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ORIGINAL ARTICLE

A novel preparation for siderophore-assisted copper and zinc enrichment in yeast

Xiao-ying Fan[1] | Zi-yu Liu[1] | Zhi-peng Jia[1] | Ya-ru Wei[1] | Dong-dong Xie[1] | Ji Zhang[2] | Bei Wang[1] | Xin-guo Zhang[1]

[1]School of Life Science and Engineering, Key Laboratory of Herbal-Tebitan Drug Screening and Deep Processing of Gansu Province, Lanzhou University of Technology, Lanzhou, China

[2]College of Life Sciences, Northwest Normal University, Lanzhou, China

Correspondence
Xin-guo Zhang, School of Life Science and Engineering, Lanzhou University of Technology, No.287 Langongping Road, Qilihe District, Lanzhou 730050, Gansu Province, China.
Email: biodrug@163.com

Bei Wang, School of Life Science and Engineering, Lanzhou University of Technology, No. 287, Langongping Road, Qilihe District, Lanzhou 730050, Gansu Province, China.
Email: 411406770@qq.com

Ji Zhang, College of Life Science, Northwest Normal University, No. 967, Anning East Road, Anning District, Lanzhou 730070, Gansu Province, China.
Email: zhangj@nwnu.edu.cn

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Abstract

Copper and zinc are essential trace elements for several biological activities and play an important role in living organisms. In this study, the role of siderophores obtained from 16 microorganisms isolated from an iron-rich environment was evaluated in the transport of zinc and copper in yeast. In addition, siderophores showing relevant transport activity were used in the preparation of metal-enriched yeast. Siderophores TZT-SH5I and TZT-ZTH2X significantly improved tolerance of *Saccharomyces cerevisiae* during growth under high concentrations of zinc/copper. Strains producing siderophores TZT-SH5I and TZT-ZTH2X were identified as *Aspergillus* sp and *Penicillium* sp, respectively. The orthogonal method was used to determine optimized conditions for siderophore-assisted copper and zinc enrichment of *S. cerevisiae*. The final intracellular content of organic Cu and Zn in *S. cerevisiae* grown in a siderophore-containing medium was 60.76 and 44.22 mg/g. This study provides a convenient and feasible new strategy for the preparation of supplements rich in organic trace elements.

Novelty impact statement: Bioaccumulation of essential trace elements (TEs) by microorganisms is one of the most important ways to TEs from inorganic into organic forms which can be more efficiently absorbed by humans. In the current study, a novel method for preparing copper and zinc-enriched yeast using siderophores from high iron environment microorganisms has been optimized and established. Significantly higher intracellular content of organic Cu and Zn in *Saccharomyces cerevisiae* grown in a siderophore-containing medium was obtained, which would provide a new idea and platform for the preparation of organic trace elements.

1 | INTRODUCTION

Essential trace elements (TEs) are indispensable for human nutrition and health (Fraga, 2005). Among TEs, zinc (Zn) and copper (Cu) are known to play a crucial role in many biological functions: Zn is an important structural component of many transcription factors and enzymes, being also involved in the regulation of intracellular signal transduction (Tiefenbach, 1986); Cu is part of the structure and participates in the activation of several enzymes which perform important metabolic functions and maintain homeostasis (Vetchý, 2018). Copper and zinc are thus important trace elements that play a vital role in human health.

Demands for TS very widely at different ages, usually high in Children than in adults, to support growth and development (Zemrani & Bines, 2020). Zn deficiency in children can lead to stunting or dwarfism, impaired immune function, hypogonadism, and endocrine

Xiao-ying Fan and Zi-yu Liu contributed equally to this work.

dysfunction (Hambidge, 2000; Rosado, 2003). Cu deficiency can decrease plasmatic levels of lipoprotein lipase and lecithin:cholesterol transacylase activity, as well as increase cholesterol and triglyceride levels in plasma, hence, leading to hypertriglyceridemia (Allen & Klevay, 1978; Klevay, 1987).

Supplementation of TEs is an important strategy to prevent deficiencies. Appropriate Zn supplementation can limit early fetal growth delay and increase newborn weight, significantly reduce the incidence of respiratory infections, and be used to treat attention deficit and hyperactivity disorder (Aminisani et al., 2009; Arnold & Disilvestro, 2005; Goldenberg et al., 1995). Abnormal Cu metabolism can cause organizational structure and function abnormalities (Bost et al., 2016; Harris, 2003; Trocello et al., 2010). Cu can also directly participate in the body's immune function and indirectly affect tumor genesis, hence, supplementation with sufficient amounts of Cu can help regulate the function of natural killer cells, macrophages, lymphocytes, and other reactions that limit tumor growth (Gupte & Mumper, 2009; Lin et al., 2002). Copper or zinc deficiency can be treated through the use of different inorganic salts, such as zinc sulfate, zinc oxide, and copper sulfate. At present, zinc sulfate alters food sensorial characteristics, rendering food unpalatable, while zinc oxide is nearly insoluble and poorly absorbed (Salgueiro et al., 2000). Moreover, intake of copper sulfate as a supplement has several side effects, such as nausea, diarrhea, and reflux (Jin & Xu, 1993; Pratt et al., 1985). Therefore, there is a need for finding novel strategies to produce supplements where TEs are found in organic form.

