

Improvement of nucleotide content of *Cordyceps tenuipes* by *Schisandra chinensis*: fermentation process optimization and application prospects

Anni Li

Jiangsu Ocean University

Qiang Zhu

Jiangsu Ocean University

Yuting Li

Jiangsu Ocean University

Lu Yang

Jiangsu Ocean University

Zhihao Chen

Jiangsu Ocean University

Xiang Zhou

Jiangsu Ocean University

Yanqiu Xia

xyq78412@163.com

Jiangsu Ocean University

Research Article

Keywords: *Cordyceps tenuipes*, *Schisandra chinensis*, Fermentation process, Nucleotide, AChE, Antioxidant

Posted Date: March 22nd, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4127978/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Archives of Microbiology on May 13th, 2024. See the published version at <https://doi.org/10.1007/s00203-024-03988-4>.

Improvement of nucleotide content of *Cordyceps tenuipes* by *Schisandra chinensis*: fermentation process optimization and application prospects

Anni Li ^{1,2}, Qiang Zhu ^{1,2†}, Yuting Li ^{1,2†}, Lu Yang ^{1,2†}, Zhihao Chen ^{1,2†}, Xiang Zhou ^{1†}, Yanqiu Xia ^{1,2*}

¹Jiangsu Key Laboratory of Marine Bioresources and Environment/Jiangsu Key Laboratory of Marine Biotechnology, Jiangsu Ocean University, Lianyungang 222005, China

²Co-Innovation Center of Jiangsu Marine Bio-industry Technology, Jiangsu Ocean University, Lianyungang 222005, China

*Corresponding authors. E-mail address: xyq78412@163.com;

Contributing authors: 2021221230@jou.edu.cn; zq7685@163.com; 2021221235@jou.edu.cn; luyang@jou.edu.cn; zhihaochen@jou.edu.cn; zx874871597@163.com;

† These authors contributed equally to this work.

Abstract

Nucleotides are important components and the main indicators for judging *Cordyceps* quality. To increase the nucleoside content of *Cordyceps tenuipes* for enhancing the efficacy activity. In this paper, the mixed fermentation process of *Schisandra chinensis* and *C. tenuipes* was systematically studied, and it was proposed that the fermentation products aqueous extract (S-ZAE) had antioxidant activity and anti-AChE ability. Herein, the results of a single factor showed that *S. chinensis*, yeast extract, inoculum amount, and pH had significant effects on nucleotide synthesis, and these four factors were used as the factor of response surface optimization. The results of response surface optimization were 3% glucose, 0.25% KH₂PO₄, 2.1% yeast extract, and *S. chinensis* 0.49% (m/v), the optimal fermentation conditions were 25°C, inoculum 5.8% (v/v), pH 3.8, 6 d. The yield of total nucleotides in the scale-up culture was 0.64 ± 0.027 mg/mL, which was 10.67 times that before optimization, and S-ZAE has good antioxidant and anti-AChE activities. This fermentation method has the advantage of industrialization, and its fermentation products have the potential to become good functional foods or natural therapeutic agents.

Keywords: *Cordyceps tenuipes*; *Schisandra chinensis*; Fermentation process; Nucleotide; AChE; Antioxidant

1. Introduction

Cordyceps tenuipes is a medicinal fungus that parasitizes the pupal and larvae of *Lepidoptera*, and currently, wild and farmed *Cordyceps* are mainly parasitic on silkworm larvae (Kang et al. 2010; Ji et al. 2011). However, *C. tenuipes* currently serves as an alternative that East Asian countries use extensively for raw materials from natural health foods or traditional medicine for treating ailments. Researchers from Korea and Japan have investigated *C. tenuipes* as a viable substitute for *Cordyceps sinensis*, yielding notable findings. *C. tenuipes* contains fungal polysaccharides (Lu et al. 2016), alkaloids (Bunyapaiboonsri et al. 2009), and terpenoids (Kikuchi et al. 2004). Studies have reported that its extract has antitumor (Han et al. 2004), immunomodulatory (Chen et al. 2008), antidepressant (Liu et al. 2017), antifungal (Haritakun et al. 2007), hypoglycemic (Liu et al. 2017), hypolipidemic (Lee et al. 2006), neuroprotective (Suzuki et al. 2017), and other pharmacological effects. Regular intake of bioactive components in herbal

supplements and functional foods has significant long-term health-promoting effects; however, the medicinal potential of many wild plants is poorly understood (Qurat-ul-Ain et al. 2023).

S. chinensis is a traditional Chinese medicine (TCM) with a rich and rich nutritional and medicinal value. *S. chinensis* itself contains lignans, polysaccharides polyphenol organic acids, and other components that can provide nutrients to the fungus during fermentation (Yang et al. 2021). It is revealed that *S. chinensis* has antitussive (Zhong et al. 2015), prostate relaxation (Choo et al. 2014), hepatoprotective (Zhu et al. 2019).

Modern pharmacological studies have revealed antitussive (Zhong et al. 2015), cardioprotective (Kim et al. 2011), and prostate relaxation (Choo et al. 2014) that provide the basis for traditional applications. Additionally, *S. chinensis* has hepatoprotective (Zhu et al. 2019), anticancer (Zhao et al. 2014), anti-inflammatory (Bae et al. 2012), and hypoglycemia (Kwon et al. 2011). Alzheimer's disease (AD) is a progressive neurological disorder that causes atrophy of the hippocampus and cortex. Although the exact etiology of AD is still unclear, the cholinergic hypothesis that the neurotransmitter acetylcholine (ACh) is associated with memory deficits in the brain has received much attention, and thus the normal level of ACh in the synaptic cleft is essential for cholinergic neural signaling (Kumar et al. 2016).

The fermentation of medicinal fungi and TCM is essentially a process of microbial transformation. After fermentation, microorganisms use certain components of TCM as carbon sources, part nitrogen sources, metal ions, etc., to achieve the purpose of mutual synergy (Wu et al. 2013). Nucleotides are an important component of *Cordyceps* and are the main indicators for judging the quality of *Cordyceps*. Therefore, it is necessary to increase the nucleotide content by optimizing the fermentation process (Raethong et al. 2020; Iamtham et al. 2022).

Although the mixed fermentation of fungi and TCM has become a hot topic (Wang et al. 2022; Wang et al. 2023; He et al. 2024), and a large number of studies have reported that *Cordyceps* polysaccharides and polyphenols have strong antioxidant activity, but few reports have reported the effect of *Cordyceps* nucleotides on antioxidant activities (Van et al. 2022; Jiang et al. 2023; Tang et al. 2022). However, it has not been reported that increasing the nucleotide content of *C. tenuipes* by mixing fermentation with TCM and optimizing the fermentation process can improve antioxidant activity and AD. Therefore, in this study, *S. chinensis* was used as the addition substrate and the optimal fermentation process was determined to increase nucleotide content by single factor and response surface analysis. ZXC-18 was amplified and cultured in a 5-L fermenter under the optimal fermentation process, and the antioxidant activity and AChE inhibitory activity of S-ZAE were evaluated to evaluate the efficacy of improving and preventing AD.

2. Materials and Methods

2.1 Fungal activation and liquid culture

A field strain was isolated from soil-infected *Clanis bilineata* larvae at the Doudan Breeding Base (119°31'1" E 30°20'7" N) in Guanyun County, Lianyungang City, Jiangsu Province, China. *C. tenuipes* were inoculated on potato glucose agar medium and incubated at 25°C for 10 d. After mycelium grows, cut off 2–3 pieces of approximately 1 cm³ of fungus and incubate them in 500 ml conical flasks of potato glucose liquid medium at 25°C at 150 r/min for 5 days.

2.2 Identification of the fungal strain

Morphological identification and phylogenetic tree analysis were used to determine strain affiliation. Sangong Biotechnology (Shanghai, China) Co., Ltd. Complete extraction, amplification, and determination of the sequence of the strain DNA-specific steps are illustrated in Figure 1. The sequences

obtained were compared with the nucleic acid data in GeneBank using the BLAST program. The closely related strains were selected as reference strains, and the phylogenetic tree was constructed using the MEGA 7.0 software.

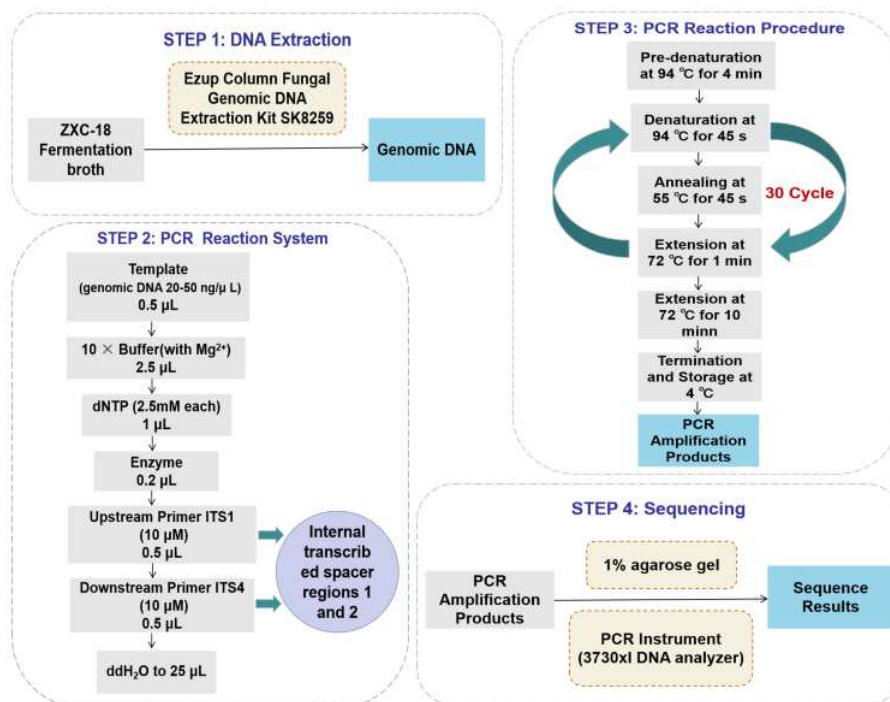


Fig. 1 Gene sequencing flow chart. The first step is DNA extraction, the second step is the polymerase chain reaction system, the third step is the PCR reaction procedure, and the fourth step is sequencing.

2.3 Medium and fermentation condition conditions for nucleotide optimization for production

2.3.1 Screening of TCM

150 ml of potato dextrose water in 250 ml conical flasks was added with 1% powder (*Rheum officinale* Baill; *Terminalia chebula* Retzius; *Radix astragali*; *Mume Fructus*; *Galla Chinensis*; *Suaeda*; *Fructus Arctii*; *S. chinensis*), sterilized for 35 min at 121°C after shaking and mixing, inoculated 10 ml of culture in this different TCM medium, and incubated at 25°C at 150 r/min for 9 days. The fermentation products were centrifuged at 12,000 rpm for 20 min, dried in an oven at 55°C to constant weight, weighed accurately, and nucleotide by high-performance liquid chromatography. Three parallel experiments were conducted in each group with the total nucleotide yield (the ratio of the total nucleotide mass to the volume of fermentation liquid) as the index.

