

2.2. Main reagent

The purity of water, acetonitrile, methanol and isopropanol is LC-MS grade, and the brand is Thermo Fisher Scientific (Waltham, USA). The purity of ammonium acetate is LC-MS grade, and the brand is Sigma Chemical Co. (St Louis, MO, USA).

2.3. Main medium

LB solid medium: 2% glucose, 0.4% beef extract, 0.5% peptone, 0.2% potassium dihydrogen phosphate and 1.6% agar powder.

LB liquid medium: 2% glucose, 0.4% beef extract, 0.5% peptone, 0.2% potassium dihydrogen phosphate.

M. tenacissima liquid medium: The medicinal material *M. tenacissima* was extracted with water and precipitated with alcohol, and then dissolved in water to make a liquid medicine with the original medicinal materials concentration of 80 g/L, 140 g/L and 200 g/L.

2.4. Main instruments

High performance liquid chromatography, Model: ExionLC Brand: AB SCIEX, High resolution mass spectrometry, Model: SCIEX Triple TOF 5600 + Brand: AB SCIEX, Chromatographic column, Model: ACQUITY UPLC HSS T3 1.8 μm(2.1 × 100 mm), Brand: Waters, Centrifuge, Model: Legend Micro 17R Brand: Thermo, vertical pressure steam sterilizer, model: LC-50LD, Brand: Binjiang Medical clean platform, Model:SW-CJ-2FD, Brand: BoXun. Constant temperature culture oscillator, Model: ZWY-100D, Brand: ZHICHENG. Electronic balance, Model: AL104, Brand: METTLER TOLEDO. Electric blast drying oven, Model:101-1AB, Brand: TAISITE.

2.5. C. sinensis activation and propagation

The frozen *C. sinensis* was inoculated into LB solid medium and was cultured in a 25 °C *incubator* for 3–5 days, The activated *C. sinensis* was inoculated into LB liquid medium, and was cultured in the shaker for 3–5 days, keeping them in 4 °C for standby.

2.6. Single factor experiment

The fermentation time, temperature and inoculation volume, drug concentration was designed as single factor variables, and other factors remained unchanged. The setting time is 2 d, 4 d, 6 d, 8 d and 10 d, the temperature is 25 °C, 29 °C, 33 °C, 37 °C and 41 °C, and the inoculation volume is 2%, 4%, 6%, 8% and 10%. Three parallel experiments were set in each group with the biomass (The ratio of the weight of dried mycelium to the volume of fermentation liquid) as index. Providing reference for subsequent BBD experiment parameter setting.

2.7. BBD experiment design

Based on the results of the single factor experiment, The concentration of raw medicinal materials, fermentation time, inoculation volume and speed were designed as the influencing factors [27]. It was carried out by using Design. Expert13.0 software to determine the best fermentation process of *C. sinensis* fermentation *M. tenacissima*. Three levels were set, including low, medium and high levels, and the biomass was the response value (Table 1).

Table 2. Response surface regression model analysis of variance.

Source	Sum of Squares	df	Mean Squares	F-value	p-value
model	8.89	14	0.63	7.54	0.00
A	1.47	1	1.47	17.45	0.00
B	2.56	1	2.56	30.43	0.00
C	0.65	1	0.65	7.68	0.02
D	1.32	1	1.32	15.70	0.00
AB	0.11	1	0.11	1.34	0.27
AC	0.03	1	0.03	0.38	0.55
AD	0.03	1	0.03	0.34	0.57
BC	0.00	1	0.00	0.05	0.83
BD	0.03	1	0.03	0.39	0.54
CD	0.00	1	0.00	0.04	0.85
A^2	0.30	1	0.30	3.53	0.08
B^2	2.45	1	2.45	29.07	0.00
C^2	0.01	1	0.01	0.14	0.72
D^2	0.38	1	0.38	4.51	0.05
Residual	1.18	14	0.08		
Lack of fit	0.84	10	0.08	1.00	0.55
Pure Error	0.34	4	0.08		
Cor Total	10.07	28			

2.8. Test sample design and extraction of metabolites

Three groups of samples to be tested are designed, namely *C. sinensis* fermented in normal state (CN) and products of two-way liquid fermentation of *C. sinensis* and *M. tenacissima* (CM). *M. tenacissima*

Table 3. BBD experiment design and results.

Number	A	B	C	D	Actual biomass (mg/mL)	Prediction Biomass (mg/mL)
1	140	5	10	120	1.22	1.33
2	140	2	10	140	1.06	0.82
3	80	5	8	120	0.70	0.69
4	80	8	8	140	0.72	0.86
5	80	5	6	140	1.12	0.82
6	140	2	8	180	0.84	0.67
7	200	5	6	140	2.07	1.69
8	140	8	8	180	2.12	1.77
9	200	5	10	140	1.97	1.98
10	200	5	8	180	1.94	2.05
11	140	5	6	180	1.44	1.53
12	200	2	8	140	0.57	0.63
13	140	5	8	140	1.93	1.74
14	140	2	6	140	0.14	0.42
15	200	5	8	120	1.21	1.22
16	140	5	8	140	1.84	1.74
17	140	5	8	140	2.06	1.74
18	80	5	8	180	1.09	1.18
19	140	5	10	180	1.83	2.05
20	140	5	8	140	1.39	1.74
21	200	8	8	140	1.71	1.90
22	140	5	8	140	1.49	1.74
23	140	2	8	120	0.14	0.18
24	80	5	10	140	1.38	1.46
25	140	8	10	140	2.00	1.81
26	80	2	8	140	0.26	0.27
27	140	5	6	120	0.95	0.92
28	140	8	6	140	0.95	1.28
29	140	8	8	120	1.06	0.93

medicinal liquid (MT). Three parallel samples were designed for each group. Making the fermented product into powder, then grinding and crushing the sample, which was weighed 10 mg into EP tube, adding 500 uL of cracking solution (MeOH: H2O = 7:3), vortexing and mixing for 1 min, Ultrasonic treatment for 30 min, and finally the sample was centrifuged at 12,000 rpm for 20 min.