Bioaccumulation is the process of transforming inorganic TEs present in the environment into their organic forms, with various organelles and complex biological states in cells potentially and extensively providing binding sites for TEs (Bird & Wilson, 2020; Zhang, Zhang, et al., 2014). Currently, the use of microorganisms is the most important way to transform TEs from inorganic into organic forms, which are more readily absorbed by humans. In this context, dietary yeast is the most representative substrate for TE enrichment (Korhola & Edelmann, 1986). In particular, *Saccharomyces cerevisiae* has been widely used for iron (Fe) (Fernanda et al., 2011), selenium (Egressy-Molnár et al., 2016), Zn (Wang et al., 2011), Cu (Sillerová et al., 2012), and chromium (Batic & Raspor, 2000) enrichment. With bioconversion and bioaccumulation, the limitation of the use of inorganic TE supplements can be circumvented and such supplements are now being placed at the forefront of the field of organic microelement supplement development.

Although *S. cerevisiae* is a rich dietary source of organic Fe obtained through microbial transformation, wild-type *S. cerevisiae* does not normally grow in medium containing Fe at high concentration (≥600 μg/ml); thus, enrichment and transformation of TEs in such conditions are limited (Zhang et al., 2016). Siderophores are low molecular weight iron-chelators produced by bacteria and fungi under conditions of low iron stress. Studies have shown that siderophores are involved in Fe transport in Fe-deficient as well as in Fe-rich environments (Labbé et al., 2007). It has been considered that the transport of TEs by microorganisms such as *S. cerevisiae* in Fe-, Zn-, and Cu-rich environments occurs via siderophores (Fernanda et al., 2011; Sillerová et al., 2012 Wang et al., 2011). Thus, the addition of siderophores to the culture medium would help increase tolerance to trace elements by such microorganisms and improve enrichment with TEs.

The aim of this study was to increase tolerance of *S. cerevisiae* to high levels of Zn and Cu by the incorporation of siderophores to the culture medium. A novel siderophore-assisted preparation for obtaining yeast enriched with organic Zn and Cu has been developed, which would offer practical insights and future directions for organic TE production.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Sodium diethyldithiocarbamate and sodium thiosulfate were purchased from Shanghai Hengsheng Chemical Co., Ltd. The chrome azurol S (CAS) was purchased from Shanghai Yiyan Biotechnology Company. Copper sulfate, copper powder, zinc sulfate, and zinc powder were purchased from Qingdao renhexing Experimental Technology Co. Ltd. (Shandong, China). Other reagents were analytically pure.

2.2 | Isolation and determination of the concentration of siderophores

Siderophore-producing strains obtained from Fe-rich environments were inoculated in Rose Bengal liquid medium (peptone 5 g/L, glucose 10 g/L, potassium dihydrogen phosphate 1 g/L, magnesium sulfate 0.5 g/L, tiger red 0.03 g/L, chloramphenicol 0.1 g/L, agar powder 20 g/L, pH 7.2–7.4, ferrous ion concentration 1,000 μg/ml), and incubated at 28°C under gentle agitation (180 rpm) for 4 days. Supernatants were collected by centrifugation at 30,000 rpm for 10 min. Siderophore concentration was determined using a method previously reported (Milagres et al., 1999; Schwyn & Neilands, 1987). The same aliquot of deferoxamine mesylate solutions of different concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 μg/ml) was taken and mixed with fresh CAS blue dye solution to obtain standards. After standing for 1 hr, the absorbance of standard solutions was determined at 630 nm. A standard curve was made and the content of the siderophore was determined by atomic absorption spectrometry (Milagres et al., 1999; Schwyn & Neilands, 1987).