2.3.2 Single-factor tests

Based on total nucleotide content, the optimal growth factors, the amount added, and fermentation conditions were selected. Carbon sources (soluble starch, glucose, cane sugar, maltose, fructose), nitrogen sources (peptone, yeast extract, NH_4Cl , $(\text{NH}_4)_2\text{SO}_4$, and urea), and inorganic salts (CaCl_2 , FeSO_4 , KH_2PO_4 , MgSO_4 , NaNO_2) were selected as single factors to identify the most suitable growth factors. Carbon sources (1, 2, 3, 4, 5%), nitrogen sources (0.5, 1.0, 1.5, 2.0, 2.5%), inorganic salts (0.1, 0.15, 0.2, 0.25, 0.3%), VB1 (0, 0.05, 0.1, 0.15, and 0.2%) and *S. chinensis* (0.17, 0.33, 0.67, 1, 1.3%) were selected as single factors to obtain the optimal amount of addition. The amount of inoculation (2, 4, 6, 8, 10%), fermentation days (3, 4, 5, 6, 7, 8, 9, 10, 11, 12 d), temperature (21, 23, 25, 27, 29°C) and pH (3.5, natural,

4.5, 5.5, 6.5, 7.5) were selected as single factors to obtain optimal fermentation conditions. Furthermore, 2% glucose, 1% peptone, 0.15% KH₂PO₄, 0.1% VB1, and 1% *S. chinensis* were used as the basic conditions, and the liquid medium in a 250 ml Erlenmeyer flask was 150 ml. Three parallel experiments were set in each group and sterilized at 121°C for 35 min. The effects of various factors on nucleotide production in mycelium were investigated while other conditions remained unchanged.

2.3.3 Responsive surface design of experiments (RSM)

This study sought to optimize the medium and fermentation conditions for the mixed fermentation of ZXC-18 and *S. chinensis* to obtain the maximum yield of nucleotides. Based on the results of the one-way test, according to the design principle of the Box–Behnken center, the total nucleotide content was used as the response value, and the amount of yeast extract and *S. chinensis* added to the amount of inoculation, and the pH was used as independent variables. Design-Expert 13.0 software (StatEase, Inc., Minneapolis, USA) was used to optimize and verify the fermentation process of *C. tenuipes* with the total nucleotide content as the response value.

2.3.4 5-L fermentor enlargement culture fermentation

The 5-L fermentation tank (Bailun Biotechnology (Jiangsu) Co., Ltd.) was used with a fermentation process optimized by a single factor and response surface (3% glucose, 0.25% KH₂PO₄, 2.1% yeast extract and 0.49% *S. chinensis*, and optimal fermentation conditions were 25°C, inoculation amount 5.8%, pH 3.8, and 6 days). Samples (20 ml) were taken every 12 h of fermentation and biomass, pH, and residual sugar in the fermentation broth were measured.

2.4 Antioxidant activity in vitro

2.4.1 DPPH radical scavenging activity

Referring to the method of Xu et al. (2018) with slight modifications. First, 0.4 mg of DPPH was accurately weighed and prepared with 95% ethanol to obtain a concentration of 0.4 mg/L DPPH, which was then stored in the dark for backup purposes. Next, we added 100 uL of sample solution (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/mL) and 100 uL of 0.4 mg/mL DPPH test solution into a single well of a 96-well enzyme plate in triplicate fashion. After adding the sample, we shook it for approximately 30 seconds and determined its absorbance value (A_1) at a wavelength of 517 nm and a temperature maintained at 37 °C; simultaneously, we also determined the blank absorbance value (A_2) for samples without DPPH as well as the absorbance value (A_0) for samples without both DPPH and water instead of sample using the same procedure described earlier.

$$DPPH\ radical\ scavenging\ rate\ \% = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100$$

2.4.2 Hydroxyl radical antioxidant activity

Referring to the method of Smironff et al. (1989) with slight modifications. 1.1 mL 8.8 mmol/L H₂O₂, 10.0 mmol/L FeSO₄, 10.0 mmol/L salicylic acid and 1.0 mL sample solution. In a water bath at 37 °C for 30 min, water was used as a blank control and ascorbic acid aqueous solution as a positive control, and the absorbance of the reaction solution was measured at a wavelength of 510 nm. Three samples were set.

$$Hydroxyl\ radical\ scavenging\ rate\ \% = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100$$

A_0 = 1.0 mL pure water + 1.0 mL H₂O₂ + 1.0 mL FeSO₄ + 1.0 mL salicylic acid + 1.0 mL H₂O₂ reaction system, A_1 = 1.0 mL sample solution + 1.0 mL H₂O₂ + 1.0 mL FeSO₄ + 1.0 mL salicylic acid + 1.0 mL H₂O₂ reaction system, A_2 = 1.0 mL sample solution + 1.0 mL of the H₂O₂ reaction system.

2.4.3 Superoxide radical antioxidant activity

Referring to the method of Zhu et al. (2020) with slight modifications. 1.0 mL ZAE, S-ZAE sample solution (0.5, 1, 1.5, 2, 2.5, 3 mg/mL), 8 M hydrochloric acid solution (0.1 mL), deionized water (0.5 mL) and 0.05 M Tris-HCl buffer solution, pH=8.2 (4.5 mL). Absorbance was measured at 325nm with ascorbic acid as a positive control and DMSO as a blank control. Three samples were set.

$$\text{Superoxide radical scavenging rate \%} = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100$$

Where A_1 is the absorbance of the sample group, A_2 is the absorbance value of the 2.5 mM 1,2,3-Hydroxybenzene replaced by deionized water, A_0 is the absorbance value of 2.5 mM 1,2,3-Hydroxybenzene and DMSO instead of deionized water and the polysaccharide sample solution.

2.5 In vitro AChE inhibitory activity

AChE inhibitory activity was tested using the method of Ellman et al. (1961). In addition to the sample group to be tested, a blank negative control group and a donepezil positive control group were established and enzyme inhibitory activity was tested by a microplate reader using the 96-well plate method. The experiment was repeated three times under the same conditions. The specific steps were as follows: Aqueous extract of mixed fermentation product (S-ZAE), *S. chinensis* (SAE), and ZXC-18 (ZAE) were prepared into aqueous solutions with different mass concentrations (0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 mg/mL), the detection wavelength of the microplate reader was set to 412 nm, the temperature was 37°C, the interval between the detection of absorbance was 30 s, and the number of detections was 11. Additionally, 20 μ L of acetylcholinesterase solution (0.5 U/ml), 130 μ L of phosphate buffer, and 20 μ L of chromogen were added to a 96-well plate, 10 μ L of methanol was added to the blank group, 10 μ L of donepezil solution was added to the positive control group, 10 μ L of sample solution was added to the sample group to be tested, and 20 μ L of substrate solution was quickly added to each well for detection in a microplate reader. The smaller the slope of the curve, the better the cholinesterase inhibition activity, and the cholinesterase inhibition rate was determined using the following formula:

$$\text{Inhibition rate \%} = \left(1 - \frac{k_i}{k_0}\right) \times 100$$

Where k_i is the slope of the sample and k_0 is the slope of the blank.

2.6 Analytical methods

2.6.1 Determination of mycelial dry weight

The mycelium of the fermentation broth was centrifuged and washed twice with distilled water, dried to a constant weight in an oven at 55°C, as the dry weight, and then homogenized into a powder (80 mesh).

2.6.2 Measurement of nucleotides

The fermentation products were centrifuged at 12,000 rpm for 20 min, dried to constant weight in an oven at 55°C, and ground into powder through a 40 mesh sieve. Next, 0.3 g of fermentation product powder S-ZAE was precisely weighed, 9 ml of pure water was added, and the fermentation was extracted ultrasonically at 50°C and 350 W for 1 h. After treatment, the supernatant was centrifuged at 12,000 rpm for 15 min, and the supernatant was filtered through a 0.22 μ m microporous membrane.

The nucleotides of the fermentation products were determined and analyzed by HPLC. Samples and

standards were chromatographically separated using a high-performance liquid chromatograph system (Chromeleon Thermo Fisher Scientific, China) using a DVOTO C18 column (250 mm × 4.6 mm, 5 μm) at 35°C, and eluted as a mobile phase gradient consisting of water (A) and acetonitrile (B) using a solvent elution procedure as described in Table 1. The DAD detector was set between 254, 260, 200, and 400 nm, with a sample injection volume of 50 μL, and a flow rate of 1 ml/min.

Weigh 5 mg of guanine nucleoside, uracil nucleoside, cytosine nucleoside, β-thymidine, inosine, adenine nucleoside, and cordycepin standards, dissolve them in water at 50 ml at a concentration of 100 μg/ml, prepare them at concentrations of 10, 20, 40, 60, and 80 μg/ml, and store them in a refrigerator at 4 °C.

Table 1 Solvent elution program for high performance liquid chromatograph.

Step	Time(min)	Water (%)	Acetonitrile (%)
1	5	95	5
2	15	92	8
3	20	95	5

2.6.3 Determination of the reducing sugar content

The LaneEynon method was used to determine the reducing sugar content in the fermentation broth. The titration endpoint is determined based on the color change, and the reducing sugar content can be calculated based on the sample consumption.

$$X \text{ g}/100\text{g} = \frac{V_1}{V_2 \times 1000} \times 100$$

X is the reducing sugar content (g/100g), V_1 is the calibrated glucose consumption (mg), V_2 represents the sample consumption (mg), and 1000 is the conversion factor.

2.7 Statistical Analysis

Samples from the above tests were tested in triplicate. The results obtained are expressed as mean ± SD. Data were analyzed using Origin 2022. And IC₅₀ values were obtained from nonlinear regression.

3. Results and Discussion

3.1 Morphological description of the fungus

C. tenuipes exhibit the following characteristics: Insects are covered with grayish or white mycelium and have many fruiting bodies, and tips have multiple branches and are covered with white spore powder. The stalk of the fruiting body is slender and smooth, yellowish white or beige, and turns yellowish-brown to dark brown when dry (Fig. 2a). PDA inoculated with mycorrhizal blocks on appressed medium-growth, colonies in dark light mycelium white and cottony and fluffy, mycelium along the medium in whorls and radial growth, grow under conditions of light, mycelium is pale yellow and attached spores. (Fig. 2b). The fruiting body on wheat media is not only dense but also very thick, with bright yellow 5–8.5 cm long cables, which have the characteristic of growing in light and oxygen (Fig. 2c). In the case of sufficient oxygen, many white spores will be produced on the surface of the fruiting body, which resemble white snow (Fig. 2d). As seen through scanning electron microscope (Fig. 2e), the spores of ZXC-18 are smooth ovals 5.35 μm long and 2.38 μm wide, with a concave and slightly curved middle. These features are consistent with those of *Isaria tenuipes*.