2.9. Chromatographic separation

The sample was placed in the automatic injector for injection, column temperature: 40 °C, mobile phase: A-water (containing 0.1% FA), B-acetonitrile (containing 0.1% FA), flow rate: 0.3 mL/min, injection volume: 3 μ L. The gradient elution conditions were: 0–2 min, 5% B, 2–14 min, 5% B-98%B, 14–17 min, 98%B, 17–17.1 min, 98%B-5% B, 17.1–20 min, 5% B [28].

2.10. Mass spectrometer conditions

In the both negative ion acquisition mode and positive ion acquisition, IDA high sensitivity scanning mode was adopted, dynamic background subtraction was adopted, and the ion source parameters were set as: sheath gas flow rate 35, gas1 flow rate 55, gas2 flow rate 55, temperature 550, and spray voltage −4.5 kV(−)/5.5 kV(+). The scanning time is 20 min; The primary scanning range is 100–1200 M/Z, each primary scanning is followed by 12 secondary scanning, the secondary scanning range is 50–1200 M/Z, the secondary accumulation time is 0.05 s, the collision energy is – 40(−)/40(+), and the collision energy range is the theoretical frequency ±20 [29].

2.11. Data analysis

The influence plot of single factor on biomass was drawn by GraphPad Prism 9.4 (State of California, USA) software. Response surface plot of interaction among factors on mycelium biomass was drawn by Design Expert 13.0 (State–Ease, Minnesota, USA). Principal component analysis (PCA) [30], and orthogonal partial least squares discriminant analysis (OPLS-DA) [31] were carried out by SIMCA 14.1 (Sweden). Differential metabolites were screened out according to P-value, VIP-value and CM-value. Differential metabolites were identified based on self-built database TCM MS/MS library 2.1 (Beijing, China). And related differential metabolic pathways were enriched based on KEGG database (Kyoto University, Japan) by MetaboAnalyst online website (https://www.metaboanalyst.ca).

3. Results and discussion

3.1. Single factor experiment

The influence of single factor on biomass is shown in Figure 1, the significance of single factor change was analyzed. According to the relevant literature, design the appropriate single factor level. The results showed that the best drug concentration is 200 g/L (Figure 1 (A)), the best fermentation time is 6 d–8 d (Figure 1 (B)), the optimal inoculation volume is 6%–8% (Figure 1 (C)), the optimum speed is 135 rpm–165 rpm (Figure 1 (D)), the optimal growth temperature for *C. sinensis* fermentation is 33 °C (Figure 1 (E)).

It can be seen from the single factor experiment that temperature has a significant impact on the growth of *C. sinensis*. When the temperature exceeds 33°, the growth of *C. sinensis* significantly slows down, When the temperature is up to 41 °C, the enzyme activity is greatly reduced, dehydration, water imbalance, biochemical reaction is too fast, but the metabolism is too fast, resulting in more insufficient energy supply, and ultimately the overall metabolic function and the overall microbial are seriously damaged. When the fermentation time is from the second day to the fourth day, the biomass of the *C. sinensis* increases significantly, which indicates that the *C. sinensis* has reached the logarithmic growth

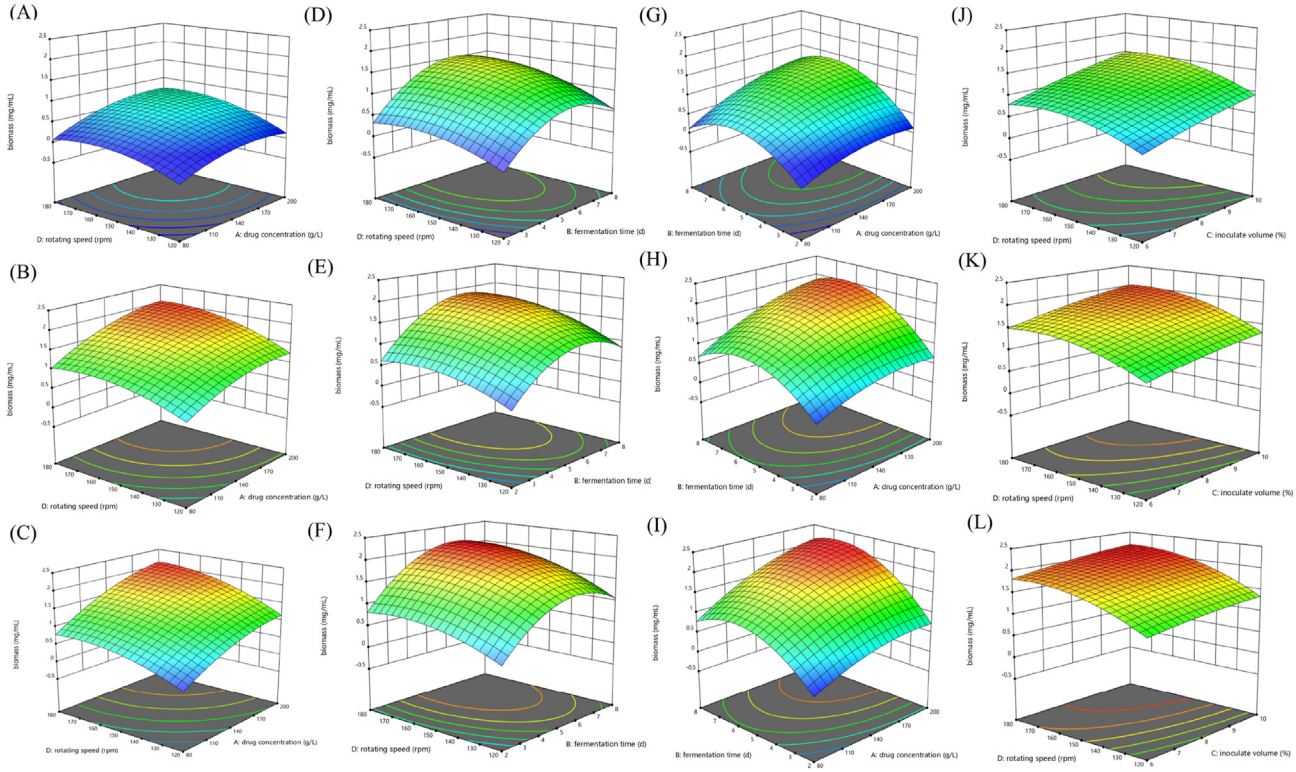

Figure 2. Effects of drug concentration and rotating speed on biomass at fermentation time of 2 days (A), 5 days (B) and 8 days (C) Effects of fermentation time and rotating speed on biomass when the inoculation volume was 6% (D), 8% (E) and 10% (F) The effect of drug concentration and fermentation time on biomass at rotating speed 120 rpm (G), 150 rpm (H), 180 rpm (I) Effects of inoculation volume and rotating speed on biomass at drug concentrations of 80 g/L (J), 140 g/L (K) and 200 g/L (L).