2.3 | Determination of Cu content

Cu content was determined using the diethyldithiosulfate spectrophotometric method according to the Chinese

Pharmacopoeia (Ministry of Health, GB/T 5009.13-2017, 2017). For the preparation of the standard curve, 125 ml of Cu standard solution were prepared at different concentrations (0, 5.0, 10.0, 15.0, 20.0, and 25.0 μg); 5 ml of ammonium citrate and ethylenediaminetetraacetic acid disodium solution, 50 μl of phenol red solution were mixed and adjusted to red with ammonia; subsequently, 2.0 ml of Cu reagent and 10.0 ml of carbon tetrachloride solution were incorporated and the mixture was shaken vigorously for 2 min. After the mixture was filtered with absorbent cotton, the absorbance of the resulting filtrate was measured at 440 nm. (UV-9200, Beijing Ruili Analytical Instrument Company).

Fermented broth samples were submitted to centrifugation (5,000 rpm for 5 min) and harvested cells were washed with deionized water until no Cu ions were detected, then dried at 60°C until a constant weight was achieved. Further, cells were digested with a mixture of 10 M perchloric acid and 14 M nitric acid (1:4, v/v) until a clear solution was obtained. After the volume was fixed, the intracellular Cu content was determined by absorbance measurements (Ministry of Health, GB/T 5009.13-2017, 2017).

2.4 | Determination of Zn content

Zn content was determined according to the method recommended by the Chinese Pharmacopoeia (Ministry of Health, GB/T 5009.14-2017, 2017). For the preparation of the standard curve, 10 ml of Zn standard solutions (0, 1.0, 2.0, 3.0, 4.0, and 5.0 μg) were prepared at different concentrations; 20 μl of methyl orange indicator solution was added, followed by the addition of ammonia water to alter the medium color from red to blue; 5 ml of acetate buffer solution and 1 ml of sodium thiosulfate solution (250 g/L) were then added to the mixture followed by thorough homogenization; 10 ml of dithiocarbazone-carbon tetrachloride solution (0.01 g/L) was then added and vigorously mixed for 4 min. After standing, the carbon tetrachloride layer was filtered with absorbent cotton into a colorimetric cup, and absorbance at 530 nm was measured. Fermented broth samples were submitted to centrifugation and determination of Zn content in cells was performed using the method described above for Cu content.

2.5 | Siderophore-assisted the growth of *S. cerevisiae* in the presence of Cu and Zn

S. cerevisiae was inoculated (1%, v/v) in Wort liquid medium with 10 °Brix containing 1,000 μg/ml of Cu or Zn. Siderophores obtained from 16 strains characterized above were added separately to the medium in equal amounts. After incubation at 28°C for 4 days, the biomass of *S. cerevisiae* was determined (OD_{600}), and intracellular Cu and Zn contents were determined by the method described above.

2.6 | Evaluation of siderophore-assisted Cu and Zn enrichment in *S. cerevisiae*

To determine the optimal amount of siderophore to be added to the medium, *S. cerevisiae* was inoculated in a Wort liquid medium containing a fixed concentration (1,000 μg/ml) of Cu or Zn, and 2 ml of siderophore at different concentrations (CAS 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.1) was added to the medium. After incubation at 28°C for 4 days, the biomass of *S. cerevisiae* was determined (OD600), and intracellular Cu and Zn contents were determined by the method described above.

Two hundred microliters of *S. cerevisiae* culture were inoculated in a Wort liquid medium containing different concentrations of Cu (500, 600, 1,500, and 2,000 μg/ml) in the presence of 2 ml of a siderophore TZT-SH5I (CAS 0.04). In addition, *S. cerevisiae* was cultivated in a Wort liquid medium containing different concentrations of Zn (800, 900, 3,500, and 4,000 μg/ml) in the presence of 2 ml of siderophore TZT-ZTH2X (CAS 0.05). Biomass of *S. cerevisiae* and intracellular Cu and Zn contents were determined by the method described above with incubation at 28°C for 48 hr.

2.7 | Identification of siderophore-producing strains

Total DNA of strains SH5I and ZTH2X was extracted using a previously described method (Lu et al., 2004), with minor modification: SH5I and ZTH2X strains were inoculated in a Wort liquid medium and incubated at 28°C for 4 days. After centrifugation, cells in precipitates were washed with PBS and distilled water. Total DNA was extracted with the Ezup genomic DNA extraction kit. 18SRDNA was amplified using 2234C and 3126T oligonucleotides. PCR reaction parameters were: 94°C for 3 min; 32 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 50 s; followed by the final extension at 72°C for 7 min (Becerril-Espinosa et al., 2013). PCR products were separated in 1% agarose gel electrophoresis and sequenced at Shanghai Biotechnology Engineering Service Center. Obtained sequences were identified using nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared against the NCBI database. Sequences were aligned using ClustalX32 (Hall & Brown, 2001), and phylogenetic trees were constructed using the neighbor-joining method on MEGA5 (Tamura et al., 2007).