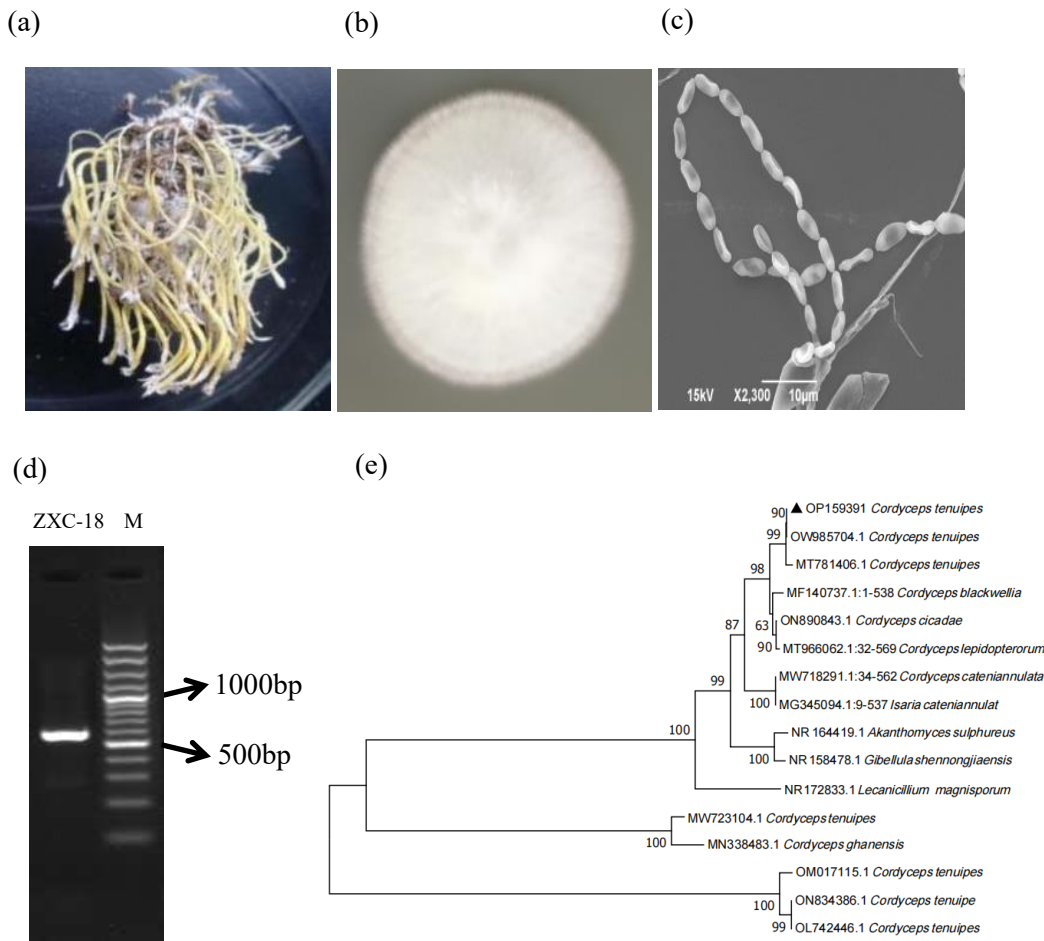


Fig. 2 Morphological characteristics of *C. tenuipes*. On *Clanis bilineata* larvae (a). Colony morphology of strain ZXC-18 (b). Spore morphology of ZXC-18 under electron microscopy (c). PCR results electrophoretogram (d). ITS gene sequence-generated phylogenetic tree (e).

3.2 Impacts of TCM on the nucleotide production of *C. tenuipes*

During fermentation, *Galla Chinensis* and *Suaeda* inhibited the growth of *C. tenuipes* (Fig. 3a). ZXC-18 contains cytidine, uridine, guanosine, and adenosine by comparing retention times with standards (Fig. 3b). As shown in Figure 3b, the total nucleotide content of ZXC-18 added with *S. chinensis* had the highest total nucleotide content of 0.0788 ± 0.002 mg/mL. The addition of *Rheum officinale* Baill also significantly increased the nucleotides of ZXC-18 but had a significant inhibitory effect on the cytidine of *C. tenuipes*, and the total nucleotide content was not as high as that of *S. chinensis*.

Therefore, *S. chinensis* was selected as the fermentation substrate, because the polysaccharides polyphenol and organic acids in *S. chinensis* provide nutrients and energy for the fermentation of fungus to some extent, and the organic acids provide a suitable growth environment for them. On the other hand, medicinal fungi use their powerful enzyme system, and many TCMs are used to break the walls, degrade biological macromolecules, and even transform certain components (Wu et al. 2013). Some studies report that *Suaeda nudiflora* can be sold as plant salt (Buhmann et al. 2013), *Suaeda* grows vigorously in saline-alkali soils, and has an enrichment effect on heavy metal elements such as Cu, Zn, Pb, Cd, etc. (Lu

et al. 2019). Therefore, excessive salt and heavy metal content lead to the difficult growth of fungi. Other studies have shown that gallic acid in *Galla chinensis* can inhibit the growth of certain types of fungi at a concentration of only 30 mg/mL (Wang et al. 2006).

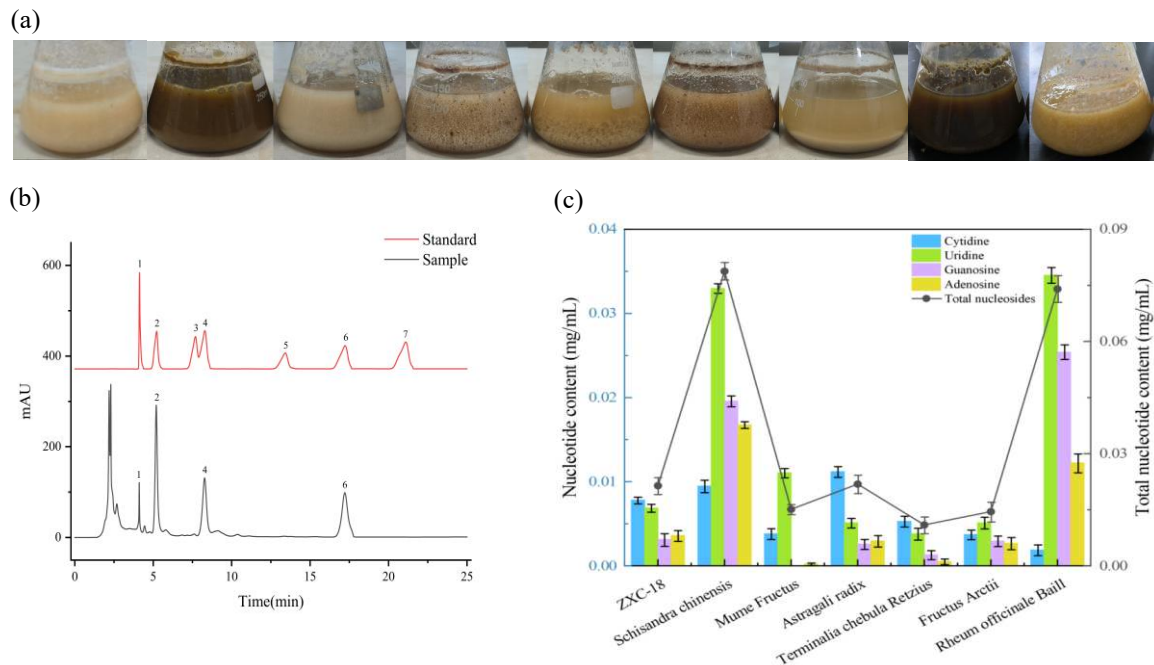


Fig. 3 Photograph of cofermentation of different Chinese herbs with *C. tenuipes*, from left to right: Control, *Rheum officinale Baill*, *Radix astragali*, *S. chinensis*, *Terminalia chebula Retzius*, *Mume Fructus*, *Galla Chinensis*, *Suaeda*, and *Fructus Arctii* (a); Retention time of standards and ZXC-18 nucleotides (b) (1) cytidine, (2) uridine, (3) inosine, (4) guanosine, (5) thymidine, (6) adenosine, (7) cordycepin; Nucleotide content of cofermentation between different TCM and *C. tenuipes* (c).

3.3 Single-factor experimental design of the *C. tenuipes* fermentation process

3.3.1 Media optimization

Single-factor test on the fermentation design of *S. chinensis*-*C. tenuipes*. Different media, dosages, and fermentation conditions determine the growth rate of microorganisms and the synthesis efficiency of the active substances. Therefore, the effects of these factors on the total nucleotide content of *C. tenuipes* are investigated in this article.

As shown in Fig. 4a, glucose promotes nucleotide synthesis even more, and Mao et al. (2005) confirmed that carbohydrates, especially glucose, are good carbon sources to produce important substances. It can be seen from Fig. 4b that inorganic nitrogen is detrimental to mycelial growth (Xu et al. 2009), which is consistent with the findings of Zhang et al. (2007) that a single amino acid is not a good source of nitrogen for mycelial growth in several fungal genera. Glucose and yeast extract provided most of the energy for fungi as carbon and nitrogen sources (Takano et al. 2005). Fig. 4c shows that the addition of KH_2PO_4 can effectively increase the nucleotide content, but the lack of micronutrients led to abnormal growth of *Cordyceps* (Kaewkam et al. 2021). When 33% *S. chinensis* powder was added to 150 ml of medium, the total nucleotide content reached 0.27 mg/mL. In the range of 0.17%–0.33%, the total nucleotide content was directly proportional to the amount of *S. chinensis* and inversely proportional to more than 0.33% (Fig. 4d). The results showed that excessive *S. chinensis* affected the efficiency of

nucleotide production in the fermentation of *C. tenuipes*, which may be because *S. chinensis* provides nutrients and energy for the fermentation of *C. tenuipes* to a certain extent and can also reduce the growth and metabolism of *Cordyceps*.

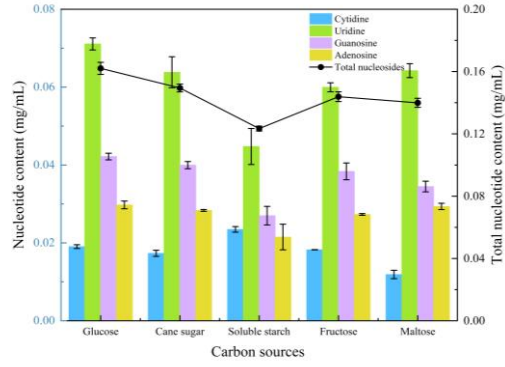
The nucleoside content of *Cordyceps* is also dependent on the concentration of carbon and nitrogen sources that are components of the culture medium (Lee et al. 2019; Patthanajuck et al. 2021). The total nucleotide content reached the maximum value of 0.21 mg/mL at 3% (Fig. 4e), which began to decline after 3%. The results show that (Fig. 4f), the total nucleotide content increased by 0.5–2% with yeast extract added to the medium, reaching a maximum of 0.29 mg/mL at 2% and then decreasing. This may be because if the nitrogen source is insufficient, the amount of fungal reproduction will be small, which will affect the yield. Excess nitrogen will make the growth of the fungus too vigorous, and the pH will be too high, which is not conducive to the accumulation of metabolites. From the results, it can be seen that the total nucleotide content of 0.25% KH_2PO_4 reached 0.21 mg/mL (Fig. 4g), and 0.25%–0.3% of the total nucleotides decreased, possibly because the addition of KH_2PO_4 would cause the pH of the medium to drop beyond the optimal pH. When no VB_1 was added, the total nucleotide content of *C. tenuipes* was the highest at 0.21 mg/mL (Fig. 4h). Overall, the total nucleotide content of *C. tenuipes* reached its maximum when the medium was 3% glucose, 2% yeast extract, 0.25% KH_2PO_4 , and 0.33% *S. chinensis*.