period. At this time, the strain has strong vitality and abundant nutrients, which is most suitable for the growth of the *C. sinensis*. The increase of rotating speed will increase the dissolved oxygen and promote the growth of *C. sinensis* [32]. However, when the rotating speed reaches 135 rpm, the increase of rotating speed has little impact on the growth of *C. sinensis*. This is because the rotating speed is too high and the shear force is too large, which limits the growth of *C. sinensis* [33]. The growth of *C. sinensis* is more vigorous with the increase of substrate concentration. When the substrate concentration is too high, which reach 200 g/L, the difference between the internal and external osmotic pressure of *C. sinensis* is too large, which will lead to its water loss and death. The inoculation volume is also related to the growth of *C. sinensis*. When the inoculation volume is less than 8%, there is a positive correlation between them. When the inoculation volume is increased again, the growth of *C. sinensis* will also be limited due to insufficient dissolved oxygen.

3.2. RSM experiment results

3.2.1. ANOVA analysis

ANOVA was used to evaluate the quality of the fitted model (Table 2), the *p* value of the model is 0.0003, less than 0.01, which is extremely significant, and the *p* value of the lack of fit is 0.5497, which is not significant. The corrected R2 value is 0.7658, indicating that the model has 76.58% reliability, and the absolute R2 value is 0.8829, indicating that the fitting degree of the model is good, and the regression model can be used to analyze and predict the actual value of biomass. It can be seen from Table 2 that the fermentation time has the greatest impact on the biomass of mycelium (*p* value is less than 0.0001, which is extremely significant), followed by the drug concentration of *M. tenacissima*, followed by the rotating speed of shaker, and finally the inoculation volume. BBD experiment design and results are shown in Table 3, According to the experimental conditions, the biomass ranged from 0.13533 to 2.11867 (Table 3).

The biomass was fitted to the drug concentration, fermentation time, inoculation volume and rotating speed, and the coding equation

$$Y = -9.68536 + 0.016724A + 0.511784B + 0.291633C + 0.076322D + 0.000934AB - 0.000743AC + 0.000047AD + 0.005333BC + 0.001009BD + 0.000472CD - 0.000059A^2 - 0.06826B^2 - 0.010564C^2 - 0.000269D^2$$

3.2.2. Interaction among influencing factors

The fermentation time is the shortest, and the drug concentration and rotating speed have little impact on the biomass. This may be because the fermentation time is too short, *C. sinensis* has not adapted to the new environment, and cannot make full use of the nutrients in the culture medium. Increasing the rotating speed which means increasing the amount of dissolved oxygen, and increasing matrix concentration has little impact on the biomass. When the fermentation time is long, the drug concentration and rotation speed have a great impact on the biomass, indicating that the metabolic activity of *C. sinensis* becomes vigorous, the oxygen demand increases, and the demand for nutrients increases at the same time (Figure 2 (A)–(C)).