2.8 | Effect of sugar content, pH, and fermentation time on siderophore-mediated Cu and Zn enrichment of *S. cerevisiae*

S. cerevisiae was inoculated in 50-ml Erlenmeyer flasks in Wort liquid medium (12.5 °Brix; pH 6.5) containing 2 ml of siderophore (TZT-SH5I CAS 0.04 for Cu; TZT-ZTH2X CAS 0.05 for Zn) and different concentrations of Cu (500, 1,000, 1,500, 2,000, and 2,500 μg/ml) or Zn (1,000, 2,000, 3,000, 4,000, and 5,000 μg/ml) were added separately to the mixture. The effect of the following

parameters on Cu and Zn enrichment of *S. cerevisiae* grown in Wort liquid medium containing 1,000 μg/ml of either Cu or Zn were evaluated: pH (5.5, 6.5, 6.5, 7.0, and 7.5) and sugar content (6, 8, 10, 12, and 14 °Brix); after incubation at 28°C for 96 hr, the biomass of *S. cerevisiae* was determined (OD 600 nm) and intracellular Cu and Zn contents were determined as described elsewhere (Lottermoser, 2009). In addition, the effect of different fermentation times (84, 96, 108, 120, and 132 hr) on siderophore-mediated Cu and Zn enrichment of *S. cerevisiae* was evaluated in a Wort liquid medium containing 1,000 μg/ml of either Cu or Zn (pH 6.5, 12.5 °Brix); cell biomass and intracellular Cu and Zn contents were determined as described above.

2.9 | Determination of optimized preparation for siderophore-mediated Cu and Zn enrichment of *S. cerevisiae*

To determine the optimal growth conditions and maximum intracellular copper/zinc concentration, different factors such as copper/zinc concentration, fermentation temperature, and pH were considered and their effects were analyzed by an orthogonal experiment. An orthogonal table testing of four factors and three levels was designed to determine the influence on siderophore-mediated Cu and Zn enrichment of *S. cerevisiae*. *S. cerevisiae* was cultured following the conditions specified in Table S1, and intracellular Cu and Zn contents were measured by the above-mentioned method. Optimal preparation for Cu and Zn enrichment in *S. cerevisiae* through the addition of siderophores was determined by comparison. ANOVA was performed in IBM SPSS Statistics v. 26 and comparisons with $p < .05$ were considered as significantly different.

3 | RESULTS

3.1 | Correlation of siderophore production and intracellular Cu and Zn contents in microorganisms resistant to Fe-rich conditions

Standard curves for Cu/Zn are shown in Figure S1. Correlation analysis for Cu ($R^2 = .9962$)/Zn ($R^2 = .9940$) showed that determination of Cu/Zn contents met the requirements established for this study. As shown in Figure 1, the biomass of *S. cerevisiae* remained unchanged and growth was stable in samples without the addition of siderophore; intracellular accumulation of Cu/Zn in such conditions was negligible. Interestingly, the incorporation of most of the siderophores produced by microorganisms isolated from Fe-rich environments promoted the growth of *S. cerevisiae* and increased intracellular accumulation of both Cu and Zn; in particular, the siderophore TZT-SH5I produced by strain SH5I and the siderophore TZT-ZTH2X produced by strain ZTH2X showed the most positive effects on the growth of *S. cerevisiae* and on Cu and Zn enrichment, respectively, being, therefore, selected for further investigation.

3.2 | Effect of siderophore TZT- SH5I on growth and Cu enrichment of *S. cerevisiae*

The effect of the addition of siderophore TZT-SH5I to a solid medium containing a high concentration of Cu on the growth of *S. cerevisiae* is shown in Figure 2 *S. cerevisiae* grew well in the medium with 500 μg/ml of Cu in the absence of siderophore (Figure 2a). No growth of *S. cerevisiae* was observed in a medium containing 600 μg/ml of Cu without a siderophore (Figure 2b), indicating that high Cu levels inhibit *S. cerevisiae* growth. Moreover, compared with growth in medium with no added siderophore (Figure 2d,f), the growth of *S. cerevisiae* was satisfactory when Cu concentration in the medium was 1,500 μg/ml in the presence of siderophore TZT-SH5I (Figure 2c). Interestingly, *S. cerevisiae* did not grow on a medium containing 2,000 μg/ml of Cu in the presence of siderophore (Figure 2e), indicating that tolerance of *S. cerevisiae* to Cu is below 2,000 μg/ml in a siderophore-containing medium.

In addition, as shown in Figure 2g, *S. cerevisiae* could not grow in high concentrations of Cu if no siderophore is added to the culture medium. However, in the presence of siderophore, *S. cerevisiae* resumes normal growth in a medium with a high concentration of Cu. With increasing siderophore concentration, the growth of *S. cerevisiae* and intracellular Cu content also increased. However, high concentrations of siderophores (CAS value > 0.05) inhibited the growth of *S. cerevisiae* and impaired intracellular accumulation of Cu. Therefore, the siderophore TZT-SH5I with a CAS value of 0.04 was used in subsequent experiments.