3.3.2 Optimization of fermentation conditions

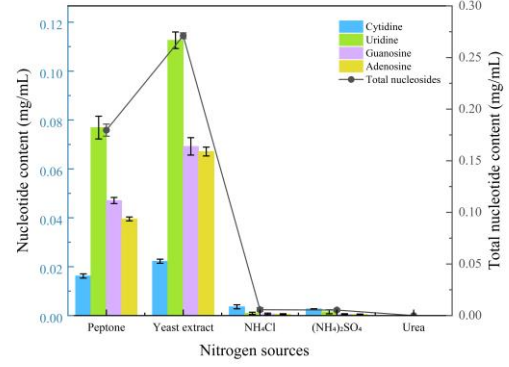
At 21–25°C, the total nucleotide content was proportional to the temperature (Fig. 5a), reaching a maximum of 0.26 mg/mL at 25°C. The results showed that the appropriate temperature promoted the synthesis of nucleotides. When the amount of inoculation was 2%–6%, the total nucleotide content increased with the increase of the inoculation amount, reaching the highest value of 0.29 mg/mL at 6%. The total nucleotide content decreased with an increase in the amount of inoculation at 6%–10% (Fig. 5b). Therefore, if the amount of inoculum is too large, the mycelium grows too fast, and the viscosity of the culture medium increases, resulting in insufficient dissolved oxygen. If the inoculum amount is too small, the lag period of cell growth is too long, which is not conducive to the growth and metabolism of cells in a short time.

When the pH was natural (3.7 ± 0.02), the total nucleotide content was up to 0.22 mg/mL and the total nucleotide content increased when the pH value was $3.5\text{--}3.7 \pm 0.02$ (Fig. 5c). Acidic pH has been reported to be more suitable for mycelial growth for various kinds of ascomycetes, including *Cordyceps* sp. (Hsieh et al. 2005). The total nucleotide content increased with the extension of the fermentation time at 3–6 d of fermentation and decreased with the extension of the fermentation time at 6–12 d, reaching a maximum of 0.27 mg/mL on 6 d (Fig. 5d). The reason may be that after 6 d, the mycelium appears to age and autolyze. The residual sugar of the fermentation broth decreased rapidly at 5–6 d and then tended to be stable, which may have been a period of rapid growth during this period, and the stage with the highest fungus and nucleotide content on 6 d. In summary, the total nucleotide content reached the highest value when the fermentation conditions were 25°C, 6% inoculum, natural pH (3.7 ± 0.02), and the incubation time was 6 d.

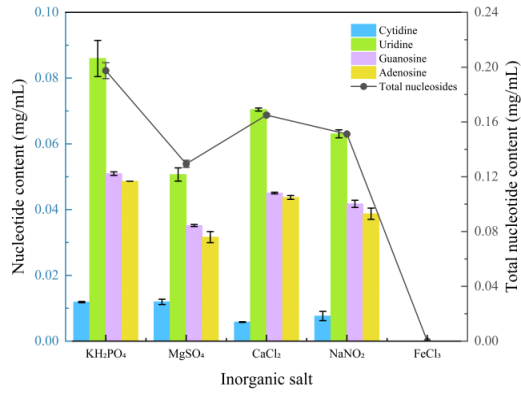
(a)



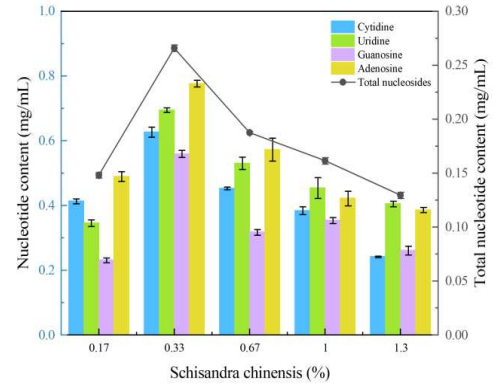
(b)



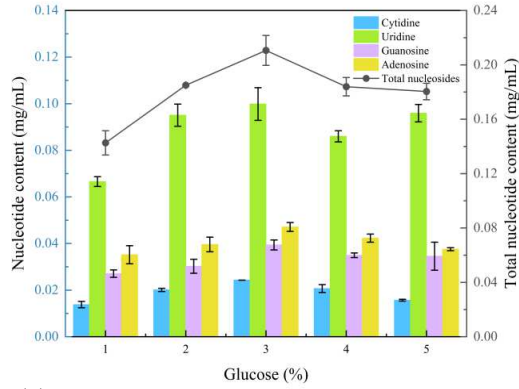
(c)



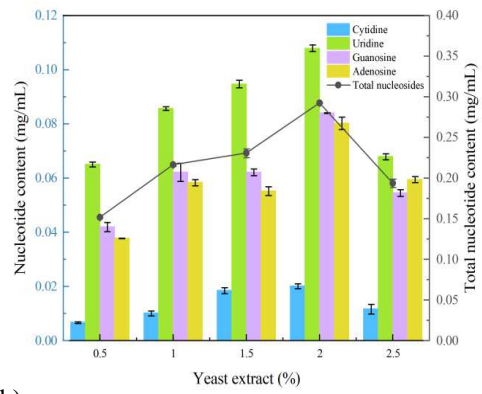
(d)



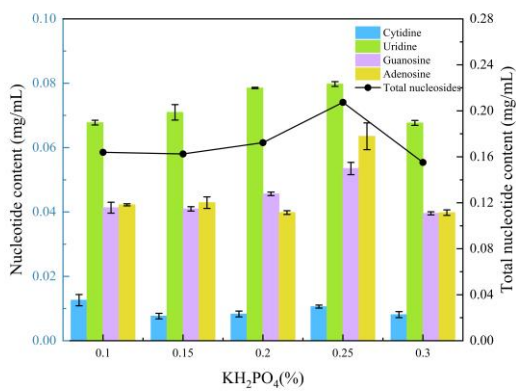
(e)



(f)



(g)



(h)

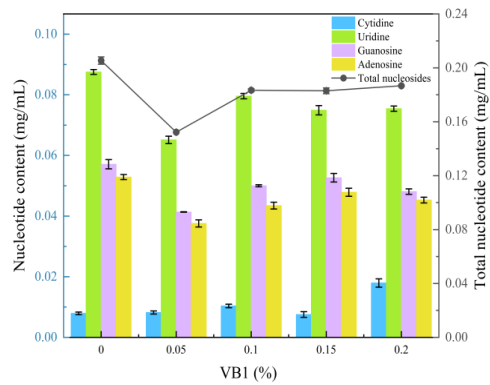


Fig. 4 Effects of carbon sources (a), nitrogen sources (b), inorganic salt (c), amount of *S. chinensis* (d), glucose amount (e), yeast extract amount (f), KH_2PO_4 amount (g), VB1 amount (h) on nucleotide content. Values are presented as the average $\pm$ standard deviation of the three replicates.

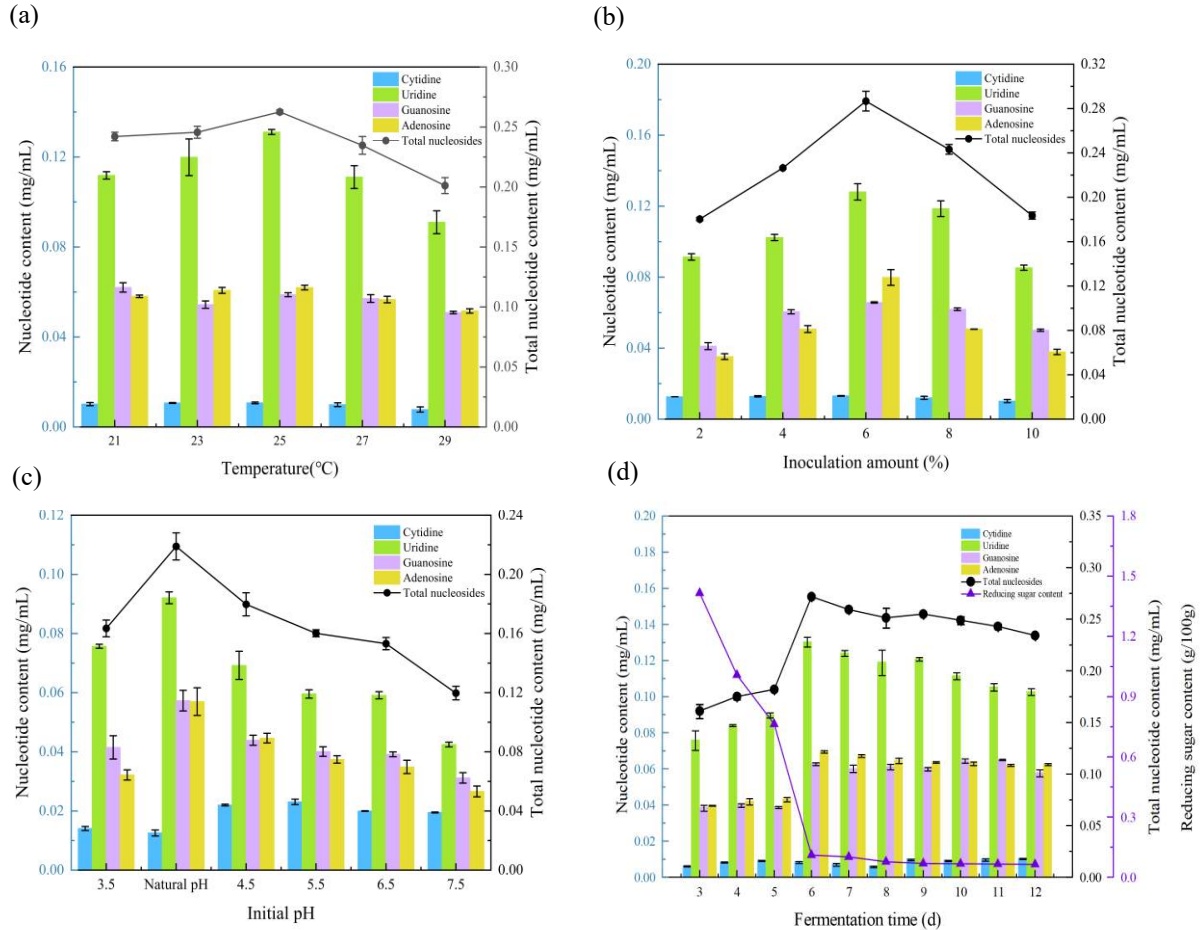


Fig. . Effects of temperature (a), inoculation amount (b), pH (c), and fermentation time (d) on nucleotide content. Values are presented as the average $\pm$ standard deviation of the three replicates.

3.4 Response surface design analysis

3.4.1 Box–Behnken design and RSM analysis

According to the results of the single-factor test, four factors, namely *S. chinensis* (A) and pH (B), yeast extract (C), and amount of inoculation (D), were selected to optimize the response surface of the experimental design. Factors and levels are shown in Table 2, and the experimental design and results are shown in Table 3.