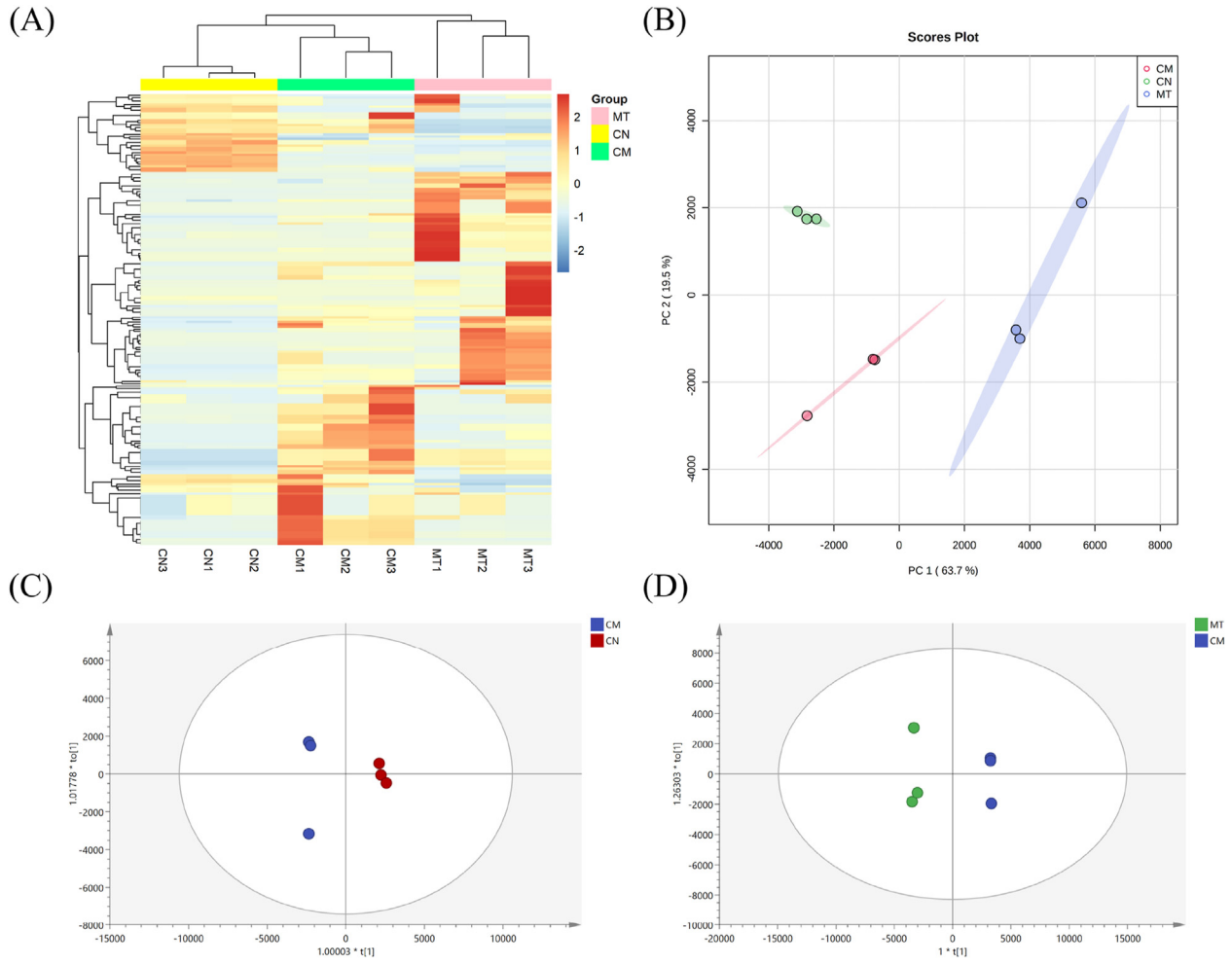

Figure 3. (A) Hierarchical Clustering Analysis (HCA) of CM, CN, MT. (B) Principal component analysis (PCA) score plot of CM, CN, MT. (C) Orthogonal partial least squares discriminant analysis (OPLS-DA) score plot of CM and CN. (D) Orthogonal partial least squares discriminant analysis (OPLS-DA) score plot of MT and CM.

Compared with the lower inoculation volume, the higher inoculation volume requires higher rotation speed to reach the maximum biomass, because the number of fungi increases and the oxygen demand also increases. The fermentation time remained unchanged for about 5 days (Figure 2 (D)–(F)).

With the increase of rotating speed, it is necessary to increase the substrate drug concentration and extend the fermentation time to obtain the maximum biomass. This may because the consumption of nutrients by *C. sinensis* increases with the extension of fermentation time and the increase of rotating speed. Therefore, it is necessary to increase the substrate drug concentration to maintain the consumption of nutrients (Figure 2 (G)–(I)).

When the drug concentration is 80 g/L or 140 g/L, the biomass can be increased by increasing the inoculation amount and rotating speed. When the concentration of matrix drug reached 200 g/l, increasing the rotating speed had a greater impact on the biomass (Figure 2 (J)–(L)).

3.2.3. Optimal fermentation conditions

According to the regression model, the optimal fermentation conditions of *C. sinensis* for *M. tenacissima* are as follows: the concentration of medicinal materials is 164.029 g/L, the fermentation time is 6.368 days, the inoculation volume is 9.260%, and the rotating speed is 177.339 rpm. Under these conditions, the biomass is 2.265 mg/mL. Combined with the actual situation, the parameters are adjusted appropriately. The adjusted fermentation conditions are as follows: the concentration of medicinal materials is 160 g/L, the fermentation time is 6 days, the inoculation volume is 9.5%, and the rotating speed is 170 rpm. Under this condition, the actual biomass is 2.162 mg/mL, The deviation between the actual value and the theoretical value is less than 5%, indicating that the model is true and reliable.

Response surface methodology (RSM) overcomes the limitations of traditional methods, optimizes and evaluates the level of factors affecting the growth of *C. sinensis* and their interactions, and quickly and effectively determines the optimal conditions of the multifactor system in the fermentation process of *C. sinensis* [34]. From the interaction between factors, it can be seen that at the same parameter level, other parameters have different effects on the growth of *C. sinensis*. For example, when the inoculation amount is 10%, choosing to extend the fermentation time can promote the growth of *C. sinensis* more than increasing the rotation speed. The optimum conditions can also provide reference for the fermentation of other medicinal fungi.

3.3. Metabolomic analysis results

3.3.1. Identification and analysis of metabolites

A total of 197 metabolites were annotated, including 52 flavonoid metabolites, 48 organic acid metabolites, 27 saponin metabolites, 18 alcohol metabolites, 12 lipid metabolites, 10 amino acid metabolites, 7 glycoside metabolites, 6 aldoketone metabolites, 6 nucleoside metabolites, 3 amide metabolites, 3 terpenoid metabolites,2 alkaloid metabolites, 2 quinone metabolites, 1 fatty acid metabolite (Figure 3 (A)). The total ion current plot of QC samples shows that there is a high overlap rate between QC samples, indicating the repeatability and reliability of the data (Figures S1 and S2).

3.3.2. Statistical analysis of fermentation metabolites

In the PCA plot, there are significant differences in principal components between CN and CM, MT and CM, and the distribution of components within the group is relatively concentrated, which indicates that the data are true and reliable (Figure 3 (B)). According to the OPLS-DA score plot, there are significant differences between CN and CM, MT and CM (Figure 3 (C)–(D)). All samples are within the confidence interval. The R^2X, R^2Y, Q^2 value were higher than 0.994 both in the CM and CN, MT and CM groups, which are close to 1, indicating that the data of the model are true and reliable (Table S1). A total of 119 differential metabolites were identified between CN and CM group (Table 4). They

Table 4. Differential metabolites between CN and CM.

Compound	Type	Significance
Organic acid		
Neochlorogenic acid	up	*
Chlorogenic acid	up	*
4-O-Caffeoyl Quinic acid	up	*
Cryptochlorogenic acid	up	*
Nicotinic acid	down	*
1,4-Dicaffeoylquinic acid	up	**
1,5-Dicaffeoylquinic acid	up	*
Isochlorogenic acid A	up	*
Isochlorogenic acid C	up	*
Isochlorogenic acid B	up	*
Ursolic Acid	up	**
Betulinic acid	up	**
Oleanolic acid	up	**
Isoferulic acid	down	**
Ferulic Acid	down	**
Fumaric acid	down	**
Maleic acid	down	**
Rosmarinic acid	down	**
Citric acid	down	*
L-Malic acid	up	**
Amber Acid	up	*
Succinic acid	up	*
3,5-Dicaffeoyl quinic acid	up	*
4,5-Dicaffeoylquinic acid	up	*
Norcantharidin	up	**
Vanillic acid	up	**
Madecassic acid	up	**
Protocatechuic acid	up	*
D-(−) quinic acid	up	**
Quinic acid	up	**
Glycyrrhizic acid	up	**
Caffeic acid	up	**
Gallic acid	up	**
4-Hydroxybenzoic acid	up	**
Salicylic acid	up	**
Saponins		
Tenacissoside G	up	**
11α-O-Tigloyl-12β-O-Acetyltenacigenin B	up	**
Chikusetsusponin IVa	up	*
Pedunsaponin C	up	*
Tenacissoside H	up	**
Diosgenin	up	*
Yamogenin	up	*
11α-O-Benzoyl-12β-O-Acetyltenacigenin B	up	*
Tenacissoside I	up	*
TenacigeninC	up	**
11α-O-2-Methylbutyryl-12β-O-2-TigloyltenacigeninB	up	**
11α,12β-Di-O-TigloylisotenacigeninB	up	**
11α,12β-Di-O-TigloyltenacigeninB	up	**
11α-O-Tigloyl-17β-Tenacigenin B	up	**
Tenacissoside L	down	**
Tenacigenin A	up	*
marsdenoside H	up	*
Tenacigenin B	up	*
marsdenoside J	up	*
Flavonoids		
Wogonin	up	*