3.3 | Effect of siderophore TZT-ZTH2X on growth and Zn enrichment of *S. cerevisiae*

The effect of the addition of siderophore TZT-ZTH2X to a solid medium containing a high concentration of Zn on the growth of *S. cerevisiae* is shown in Figure 3. *S. cerevisiae* grew sparingly with 800 μg/ml of Zn in the medium with no siderophore addition; with 900 μg/ml of Zn in the medium without siderophore, *S. cerevisiae* did not grow (Figure 3b), indicating that concentrations of Zn are greater than 900 μg/ml in the medium is inhibitory to *S. cerevisiae*. When siderophore is added to the medium, *S. cerevisiae* grew in the presence of 3,500 μg/ml (Figure 3c) and 4,000 μg/ml (Figure 3e) of Zn. With increasing concentrations of siderophore, the growth of *S. cerevisiae* and intracellular Zn content also increased. However, high concentrations of siderophore in the medium (CAS value ≥ 0.04) inhibited the growth of *S. cerevisiae*. Thus, considering intracellular Zn content and the growth of *S. cerevisiae*, the siderophore TZT-ZTH2X with a CAS value of 0.05 was used for further investigation.

3.4 | Effect of different sugar content, pH, and fermentation time on siderophore-assisted Cu and Zn enrichment of *S. cerevisiae*

S. cerevisiae grew in a medium containing Cu at concentrations greater than 500 μg/ml in the presence of siderophore (Figure 4a);

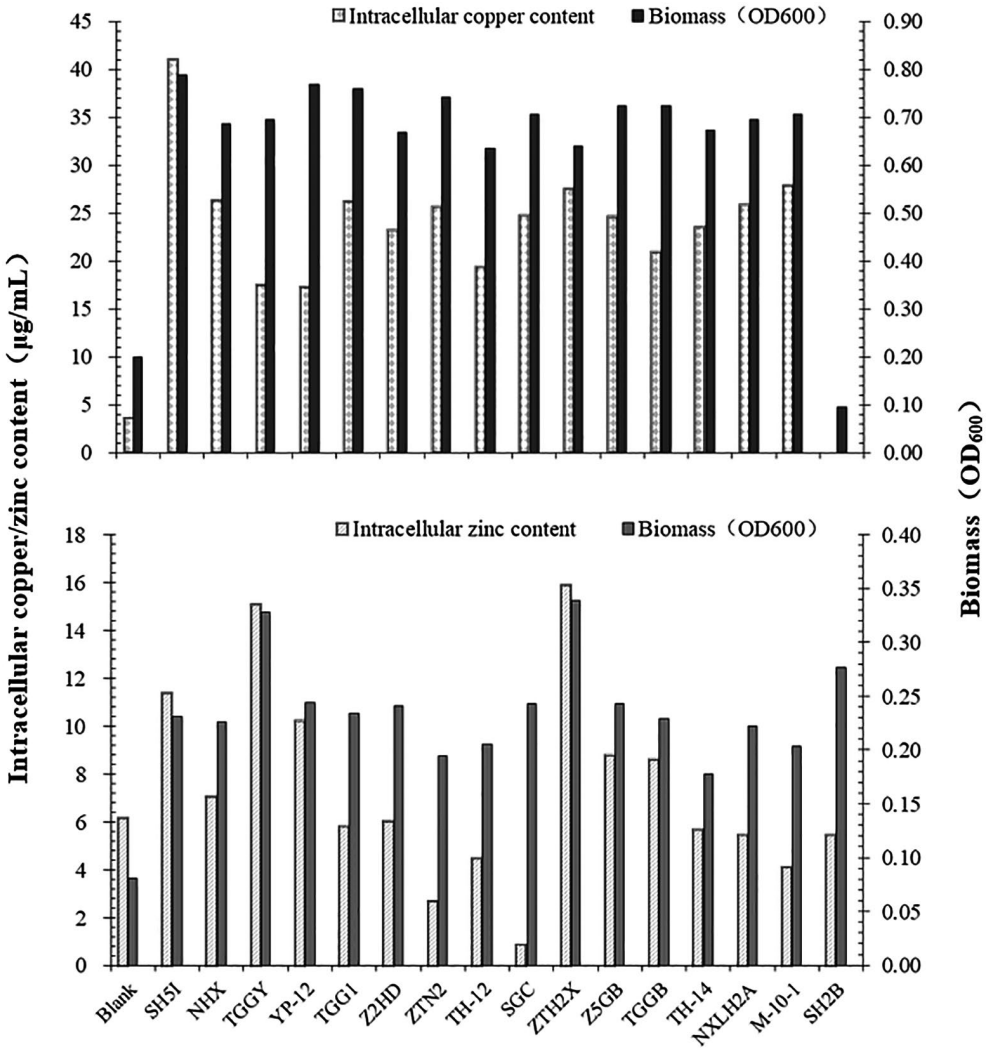

FIGURE 1 Correlation analysis of siderophore activity and intracellular copper/zinc contents in microorganisms resistant to iron-rich conditions