3.4.2 Regression fitting and analysis of variance

Design-Expert 13.0 software (State–Ease, Minnesota, USA) was used for the analysis of quadratic multiple regression fitting, and the regression models of A, B, C, and D were obtained. The linear regression equation is as follows:

$$Y = -9.842670 + 1.373310A + 3.771700B + 1.268880C + 0.390176D - 0.100917AB \\ + 0.144476AC - 0.071218AD - 0.107959BC - 0.035097BD - 0.021075CD \\ - 0.996839A^2 - 0.406798B^2 - 0.211408C^2 - 0.015316D^2$$

The analysis of variance results for this model is shown in Table 4. The correlation coefficient (R^2) of the model was 0.9591, the P -value was < 0.01 , and the misfit term was not significant ($P > 0.05$), indicating that the model could have a significant impact on nucleotide production by fermentation. The models of *S. chinensis* (A) and inoculum amount (D) and the four secondary elements (A^2 , B^2 , C^2 , and D^2) reached significant levels ($P < 0.01$), and that F value shows that the influence of each factor on the total nucleotide content was $A > D > C > B$. The results in Table 4 show that the interaction between pH and *S. chinensis* (AB) was not significant, the interaction between *S. chinensis* and yeast extract addition (AC) and amount of yeast extract addition and inoculation (CD) were significant, and the interaction between *S. chinensis* and inoculation amount (AD), pH and yeast extract (BC), and pH and inoculation amount (BD) were significant and extremely. As can be seen in Figure 6, the interaction between Figures (C~E) is extremely significant, which is consistent with the analysis results in Table 4. In conclusion, the addition of *S. chinensis* and the amount had a significant effect on the nucleotide content.

According to the prediction results provided by Design-Expert 13.0, the optimal levels of *S. chinensis*, pH, yeast extract, and inoculation amount were 0.49%, 3.8, 2.1%, and 5.8%, respectively, where the total nucleotide content reached 0.40 mg/mL. The results of the validation revealed that it was 0.39 mg/mL and insignificant compared to the predicted value.

Table 2 Factors and their corresponding levels.

Factors		Levels		
		−1	0	1
<i>S. chinensis</i> (%)	A	0.17	0.33	0.67
pH	B	3.5	3.7	4.5
Yeast extract (%)	C	1.5	2	2.5
Inoculation amount (%)	D	4	6	8

Table 3 Response surface analysis scheme

Test number	A	B	C	D	Total nucleotide content (mg/mL)
1	0	−1	1	0	0.22132
2	−1	1	0	0	0.30812
3	0	1	−1	0	0.37244
4	1	0	−1	0	0.26713
5	0	−1	0	−1	0.20285
6	0	0	0	0	0.33940
7	0	1	0	−1	0.32630
8	0	0	0	−1	0.37627
9	0	−1	0	1	0.23487

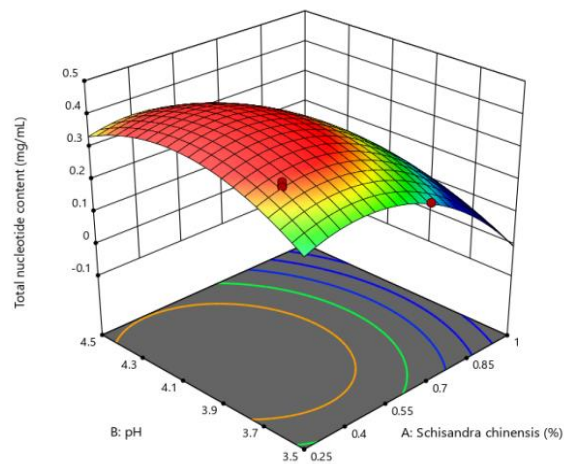
10	1	0	0	1	0.24136
11	-1	0	-1	0	0.27996
12	0	0	0	0	0.37462
13	-1	0	0	1	0.30659
14	0	0	0	0	0.38749
15	0	-1	-1	0	0.27074
16	-1	-1	0	0	0.24478
17	0	0	-1	-1	0.23743
18	1	0	1	0	0.26709
19	0	0	-1	1	0.33822
20	-1	0	1	0	0.21422
21	0	0	1	1	0.24204
22	0	1	0	1	0.24759
23	1	0	0	-1	0.28861
24	1	1	0	0	0.27816
25	0	0	1	-1	0.22555
26	0	0	0	0	0.37307
27	-1	0	0	-1	0.20452
28	0	1	1	0	0.22709
29	1	-1	0	0	0.25822

Table 4 Analysis of variance for the polynomial mode from the data of the Box–Behnken design.

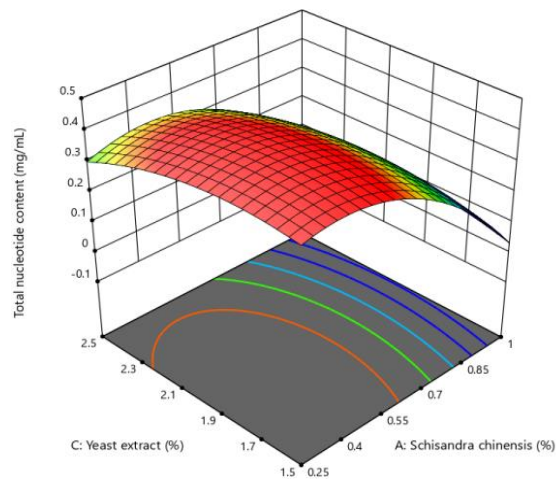
Source of variance	Sum of squares	Degrees of freedom	Mean square	<i>F</i> value	<i>P</i> value	significant
Model	0.0889	14	0.0889	23.42	< 0.0001	**
A	0.0184	1	0.0184	67.69	< 0.0001	**
B	0.0007	1	0.0007	2.42	0.1417	N
C	0.0013	1	0.0013	4.77	0.0464	*
D	0.0044	1	0.0044	16.29	0.0012	**
AB	0.0009	1	0.0009	3.15	0.0977	N
AC	0.0014	1	0.0014	5.21	0.0387	*
AD	0.0055	1	0.0055	20.24	0.0005	**
BC	0.0036	1	0.0036	13.27	0.0027	**
BD	0.0061	1	0.0061	22.44	0.0003	**
CD	0.0018	1	0.0018	6.55	0.0227	*
A ²	0.0178	1	0.0178	65.74	< 0.0001	**
B ²	0.0230	1	0.0230	84.83	< 0.0001	**
C ²	0.0181	1	0.0181	66.71	< 0.0001	**
D ²	0.0243	1	0.0243	89.64	< 0.0001	**
Residual	0.0038	14	0.0003			
Lack of Fit	0.0025	10	0.0002	0.7573	0.6727	N
Pure Error	0.0013	4	0.0003			
Cor Total	0.0927	28				

Note: ** Very significant effect. $P < 0.01$; *- Significant effect. $P < 0.05$; N- Insignificant effect. $P > 0.05$.

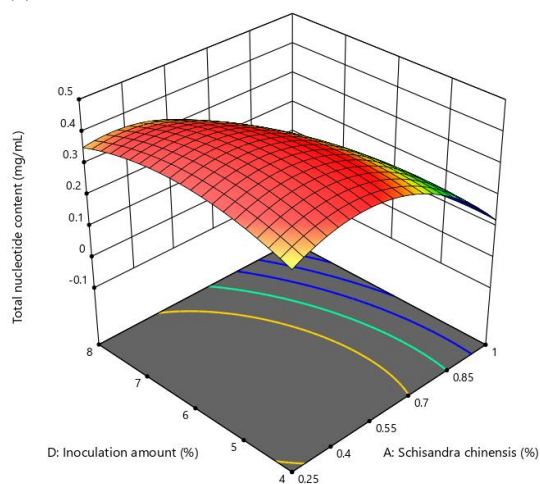
(a)



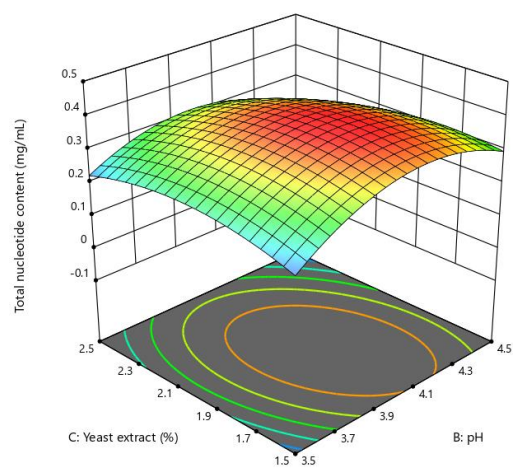
(b)



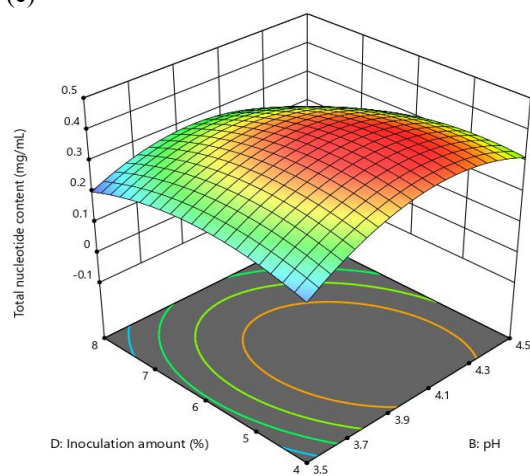
(c)



(d)



(e)



(f)

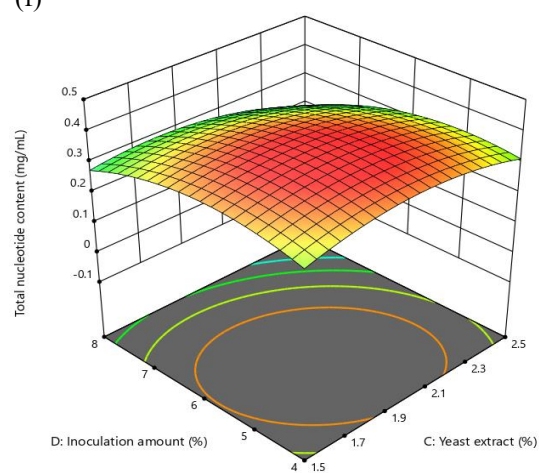


Fig. 6 Response surface plots showing the effects of inoculation amount, yeast extract, *S. chinensis*, and

pH on total nucleotide yield. Effect of the interaction between pH and *S. chinensis* (a); Effect of the interaction between *S. chinensis* and yeast extract (b); Effect of the interaction between the amount of inoculation and *S. chinensis* (c); Effect of the interaction between pH and yeast extract (d); Effect of the interaction between pH and inoculation amount (e); Effect of the interaction between yeast extract and inoculation amount (f).

3.5 Magnification of fungal culture results

During fungal fermentation, pH, microbial mass, and sugar consumption changed dynamically. Nucleotide chromatograms of *C. tenuipes* in a 5-L fermentation tank, medium fermentation conditions before and after optimization (Fig. 7a). The total nucleotide yield before optimization was 0.06 ± 0.0024 mg/mL, and the total nucleotide yield was 0.64 ± 0.027 mg/mL, which was 10.67 times the original yield (Fig. 7b). Compared to the total nucleotides of 11 batches of *C. sinensis* and related products analyzed by Qian et al. (2021), it increased by 2.71–26.73 times. Iamtham et al. (2022) reported the use of *spirulina* biomass residue and *Cordyceps militaris* fermentation, and the adenosine content of *C. militaris* was 0.71 mg/g, which was 9 times lower than that of *Cordyceps* adenosine in this study. The results showed that the fermentation method was extremely effective in increasing the yield of nucleotides and was suitable for industrial large-scale production.