(*continued on next page*)

Table 4 (*continued*)

Compound	Type	Significance
Acacetin	up	*
Maackiain	up	*
Wighteone	up	**
Demethoxycurcumin	up	**
Germacrone	up	**
Linarin	up	**
Schaftoside	up	**
Isoschaftoside	up	**
6-beta-D-Glucopyranosyl-8-beta-D-xylopyranosylapigenin	up	**
Daidzein	up	**
Calycosin	up	*
5,7,3 ′- trihydroxy-6,4′, 5 ′- trimethoxyflavone	down	**
1,7-Dimethoxyxanthone	down	**
Pinocembrin	down	**
Dihydrodaidzein	down	**
Isoliquiritigenin	down	**
Tectochrysin	up	*
Formononetin	up	*
6-hydroxydaidzein	up	**
Aloe-Emodine	up	**
Lucidin	up	**
Baicalein	up	**
Alcohols		
(−)-Pinoresinol	down	**
Curcumol	up	**
Dihydrodehydrodiconiferyl alcohol	up	**
Isolariciresinol	up	**
(+)-Lariciresinol	up	**
Triptolide	up	**
Sesamol	up	**
(−)-Pinoresinol	up	**
Matairesinol	up	**
(−)-Syringin	up	**
Syringin	up	**
Xylitol	up	**
Ambroxane	down	**
Physcion	up	*
Lipids		
Triptonide	down	**
Scopoletin	up	**
Isoscopoletin	up	**
Scopolin	up	*
Ethyl caffeate	up	*
Griffonilide	up	**
Gingerglycolipid B	up	*
Trachelogenin	up	**
Nucleosides		
Guanosine	down	**
Citicoline	down	**
Cytidine	down	**
2-Hydroxyadenosine	down	**
Uridine	up	*
amino acids		
Proline	down	**
L-valine	up	*
Histidine	up	*
L-Tryptophan	up	**
Aldoketones		
Pulegone	down	*
Curdione	up	**
Protocatechuic Aldehyde	up	**
Emodin	up	**
Glycosides		
GlycosidesDigoxin	up	*
Alkaloids		
Berberine	up	**
Betaine	up	*
Terpenoids		
Camphor	down	*
Amides		
Nicotinamide	down	**
Fatty acids		
Linolenic acid	down	**

Note: * means P value less than 0.05, ** means P value less than 0.01.

can be divided into 13 groups, mainly include organic acids (29.4%), flavonoids (19.3%), saponins (16.0%), alcohols (12.61%) (Figure 4 (A)). A total of 43 differential metabolites were identified between MT and CM group (Table S2). They can be divided into 9 groups, which mainly include organic acids (25.6%), saponins (14.0%), flavonoids (14.0%), amino acids (11.6%) (Figure 4 (B)). 26 differential metabolites were down-regulate, 93 differential metabolites were up-regulate between CM and CN (Figure. 4 (C)–(D)). 15 differential metabolites were down-regulate, 28 differential metabolites were up-regulate between CM and MT (Figure. 4 (E)–(F)).

3.3.3. Changes of components before and after fermentation

3.3.3.1. Changes of organic acids between CN and CM. 7 organic acids were down-regulated and 28 organic acids were up-regulated (Figure 5 (A)). Isochlorogenic acid A, isochlorogenic acid B, isochlorogenic acid C,1,4-dicaffeoylquinic acid, 1,5-dicaffeoylquinic acid, norcantharidin, vanillic acid, madecassic acid and protocatechuic acid were new compounds which were found in fermented *C. sinensis*, Among them, madecassic acid has been proved to have antioxidant, antidepressant, immune regulation and other functions, especially a significant inhibitory effect on tumor cells [35, 36]. Ursolic acid is a natural triterpene carboxylic acid compound. Its content increased 331 times after fermentation. It has resistance to a variety of carcinogens and cancer promoting substances. It has the characteristics of anti-inflammatory and hyperlipidemia [37], and also has significant anti-tumor activity. Studies have shown its inhibitory effect on human colon cancer cell line hct15 [38].

3.3.3.2. Changes of saponins between CN and CM. 18 saponins were up-regulate, 1 saponins were down-regulate (Figure 5 (B)). The new compounds were found such as tenacissoside G, tenacissoside H, 11α-O-Benzoyl-12β-O-acetyltenacigenin B, tenacigenin A etc. Other saponins except tenacissoside L increased significantly after fermentation. Among up-regulate metabolites, 14 saponins were polyoxypregnanes, the fold change of 11α-O-Tigloyl-17β-tenacigenin B was achieved 1140.8 times increase. Polyoxypregnanes contain such as tenacissoside H, tenacissoside G etc. Tenacissoside H has been proved to modulate inflammation and oxidative stress via TrkB pathway and down-regulate expression of proteins in the PI3K/Akt-NF-κB pathway to play the anti-cancer role [39, 40]. Tenacissoside G has been shown to inhibit the proliferation of colorectal cancer cells through atm-chk2-p53 pathway [41].