with increasing concentrations of Cu, growth of and intracellular Cu content in *S. cerevisiae* also gradually increased, reaching a tolerance of 1,000 μg/ml of Cu in the medium to support the growth of *S. cerevisiae*. As shown in Figure 4a*, *S. cerevisiae* grew under different concentrations of Zn in the medium, but optimized growth was observed with 4,000 μg/ml of Zn.

As shown in Figure 4b, high sugar content in the Wort medium impaired growth of and Cu accumulation in *S. cerevisiae*. The growth of *S. cerevisiae* was optimal and intracellular Cu content peaked in medium with 8 Brix. Similarly, high sugar content in the Wort medium also hindered the growth of and Zn accumulation in *S. cerevisiae* (Figure 4b*). The growth of *S. cerevisiae* was optimal in medium with 6 °Brix, while Zn content peaked in medium containing 10 °Brix.

Changes in pH of Wort medium affected growth and intracellular content of both Cu and Zn of *S. cerevisiae* (Figure 4c,c*). At pH 6.5, the growth of *S. cerevisiae* was optimal, and both Cu and Zn contents peaked.

In addition, the growth of *S. cerevisiae* reached a steady state after 96 hr of fermentation (Figure 4d,d*); with extended fermentation time, growth was impaired. Intracellular Cu content reached a maximum at 108 hr, while Zn content peaked after 120 hr of fermentation.

3.5 | Identification of siderophore-producing strains

Strains SH5I and ZTH2X were identified by nucleotide sequencing of 18S rDNA. The obtained sequence of SH5I had 99% homology with 18S rDNA sequence of *Aspergillus fumigatus*; ZTH2X had the highest homology (86%) with 18S rDNA sequence of *Penicillium aureum*. A phylogenetic tree was constructed using the 18S rDNA sequences (Figure 5), which showed that strain SH5I is most closely related to *A. fumigatus*, whilst strain ZTH2X is related to *P. aureum*.