The results showed that (Fig. 7c), the biomass was logarithmic in the first 60 h, and then the growth rate slowed, reaching the maximum value of 18.80 ± 0.32 mg/mL at 132 h, and then the fungus began to autolyze. The reducing sugar content in the medium slowly decreased during the logarithmic growth period of 0–60 h, and the sugar consumption was rapidly reduced at 84–108 h, and the sugar consumption gradually stopped at 0.21 ± 0.08 g/100 g. Combined with Fig. 5d, it can be seen that at the turning point when the residual sugar content decreases rapidly and then tends to be stable, the nucleotide content reaches the highest. Similarly, the fermentation can be stopped when the fermenter is incubated for 108 h. Therefore, the amount of residual sugar in the fermentation broth can be used as an indicator of the highest nucleotide content at the time of industrial production. Therefore, the amount of reducing sugar in the fermentation broth can be used as an indicator of the highest nucleotide content at the time of industrial production. With increasing fermentation time, the pH of the medium increased to 4.1 ± 0.06 at 0–48 h, which first produced alkaline metabolites and decreased to 3.48 ± 0.06 at 48–84 h, which may be due to the decomposition of *S. chinensis* to produce acidic substances, and the pH of *C. tenuipes* increased rapidly at 84–120 h and then increased to 4.56 ± 0.04 .

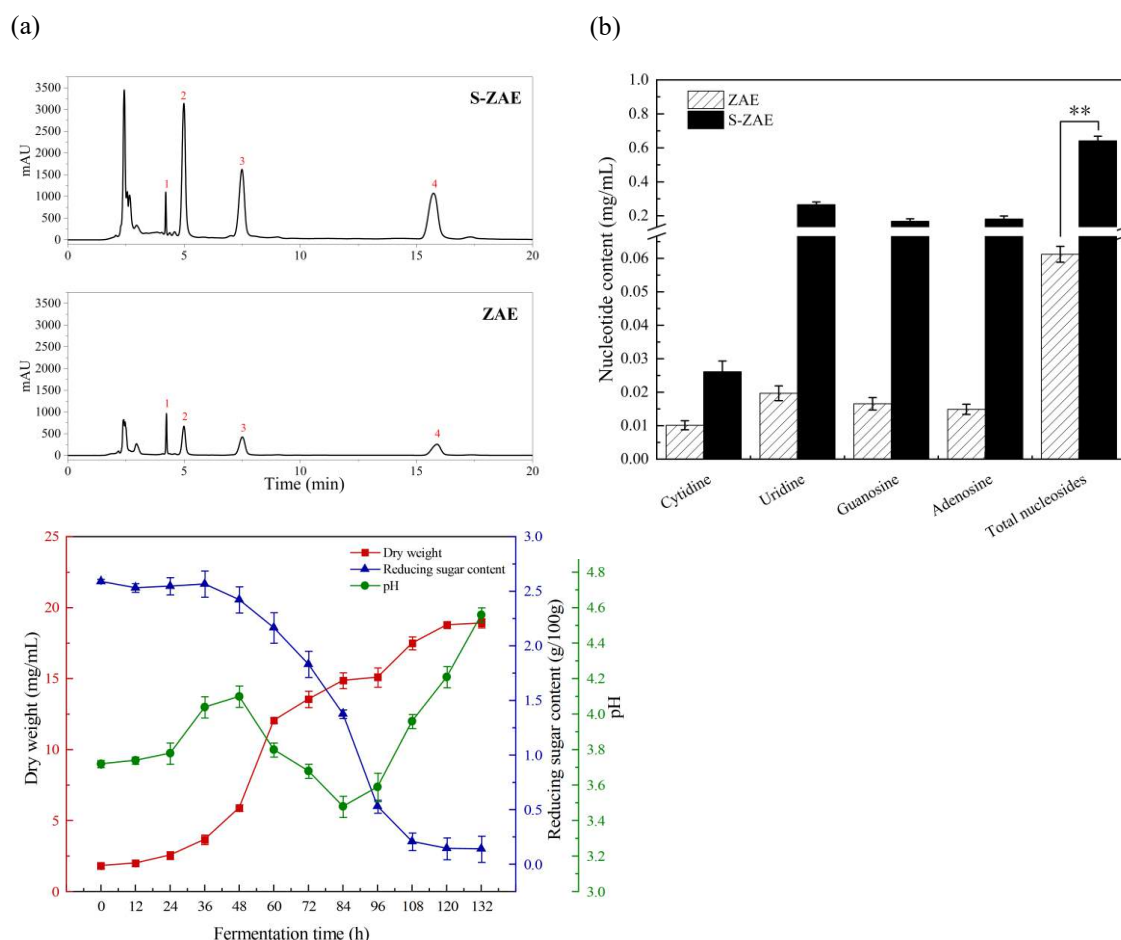


Fig. 7 Nucleotide chromatograms of *C. tenuipes* in a 5-L fermentation tank. (1) cytidine, (2) uridine, (3) guanosine, and (4) adenosine (a). Nucleotide content of enlarged fungi cultures in 5-L fermenters. (b). Growth kinetic curves of *S. chinensis* and *C. tenuipes*. Dry weight (mg/mL), reducing sugar content (g/100 g) (c).

3.6 AChE inhibitory activity assay results

AD is attributed to several pathological pathologies, such as oxidative stress (Kandimalla et al. 2017). Inhibition of AChE activity is an important means of clinical treatment of AD (Knopman et al. 2021). The results show that (Fig. 8a), in the range of 0.05–1.6 mg/mL of S-ZAE, the inhibitory effect of S-ZAE on AChE was significantly enhanced with an increase in the mass concentration, increased by 19.70%–27.79%, and showing significant dose dependence. The inhibitory activity of the *S. chinensis* aqueous extracts and ZXC-18 against acetylcholinesterase was lower than that of S-ZAE at the same mass concentration, indicating that the mixed fermentation of *S. chinensis* and *C. tenuipes* could significantly increase the inhibition rate of acetylcholinesterase of the aqueous extracts of *C. tenuipes* and *S. chinensis*. The positive control donepezil ($IC_{50} 0.028 \pm 0.005 \mu M$) showed strong AChE inhibition ability, but it is expensive and has side effects compared with natural inhibitors (Weller et al. 2018). However, the inhibition rate of AChE by the aqueous extract of S-ZAE ($IC_{50} 0.50 \pm 0.050 \text{ mg/mL}$) was higher than that of Boris et al. (2019) (IC_{50} values of $0.62 \pm 0.07 \text{ mg/mL}$ and $0.61 \pm 0.03 \text{ mg/mL}$). These results indicate that the mixed fermentation fungus *S. chinensis*-ZXC-18 has the potential to become a good

acetylcholinesterase inhibitor. The total nucleotide contents of 0.50 mg/mL of ZAE, SAE, and S-ZAE were 0.13 ± 0.005 , 0, and 0.29 ± 0.01 mg/mL, respectively (Fig. 8b). There were very few impurities in the aqueous extract of the fermentation product, and some unspecified water-soluble substances of *S. chinensis* were used or degraded during the fermentation process (Fig. 8c) (Hussain et al. 2016). S-ZAE had the highest nucleotide content, which was positively correlated with the inhibition rate of AChE. The results showed that the nucleotide content can greatly determine the inhibition ability of S-ZAE on AChE and there was a strong correlation between them. It has been reported that nucleotides can regulate AD by reducing oxidative stress response (Natchadaporn et al. 2023; Opeyemi et al. 2016).

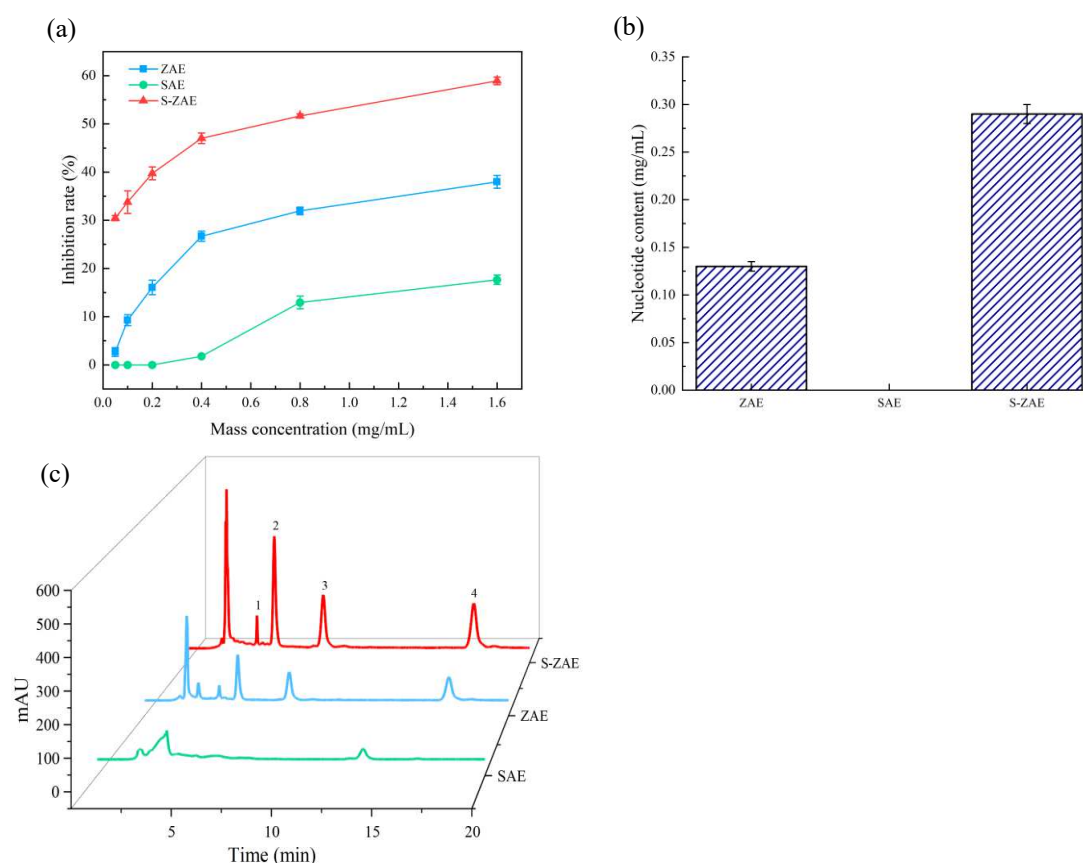


Fig. 8 Inhibition of AChE activity (a). Total nucleotide content of ZAE, SAE, and S-ZAE at 0.50 mg/mL (b). Nucleotide chromatograms of 0.50 mg/mL for SAE, ZAE, and S-ZAE: (1) cytidine, (2) uridine, (3) guanosine, and (4) adenosine (c).

3.7 Evaluation of antioxidant activity

DPPH is widely used to evaluate the antioxidant activity of substances (Wang et al. 2019). At a concentration of 0.5 mg/mL, the DPPH free radical scavenging rate was the lowest ($25.6 \pm 0.3\%$), and at 3.0 mg/mL, the scavenging rate was the highest ($77.4 \pm 0.5\%$), with IC_{50} of 1.38 ± 0.008 mg/mL (Fig. 9a). Oxidative stress is associated with reactive oxygen species (ROS) (Bierhaus et al. 2019). It can be seen from Fig. 9b that the hydroxyl radical scavenging rate was the lowest ($30.6 \pm 0.3\%$) at 0.5 mg/mL and the highest ($78 \pm 0.3\%$) at 3.0 mg/mL, with an IC_{50} of 1.31 ± 0.05 mg/mL. From Fig. 9c, it can be seen that the concentration of S-ZAE at 0.5 mg/mL was the weakest ($28.5 \pm 0.1\%$), and at 3.0 mg/mL, the scavenging ability was the strongest ($64.2 \pm 0.2\%$), with IC_{50} of 1.43 ± 0.08 mg/mL.