3.3.3.3. Changes of flavonoids between CN and CM. Flavonoids are a kind of plant secondary metabolites, which exist widely in many plants. They are not only numerous and diverse, but also complex and diverse in structural types. Flavonoids have many important physiological and

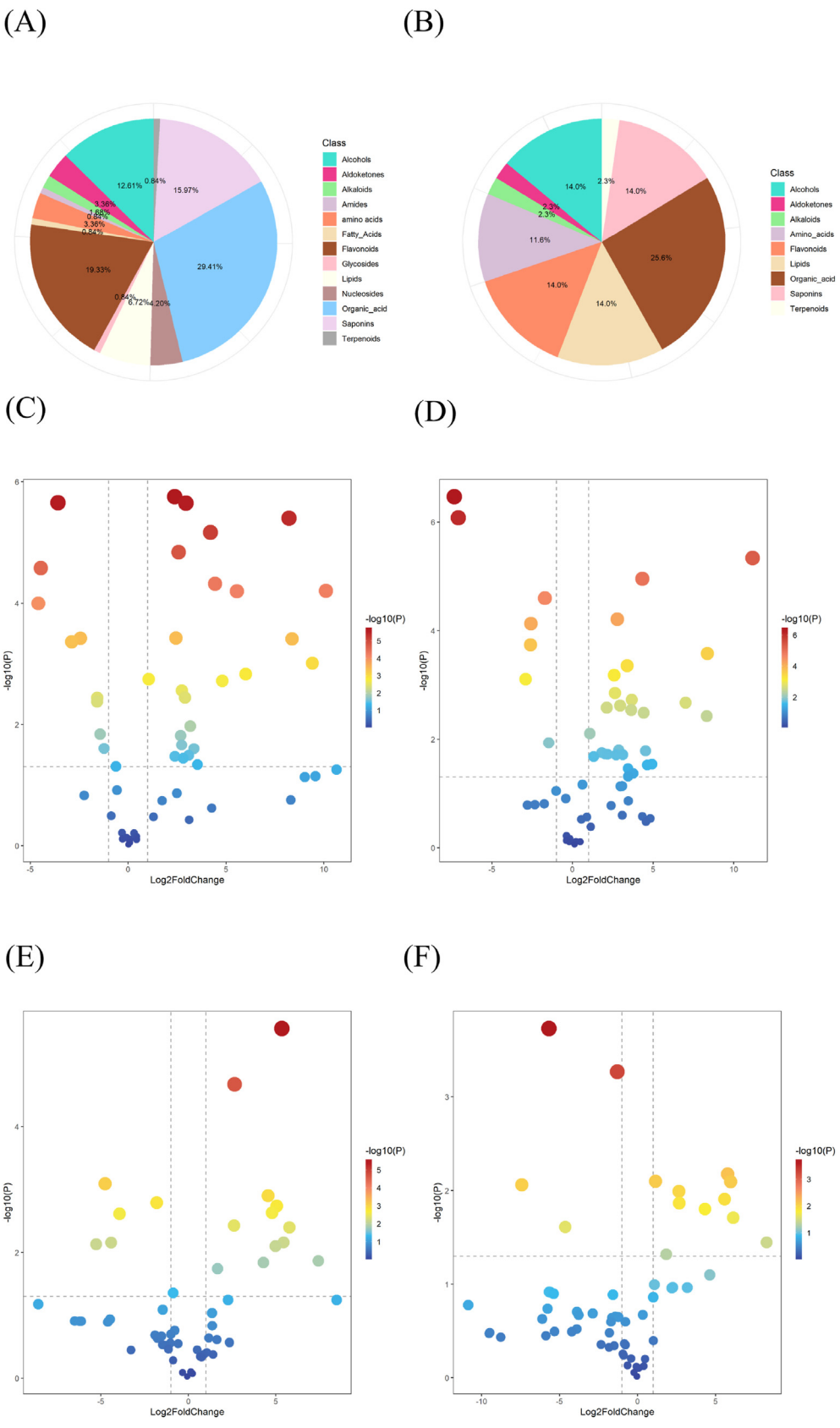

Figure 4. (A) Pie chart of different metabolite (Satisfying conditions Fold Change > 2 or Fold Change <0.5, P < 0.05 was considered as different metabolite) species between CM and CN. (B) Pie chart of different metabolite species between CM and MT. (C) Volcano plots of differential metabolites in the positive ions between CM and CN. (D) Volcano plots of differential metabolites in the negative ions between CM and CN. (E) Volcano plots of differential metabolites in the positive ions between CM and MT. (F) Volcano plots of differential metabolites in the negative ions between CM and MT.