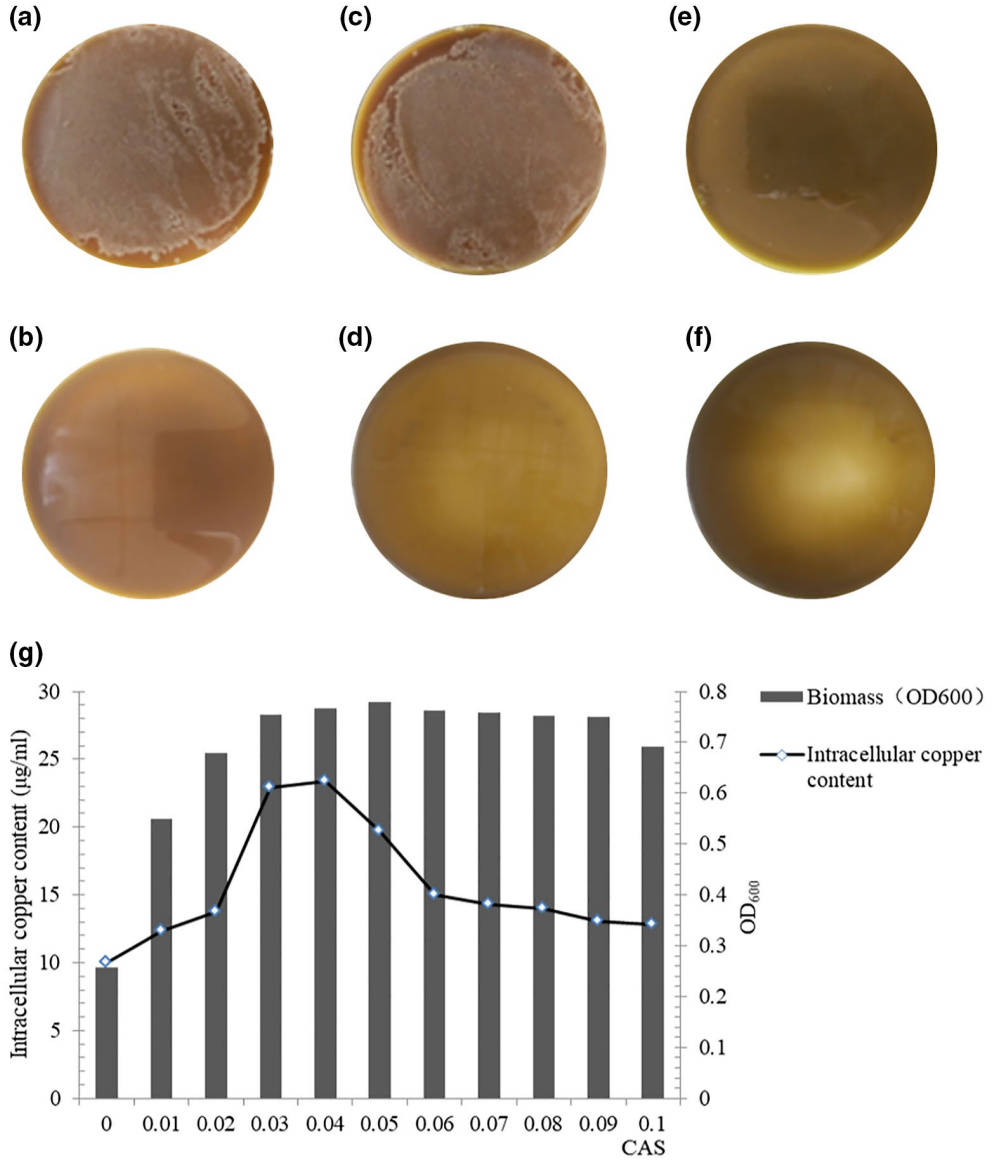

FIGURE 2 Growth of wild-type *Saccharomyces cerevisiae* in solid medium with a high concentration of copper. (a) 500 μg/ml of copper in medium without siderophore; (b) 600 μg/ml of copper in medium without siderophore; (c) 1,500 μg/ml of copper in medium with siderophore; (d) 1,500 μg/ml of copper in medium without siderophore; (e) 2,000 μg/ml of copper in medium with siderophore; (f) 2,000 μg/ml of copper in medium without siderophore; (g) effect of increasing siderophore concentration on growth and intracellular copper content of *S. cerevisiae*

3.6 | Optimized preparation for siderophore-assisted Cu and Zn enrichment of *S. cerevisiae*

To determine the best process for the preparation of siderophore-mediated Cu and Zn enrichment of *S. cerevisiae*, four factors and three levels were selected on the basis of a single factor experiment, and the content of intracellular Cu and Zn was used as the index of the orthogonal array. According to R values obtained with the method of range analysis (Table S1), the following order of effect of the four evaluated factors was determined: Cu and Zn concentration in the medium > fermentation time > pH > Brix. Cu and Zn concentration in the medium had the greatest influence on intracellular copper/zinc content, which was the determining factor for copper/zinc accumulation. Brix had less relevance, being further set as the error term (Table 1). An orthogonal array testing was performed, which indicated that the effect of Cu/Zn concentration, fermentation time, and pH significantly influenced intracellular copper/zinc content. Comparing the K value (Table S1), optimal levels $A_2B_1C_2D_3$ for Cu and $A'_2B'_1C'_1D'_3$ for Zn were determined. At 1,000 μg/ml of Cu in medium with 6 °Brix after 96 hr of fermentation at pH 7.0, *S. cerevisiae* grew well and the final organic Cu content obtained through the addition of siderophore was 60.67 mg/g; at 4,000 μg/ml of Zn in medium with 6 °Brix after 84 hr of fermentation at pH 7.0, optimal *S. cerevisiae* biomass was obtained and