Compared with other medicinal fungi, S-ZAE hydroxyl radicals were higher than *Ganoderma lucidum* spore water extract (IC₅₀ 3.573 mg/mL) (Zhang et al. 2022). The hydroxyl radical scavenging rate and superoxide ion scavenging rate of *Cordyceps militaris* polysaccharides were lower than those of S-ZAE (Zhang XL et al. 2021), but the IC₅₀ of the water extract of *Cordyceps sinensis* was 0.93 ± 0.01 mg/mL for DPPH free radical, 0.96 ± 0.06 mg/mL for hydroxyl radical, and 1.00 ± 0.05 mg/mL for superoxide ion free radical (Dong et al. 2008), which were all higher than the antioxidant activity of S-ZAE. In Fig. 9, the antioxidant activity of ZAE is lower than that of S-ZAE, indirectly indicating that the nucleotide content affects the antioxidant activity, and Jiang et al. (2011) found that the antioxidant activity of medicinal fungi is related to nucleotides. It has been reported that water extract of *Cordyceps* could reduce oxidative stress (Chu et al. 2011; Bai et al. 2018), and *Paecilomyces tenuipes* extract could also alleviate oxidative stress and have antioxidant effects (Li et al. 2019). Products using a two-way liquid fermentation process are either directly applied to fungal powder extracts, while others extract active ingredients such as polysaccharides before being used in medicine (Wang et al. 2022). Therefore, S-ZAE has the potential to be a good functional food or natural therapeutic agent.

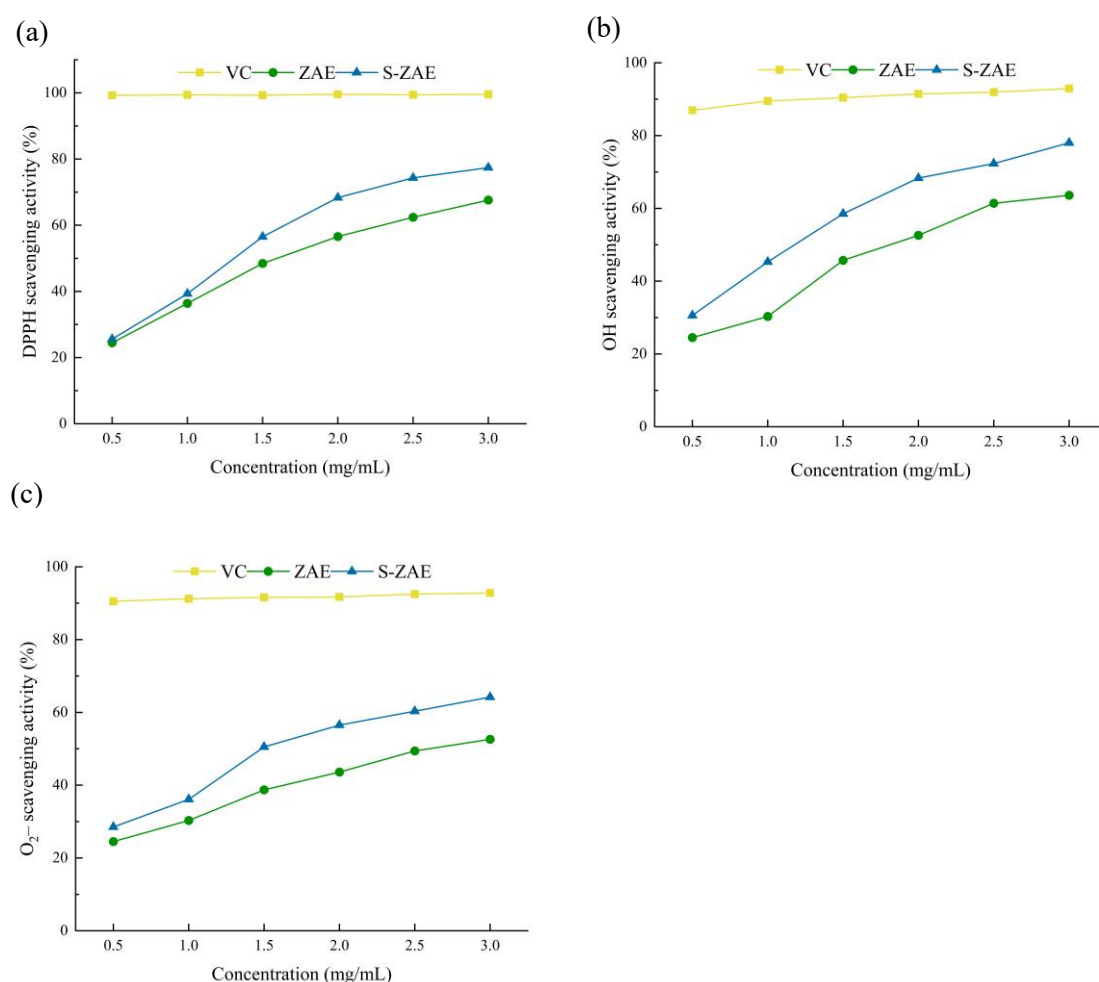


Fig. 9 Results of antioxidant activity.

4. Conclusions

We can often see the process optimization and biological activity of *C. sinensis* and *C. militaris* for the production of polysaccharides, adenosine and cordycepin. However, there are no reports that the method of mixed fermentation of *C. tenuipes* and TCM can increase the total nucleotide content and thus improve AD preliminarily. In this study, ZXC-18 was evaluated for its nucleotide production ability and potential AD-modifying effects. The total nucleotide content was 0.64 ± 0.027 mg/mL in the 5-L fermentation tank under the optimization process of single factor and response surface method, which was 10.67 times higher than the control. It has outstanding results in anti-AChE ability and obvious superiority in scaling up nucleotide production. In conclusion, the mixed fermentation of *S. chinensis* and *C. tenuipes* can significantly increase the nucleotide content of *Cordyceps* and effectively improve the AChE resistance and antioxidant capacity. This method has great potential in the field of fermentation and is of great value for the industrialization and commercial application of medicinal fungi. The fermented products have the potential to become good functional food and natural therapeutic agents.

Acknowledgments

This work was supported by the Jiangsu Key Laboratory of Marine Bioresources and Environment/Jiangsu Key Laboratory of Marine Biotechnology.

Author contributions

Anni Li: Conceptualization, Methodology, Data curation, Formal analysis, Writing—original draft, Writing—review & editing. Qiang Zhu: Supervision, Data curation. Yuting Li: Investigation, Validation. Lu Yang: Conceptualization, Investigation. Zhihao Chen: Supervision. Xiang Zhou: Formal analysis. Yanqiu Xia: Conceptualization, Writing—review & editing, Funding acquisition.

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data availability

The gene sequence and complete genome sequence of ZXC-18 are deposited in GenBank under the accession number OP159391.

Declaration

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent to publish All authors read and approved the manuscript for publication.

References

- Bierhaus A, Reddy PV, Zhu X et al (2009) Oxidative Stress in Diabetes and Alzheimer's Disease. *Journal of Alzheimer's Disease* 16 (4): 763–774.
- Bunyapaiboonsri T, Yoiprommarat S, Intereya YK, Rachtawee P, Hywel-Jones NL, Isaka M (2009) Isariotins E and F, spirocyclic and bicyclic hemiacetals from the entomopathogenic fungus *Isaria tenuipes* BCC 12625. *J Nat Prod* 72 (4): 756–759.
- Buhmann A, Papenbrock J (2013) An economic point of view of secondary compounds in halophytes. *Functional Plant Biology* 40 (9): 952–967.
- Bai X, Tang YB, Lin Y, Zhao YQ, Tan TY, Wang SY, Liu MQ, Chang ZH, Liu Y, Liu ZQ (2018) Protective effect of *Cordyceps sinensis* extract on rat brain microvascular endothelial cells injured by oxygen–glucose deprivation. *Journal of Traditional Chinese Medical Sciences* 5 (1): 64–71.
- Boris P, Kristina T, Dragica J, Sonja K, Filip S, Milena R, Maja K (2019) The polysaccharide extracts from the fungi *Coprinus comatus* and *Coprinellus truncorum* do exhibit AChE inhibitory activity. *Natural Product Research* 33 (5): 750–754.
- Chen XM, Lu JX, Zhang YD, He JT, Guo XZ, Tian GY, Jin LQ (2008) Studies of macrophage immuno-modulating activity of polysaccharides isolated from *Paecilomyces tenuipes*. *International Journal of Biological Macromolecules* 43 (3): 252–256.
- Chu HL, Chien JC, Duh PD (2011) Protective effect of *Cordyceps militaris* against high glucose-induced oxidative stress in human umbilical vein endothelial cells. *Food Chemistry* 129 (3): 871–876.
- Choo SH, Sung HH, Chae MR, Kang SJ, Han DH, Park JK, So I, Lee SW (2014) Effects of *Schisandra chinensis* extract on the relaxation of isolated human prostate tissue and smooth muscle cell. *J Ethnopharmacol* 156: 271–276.
- Dong CH, Yao YJ (2008) In vitro evaluation of antioxidant activities of aqueous extracts from natural and cultured mycelia of *Cordyceps sinensis*. *LWT - Food Science and Technology* 41 (4): 669–677.
- Ellman GL, Courtney KD, Andres VJ (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7 (2): 88–95.
- Han HC, Lindequjst U, Hyun JW, Kim YH, An HS, Lee DH, Kim HW (2004) Apoptosis induction by 4 β -acetoxyscirpentiol from *paecilomyces tenuipes* in human leukaemia cell lines. *Die Pharmazie* 59 (1): 42–49.
- Hsieh C, Tsai MJ, Hsu TH, Chang DM, Lo CT (2005) “Medium optimization for polysaccharide production of *Cordyceps sinensis*”. *Applied Biochemistry and Biotechnology* 120 (2): 145–157.
- Hussain A, Bose S, Wang JH, Yadav MK, Mahajan GB, Kim H (2016) Fermentation, a feasible strategy for enhancing bioactivity of herbal medicines. *Food Research International* 81: 1–16.
- He XJ, Fu JX, J J, Gai QY, Fu YJ, Feng X, Wang Y (2024) Semi–solid–state fermentation of *Polygonum cuspidatum* roots by a novel endophytic fungus *Penicillium rubens* with capabilities of cell wall hydrolysis and polydatin deglycosylation to improve the yield of high–value resveratrol. *Industrial Crops and Products* 209.
- Iamtham S, Kaewkam A, Chanprame S, Pan-utai W (2022) Effect of *Spirulina* biomass residue on yield and cordycepin and adenosine production of *Cordyceps militaris* culture. *Bioresource Technology Reports*: 17.
- Ji SD, Sung GB, Kang PD, Kim KY, Choi YS, Kim NS, Woo SO, Han SM, Hong IP, Ha NG (2011) Synnemata production using silkworm variety, female Yangwonjam by *Isaria tenuipes*. *Mycobiology* 39 (3): 158–163.