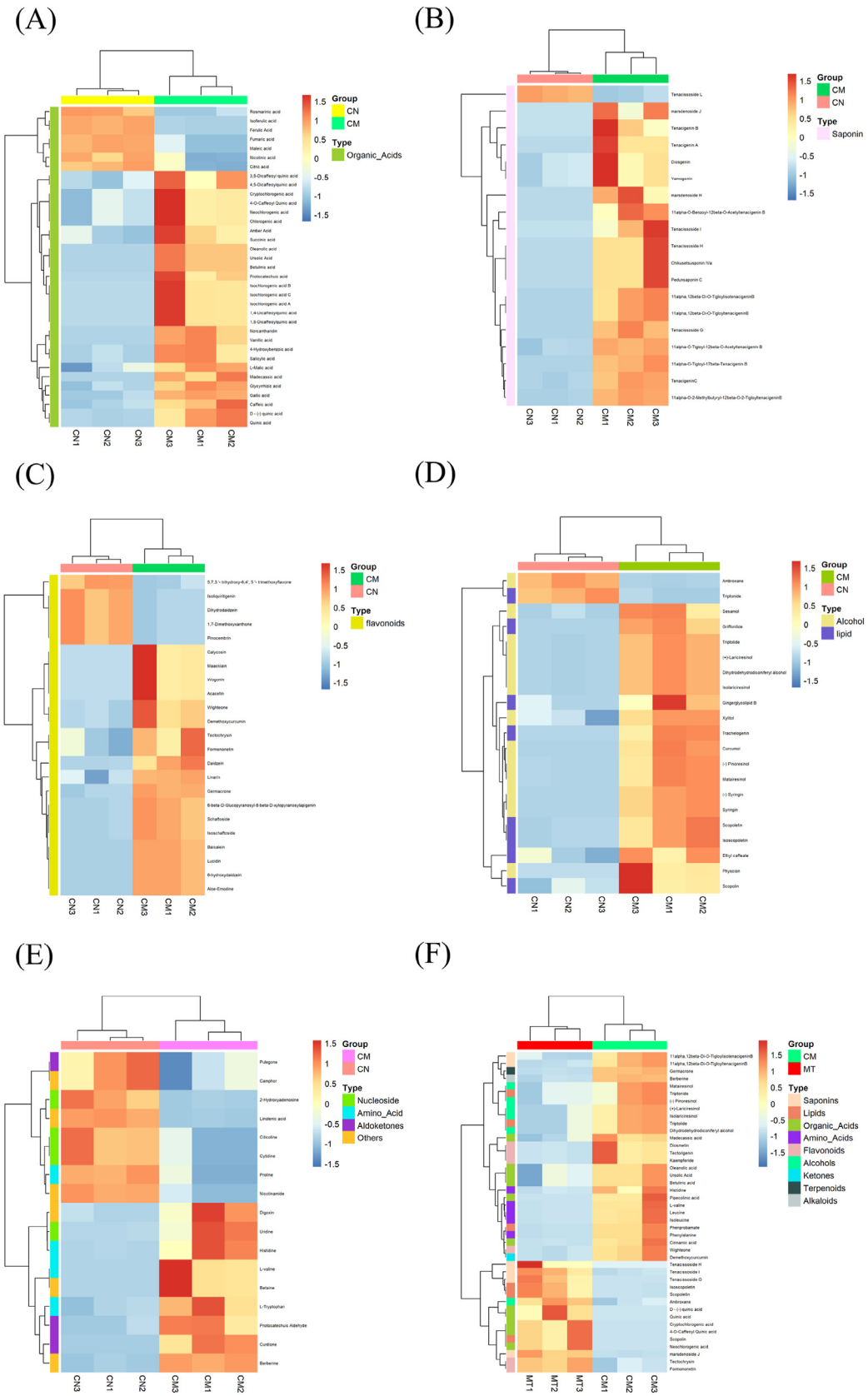

Figure 5. Heatmap of differential metabolites (A) Organic acids between CM and CN (B) Saponins between CM and CN. (C) Flavonoids between CM and CN. (D) Alcohols and lipids between CM and CN. (E) Nucleosides, amino acids, aldoketones and others between CM and CN. (F) Differential metabolites between CM and MT.

biochemical effects on mammals and other types of cells because of their unique chemical structure [42]. They are the effective components of many Chinese herbal medicines. 5 flavonoids were down-regulate, 18 flavonoids were up-regulate, acacetin, maackiain, Wighteone were new compound produced in the fermentation (Figure 5 (C)). Dihydrodaidzein, pinocembrin etc were down-regulate, lucidin, formononetin, tectochrysin, germacrone, aloe-emodine etc were up-regulate. Emodin has pharmacological activities such as anti-inflammatory, immune regulation, anti-oxidation and anti-tumor [43].

3.3.3.4. Changes of alcohols and lipids between CN and CM. Lipids are one of the important nutrients needed by the human body. They provide energy and essential fatty acids for the body. They are components of human cell tissues. The products of microbial fermentation are usually alcohols. After fermentation. (−)-pinoresinol, triptonide were down-regulate, other metabolites were all up-regulate, such as matairesinol, ethyl caffeate. Curcumol, Physcion, Griffonilide were new compounds in CM group (Figure 5 (D)). Among them, Curcumol has certain anti-tumor effect [44].

3.3.3.5. Changes of other kinds of metabolites between CN and CM. Amino acids are essential nutrients for human body, which can speed up the synthesis of human immunoglobulin, thus improving immunity. Amino acids can maintain the normal metabolism of human body. Enzymes, hormones and antibodies in the body are transformed from amino acids. Some amino acids also regulate key metabolic pathways necessary for maintenance, growth, reproduction and immunity. They are called functional amino acids and include arginine, cysteine, glutamine, leucine, proline and tryptophan [45]. The content of proline were down-regulate, the content of L-valine, histidine, L-tryptophan were up-regulate after fermentation. Nucleosides are a class of water-soluble components with extensive physiological activities, which exist in fungi such as *C. sinensis* [46]. The content of guanosine, citicoline, cytidine, 2-hydroxyadenosine were down-regulate, uridine were up-regulate. Curdione were new compound found in CM, curdione has been proved to be used in the treatment of breast cancer. It can inhibit the proliferation of breast cancer cells by inducing apoptosis [47, 48]. The content of pulegone, camphor, nicotinamide, linolenic acid were down-regulate, the content of protocatechuic aldehyde, betaine etc were up-regulate (Figure 5 (E)).

3.3.3.6. Changes of metabolites between MT and CM. Among 43 differential metabolites, 15 were down regulated and 28 were up regulated (Figure 5 (F)). The content of some organic acids such as quinic acid were down-regulate, the metabolites of some alcohols and amino acids such as L-valine, phenylalanine are up-regulated, this indicates that the activity of protein hydrolysis of *C. sinensis* is enhanced, fermentation can hydrolyze the macromolecular proteins in *M. tenacissima* into small molecular amino acids through the hydrolysis of enzymes in microorganisms, the amino acid L-valine produced by hydrolysis is a natural essential amino acid for human body, which can be used as a nutritional supplement and can also improve flavor. Among flavonoid metabolites, tectochrysin, formononetin were down-regulate, wighteone, tectorigenin, kaempferide, diosmetin were up-regulate. Diosmetin is a functional food, cosmetic and future drug with antioxidant, anti infection and anti shock effects, study found that diosmetin can inhibit tumor development and block tumor angiogenesis in skin cancer [49]. the Polyoxypregnanes are the main active components in *M. tenacissima*, the content of tenacissoside G, tenacissoside H, tenacissoside I were down-regulate, and the content of small polyoxypregnanes 11α, 12β-Di-O-tigloylisotenacigeninB, 11α,12β-Di-O-tigloyltenacigenin B were up-regulate after fermentation. It is likely to be the metabolite of *C. sinensis* using the macromolecular polyoxypregnanes of *M. tenacissima.*

3.3.3.7. Analysis of biomarkers of fermentation product. We take the four metabolites with the most significant changes as biomarkers for further analysis, namely isoferulic acid, tenacissoside H, tenacissoside G, marsdenide J. Isoferulic acid is an organic acid which belongs to cinnamic acid derivative, caffeic acid, coumaric acid, ferulic acid is one of the derivatives of cinnamic acid. Studies have shown that the derivatives of cinnamic acid have inhibitory effects on human cancer cell lines such as A549, and can be used as anti-cancer agents to participate in the development of lung and colon cancer as active ingredients [50]. Tenacissoside H, tenacissoside G, marsdenide J are all Steroidal saponins, steroidal saponin is an important natural product [51], there are many kinds of saccharides contained in steroidal saponins, among which D-glucose, D-galactose, D-xylose, L-rhamnose and L-arabinose are the most common. The saccharides contained in tenacissoside H, tenacissoside G and marsdenoside J are D-glucose. Studies show that the steroidal saponins have certain cytotoxicity and anti-inflammatory activity [52].