- Jiang Y, Wong JH, Fu M, Ng TB, Liu ZK, Wang CR, Li N, Qiao WT, Wen TY, Liu F (2011) Isolation of adenosine, iso-sinensetin and dimethylguanosine with antioxidant and HIV-1 protease inhibiting activities from fruiting bodies of *Cordyceps militaris*. *Phytomedicine* 18 (2–3): 189–193.
- Jiang W, Sui ZJ, Zhu ZY (2023) Effect of *Cordyceps militaris* polysaccharide on the solubility, stability and antioxidant properties of dihydromyricetin. *Process Biochemistry* 130: 606–613.
- Kikuchi H, Miyagawa Y, Sahashi Y, Inatomi S, Haganuma A, Nakahata N, Oshima Y (2004) Novel trichothecanes, paecilomycine A, B, and C, isolated from entomopathogenic fungus, *Paedlomyces tenuipes*. *Tetrahedron letters: The International Journal for the Rapid Publication of Preliminary Communications in Organic Chemistry* 45 (33): 6225–6228.
- Kang PD, Sung GB, Kim KY, Kim MJ, Hong IP, Ha NG (2010) Breeding of a silkworm variety for synnemata production of *Isaria tenuipes*. *Mycobiology* 38 (3): 180–183.
- Kumar A, Nisha CM, Silakari C, Sharma I, Anusha K, Gupta N, Nair P, Tripathi T, Kumar A (2016) Current and novel therapeutic molecules and targets in Alzheimer's disease. *Journal of the Formosan Medical Association* (1): 3–10.
- Kandimalla R, Reddy PH (2017) Therapeutics of neurotransmitters in Alzheimer's disease. *J Alzheimers Dis* 57: 1049–1069.
- Knopman, DS, Amieva H, Petersen RC (2021) Alzheimer disease. *Nature Reviews Disease Primers* 7 (1): 33.
- Kaewkam A, Sornchai P, Chanprame S, Iamtham S (2021) Utilization of *Spirulina maxima* to enhance yield and cordycepin content in *Cordyceps militaris* artificial cultivation. *J ISSAAS* 29: 1–14.
- Lee DH, Park T, Kim HW (2006) Induction of apoptosis by disturbing mitochondrial-membrane potential and cleaving PARP in Jurkat T cell through treatment with acetoxyscirpenol mycotoxins. *Biol Pharm Bull* 29 (4): 648–654.
- Lu R, Miyakoshi T, Tian G, Yoshida T (2006) Structural studies of *Paecilomyces tenuipes* Samson polysaccharide-part-2. *Carbohydrate Polymers* 67 (3): 343–346.
- Liu CG, Wang J, Xu SQ, An SS, Tang SY, He J, Liu Y, Lee RJ, Wang D (2017) *Paecilomyces tenuipes* extract prevents depression-like behaviors in chronic unpredictable mild stress-induced rat model via modulation of neurotransmitters. *Mol Med Rep* 16 (2): 2172–2178.
- Liu CG, Zeng XR, Li Y, Ma HL, Song JJ, Li YH, Zhou YL, Lee RJ, Wang D (2017) Investigation of hypoglycemic, hypolipidemic and anti-nephritic activities of *Paecilomyces tenuipes* N45 in diet/streptozotocin induced diabetic rats. *Mol Med Rep* 15 (5): 2807–2813.
- Lu XY, Chen Q, Cui XY, Abozeid A, Liu Y, Liu J, Tang ZH (2019) Comparative metabolomics of two saline-alkali tolerant plants *Suaeda glauca* and *Puccinellia tenuiflora* based on GC-MS platform. *Nat Prod Res* 35 (3): 499–502.
- Li YY, Han L, Lu T, Noman M, Qiang WD, Lan XX et al (2019) Antidepressant-like activities of extracts of the fungus *Paecilomyces tenuipes* M98. *Psychiatry and Clinical Psychopharmacology* (4): 872–879.
- Lee SK, Lee JH, Kim HR, Chun Y, Lee JH, Yoo HY, Park C, Kim SW (2019) Improved cordycepin production by *Cordyceps militaris* KYL05 using casein hydrolysate in submerged conditions. *Biomolecules* 9 (9): 461.
- Mao XB, Eksriwong T, Chauvatcharin S, Zhong JJ (2005) Optimization of carbon source and carbon/nitrogen ratio for cordycepin production by submerged cultivation of medicinal mushroom *Cordyceps militaris*. *Process Biochem* 40 (5): 1667–1672.

- Natchadaporn S, Nudjanad H, Wilasinee P, Phongsakorn K, Palakorn K, Nipha C, Parinya N (2023) Cordycepin, a bioactive compound from *Cordyceps* spp., moderates Alzheimer's disease-associated pathology via anti-oxidative stress and autophagy activation. *Journal of Asian Natural Products Research* 21–21.
- Opeyemi JO, Feng Y, Oyenike OO, Tang J, Ouyang Z, Su ZL, Wang DJ, Yu XF (2016) Neuroprotective effects of adenosine isolated from *Cordyceps cicadae* against oxidative and ER stress damages induced by glutamate in PC12 cells. *Environmental Toxicology and Pharmacology* 44: 53–61.
- Patthanajuck V, Bunnag S (2021) Effects of carbon and nitrogen sources on fruiting body formation and cordycepin production of *Cordyceps militaris* (L.) link. *Khon Kaen Agri J* 49: 274–283.
- Qian ZM, Wu Z, Huang Q, Wang CX, Tan GY, Li WJ, Sun WY, Lv GP, Gao H (2021) Development of an eco-friendly and fast HPLC method for quantitative analysis of four nucleosides in *Cordyceps* and related products. *Chinese Journal of Natural Medicines* 19: 954–960.
- Qurat-ul-Ain M, Saleem M, Nazir N, Riaz M, Tousif I, Tauseef S, Shah SAA (2023) Secondary metabolite profiling, antioxidant capacity, enzyme inhibitory potential and in silico studies of *Launea intybacea* (Jacq.) Beauverd: A multifunctional approach to probe into the new nutraceuticals. *Journal of Molecular Structure* 1294 (P2).
- Raethong N, Wang H, Nielsen J, Vongsangnak W (2020) Optimizing cultivation of *Cordyceps militaris* for fast growth and cordycepin overproduction using rational design of synthetic media. *Computational and Structural Biotechnology Journal* 18: 1–8.
- Smironoff N, Cumbes QJ (1989) Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry* 28 (4): 1051–1060.
- Suzuki K, Tsushima M, Goryo M et al (2017) Neuro protective properties of the fungus *Isaria japonica*: evidence from a mouse model of aged-related degeneration, in: Atta-ur-Rahman. *Frontiers in Clinical Drug Research (Alzheimer Disorders)*. Bentham Science Publishers, Sharjah, UAE 6: 154–186.
- Takano F, Yahagi N, Yahagi R, Takada S, Yamaguchi M, Shoda S, Murase T, Fushiya S, Ohta T (2005) The liquid culture filtrates of *Paecilomyces tenuipes* (Peck) Samson (= *Isaria japonica* Yasuda) and *Paecilomyces cicadae* (Miquel) Samson (= *Isaria sinclairii* (Berk.) Llod) regulate Th1 and Th2 cytokine response in murine Peyer's patch cells in vitro and ex vivo. *International Immunopharmacology* 5 (5): 903–916.
- Tang ZZ, Lin WJ, Yang J, Feng SL, Qin YH, Xiao YR, Chen H, Liu YT, Chen H, Bu TL, Li QF, Yao HP, Ding CB (2022) Ultrasound-assisted extraction of *Cordyceps cicadae* polyphenols: Optimization, LC-MS characterization, antioxidant and DNA damage protection activity evaluation. *Arabian Journal of Chemistry* 15 (8): 103953.
- Van TTT, Van TK, Lam LS, Thanh TM, Chau GDN, Kiet TT, Vinh QN, Chien CN, Hieu TL (2022) Phenolic content and antioxidant activity of *Ophiocordyceps Sobolifera* extract for renal injury prevention. *Process Biochemistry* 121: 322–329.
- Wang LP (2006) Experimental Study on Anti-tumor Effect of Gallic Acid. Jilin University.
- Wu T, Wang N, Zhang Y, Xu X (2013) Advances in the study on microbial fermentation and transformation of traditional Chinese medicine. *African Journal of Microbiology Research* 7 (17): 1644–1650.
- Weller J, Budson A (2018) Current understanding of Alzheimer's disease diagnosis and treatment. *F1000Res* 7.

- Wang L, Guo H, Liu X, Jiang G, Li C, Li X, Li Y (2019) Roles of *Lentinula edodes* as the pork lean meat replacer in production of the sausage. *Meat Science* 156: 44–51.
- Wang SQ, Lu L, Song TY, Xu XX, Yu J, Liu TX (2022) Optimization of *Cordyceps sinensis* fermentation *Marsdenia tenacissima* process and the differences of metabolites before and after fermentation. *Heliyon* 8 (12): e12586–e12586.
- Wang YC, Gao C, Long PC, Hi ZY, Zhu LS, Wang LL (2023) Dynamic changes of active substances of rice, *Pueraria* and yam fermentation by *Monascus ruber*. *LWT* 183.
- Xu QQ, Lv LX, Chen SY, Zheng J, Zheng GL, Li YQ (2009) Isolation of *Cordyceps ophioglossoides* L2 from Fruit Body and Optimization of Fermentation Conditions for Its Mycelial Growth. *Chinese Journal of Chemical Engineering* 17 (2): 278–285.
- Xu Y, Niu X, Liu N, Gao Y, Wang L, Xu G, Li XG, Yang Y (2018) Characterization, antioxidant and hypoglycemic activities of degraded polysaccharides from blackcurrant (*Ribes nigrum* L.) fruits. *Food Chemistry* 243: 26–35.
- Yang K, Qiu J, Huang ZC, Yu ZW, Wang WJ, Hu HL, You Y (2021) A comprehensive review of ethnopharmacology, phytochemistry, pharmacology, and pharmacokinetics of *Schisandra chinensis* (Turcz.) Baill. and *Schisandra sphenanthera* Rehd. et Wils. *Journal of ethnopharmacology* 284 114759–114759.
- Zhang Z, Li Y, Zhang K (2007) “Application of statistical analysis for the optimization of mycelia and polysaccharide production by *Tremella aurantialba*”. *Food Technology and Biotechnology* 45 (1): 45–50.
- Zhong S, Nie YC, Gan ZY, Liu X, Fang Z, Zhong B, Tian J, Huang C, Lai K, Zhong N (2015) Effects of *Schizandra chinensis* extracts on cough and pulmonary inflammation in a cough hypersensitivity Guinea pig model induced by cigarette smoke exposure. *J Ethnopharmacol* 165: 73–82.
- Zhu P, Li J, Fu X, Yu Z. (2019) *Schisandra* fruits for the management of drug-induced liver injury in China: a review. *Phytomedicine* 59: 152760.
- Zhu YM, Pan LC, Zhang LJ, Yin Y, Zhu ZY, Sun HQ, Liu CY (2020) Chemical structure and antioxidant activity of a polysaccharide from *Siraitia grosvenorii*. *Int J Biol Macromol* 165 (Pt B) :1900–1910.
- Zhang XL, Zhang XJ, Gu SS, Pan LC, Sun HQ, Gong EL, Zhu ZY, Wen TC, Daba GM, Elkhateeb WA (2021) Structure analysis and antioxidant activity of polysaccharide-iron (III) from *Cordyceps militaris* mycelia. *International Journal of Biological Macromolecules* 178: 170–179.
- Zhang L, Qiao HY, Liu HX, Jiang GC, Wang LY, Liu XJ (2022) Antioxidant, hypoglycemic and protection of acute liver injury activities of *Ganoderma lucidum* spore water extract. *Journal of Functional Foods* 97: 105254.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Graphicalabstract.pdf](#)