3.3.4. Pathway analysis of differential metabolites

Differential metabolites were enriched to 24 differential metabolic pathways, mainly include citrate cycle (TCA cycle), nicotinate and nicotinamide metabolism, pentose and glucuronate interconversions,

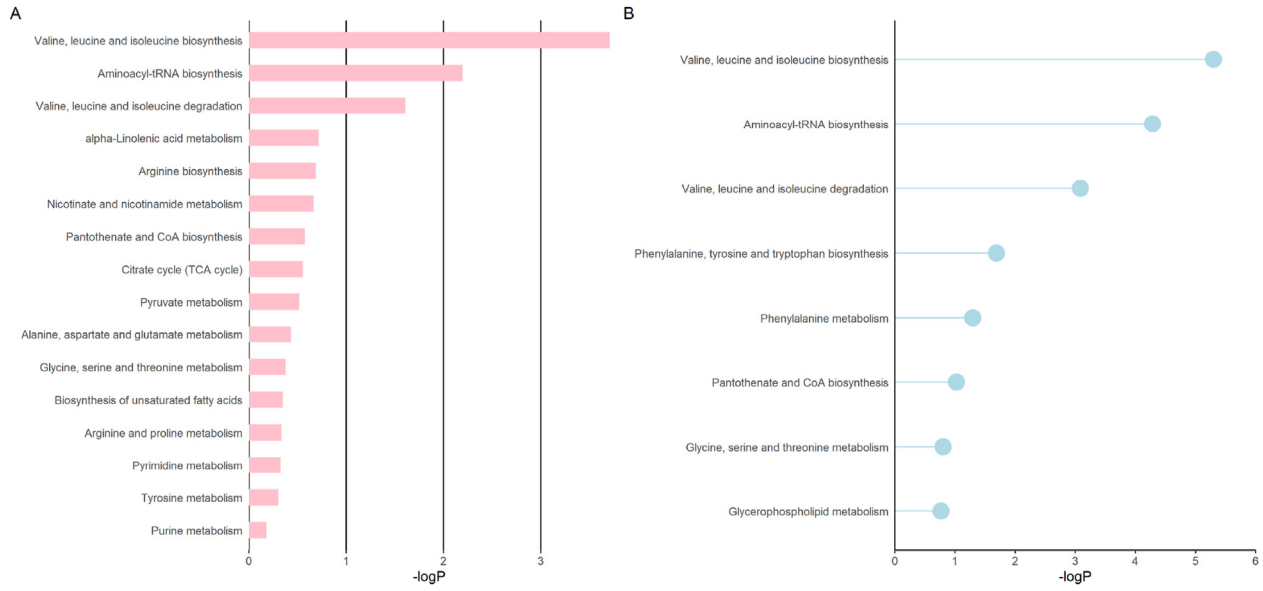

Figure 6. Enrichment of differential metabolic pathways (A) UFC and FC. (B) MT and FC.

pyruvate metabolism, glyoxylate and dicarboxylate metabolism (Figure 6 (A)–(B)). Succinate, (S)-malate, citrate, fumarate involved in citrate cycle (TCA cycle), nicotinamide, nicotinate, succinate involved in nicotinate and nicotinamide metabolism, citrate, (S)-malate, succinate involved in glyoxylate and dicarboxylate metabolism, xylitol involved in pentose and glucuronate interconversions.

It can be seen from the pathway analysis of the two groups that Valine, leucine and isoleucine biosynthesis, and Aminoacyl tRNA biosynthesis higher significance. Leucine is synthesized through valine, leucine and isoleucine biosynthesis, so this pathway is more active in the fermentation process. At present, valine and isoleucine production have been realized based on metabolic engineering [53]. Aminoacyl-tRNA synthetase is an essential enzyme family in the aminoacyl tRNA biosynthesis pathway, which plays a key role in protein synthesis [54]. It can be concluded that protein and some amino acids are actively synthesized during fermentation. As for the specific mechanism, further exploration is still needed.

4. Conclusions

In this study, *C. sinensis* was used as the strain and *M. tenacissima* as the substrate for two-way liquid fermentation for the first time. The box-behnken design (BBD) in response surface methodology (RSM) was used to analyze interaction among factors and its influence on biomass, optimizing the process to obtain the maximum biomass. The optimum fermentation conditions are as follows: the concentration of medicinal materials is 160 g/L, the fermentation time is 6 days, the inoculation volume is 9.5%, and the rotating speed is 170 rpm. Providing theoretical reference for future actual production.

The non-targeted metabolomics analysis based on ultra-high performance liquid chromatography-mass spectrum (UHPLC-MS) were established. A total of 197 differential metabolites were identified and annotated, of which 119 were differential metabolites between CM and CN, 43 differential metabolites between CM and MT.

Through the optimization of the fermentation process of *C. sinensis* and the exploration of its metabolites, we revealed the conditions suitable for the growth of *C. sinensis*, providing a reference for the fermentation conditions of medicinal fungi. We also found that many metabolites have good pharmacological effects, such as anti-cancer and anti-inflammatory, which can provide a new direction for disease research.

Declarations

Author contribution statement

Siqi Wang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Lin Lu: Performed the experiments; Wrote the paper.

Tianyuan Song; Xinxin Xu: Analyzed and interpreted the data.

Jie Yu: Contributed reagents, materials, analysis tools or data.

Tongxiang Liu: Conceived and designed the experiments; Wrote the paper.

Funding statement

Tongxiang Liu was supported by National Natural Science Foundation of China [No. 81973977], Fundamental Research Funds for the Central Universities [2021JCXK02].

Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no competing interests.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e12586.

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