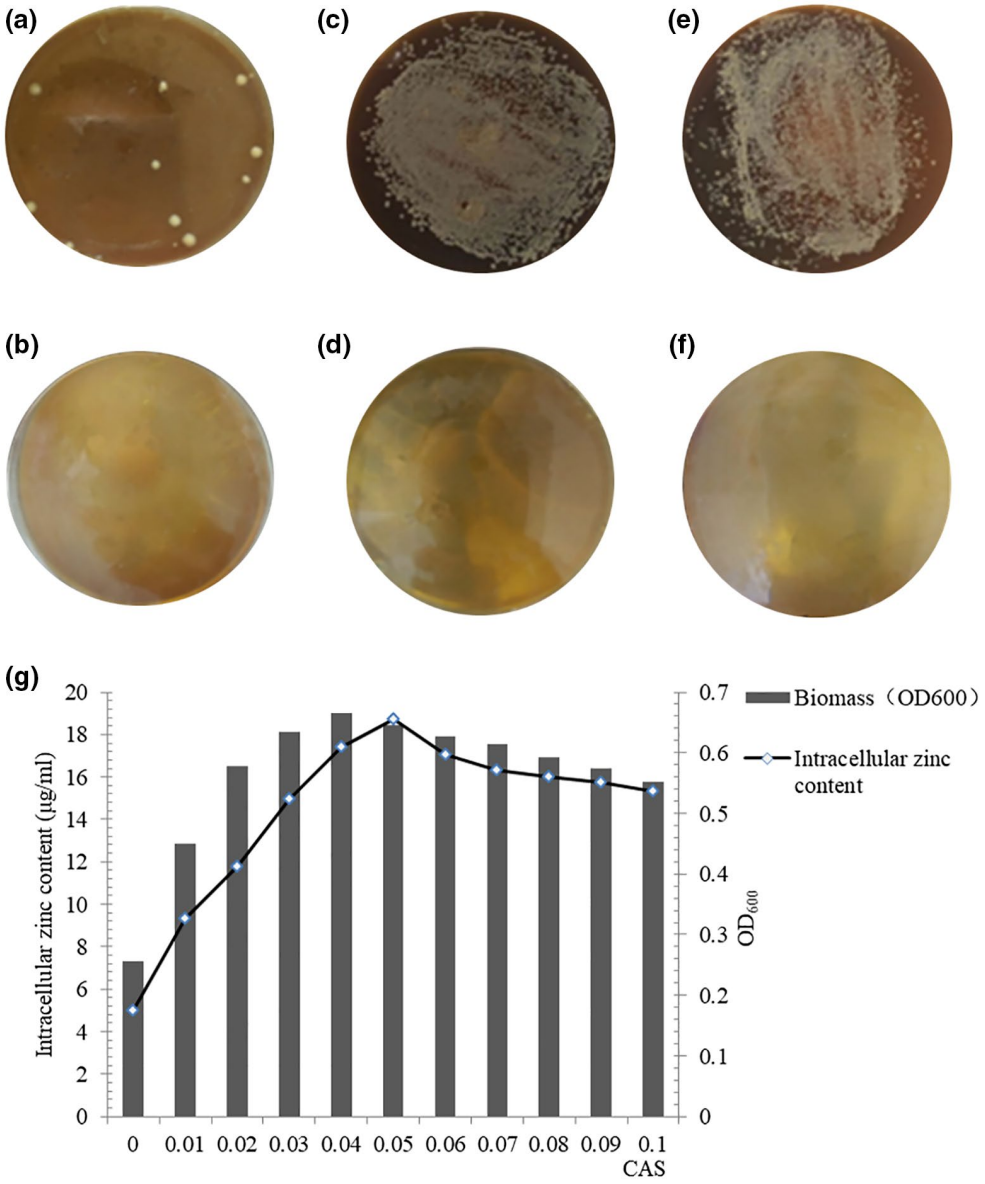

FIGURE 3 Growth of wild-type *Saccharomyces cerevisiae* in solid medium with a high concentration of zinc. (a) 800 μg/ml of zinc in medium without siderophore; (b) 900 μg/ml of zinc in medium without siderophore; (c) 3,500 μg/ml of zinc in medium with siderophore; (d) 3,500 μg/ml of zinc in medium without siderophore; (e) 4,000 μg/ml of zinc in medium with siderophore; (f) 4,000 μg/ml of zinc in medium without siderophore; (g) effect of increasing siderophore concentration on growth and intracellular zinc content of *S. cerevisiae*

final organic Zn content obtained via the addition of siderophore was 44.22 mg/g.

4 | DISCUSSION

In the present study, a novel preparation was designed for enriching the content of organic Cu and Zn in *S. cerevisiae* through the use of siderophores produced by microorganisms previously isolated from Fe-rich environments. The final intracellular content of organic Cu and Zn in *S. cerevisiae* grown in a siderophore-containing medium was 60.76 and 44.22 mg/g, respectively.

As a metal chelator, siderophores enhance the absorption of iron by microorganisms growing under iron-deficient conditions. Yeasts likely express siderophore receptors responsible for the transport of the siderophore-iron complex into the cell. Thus, the hypothesis adopted in this study was that the transport of TEs by microorganisms such as *S. cerevisiae* in Fe-, Zn-, and Cu-rich environments occurs via siderophores; hence the ability of siderophores in enhancing Cu and Zn transport in *S. cerevisiae* was explored.

The microbial transformation has been considered an increasingly important strategy to obtain organic TEs. Yeasts can accumulate TEs at high levels and increased absorption rate, as well as yield a large number of thallus proteins, polypeptides, small peptides,

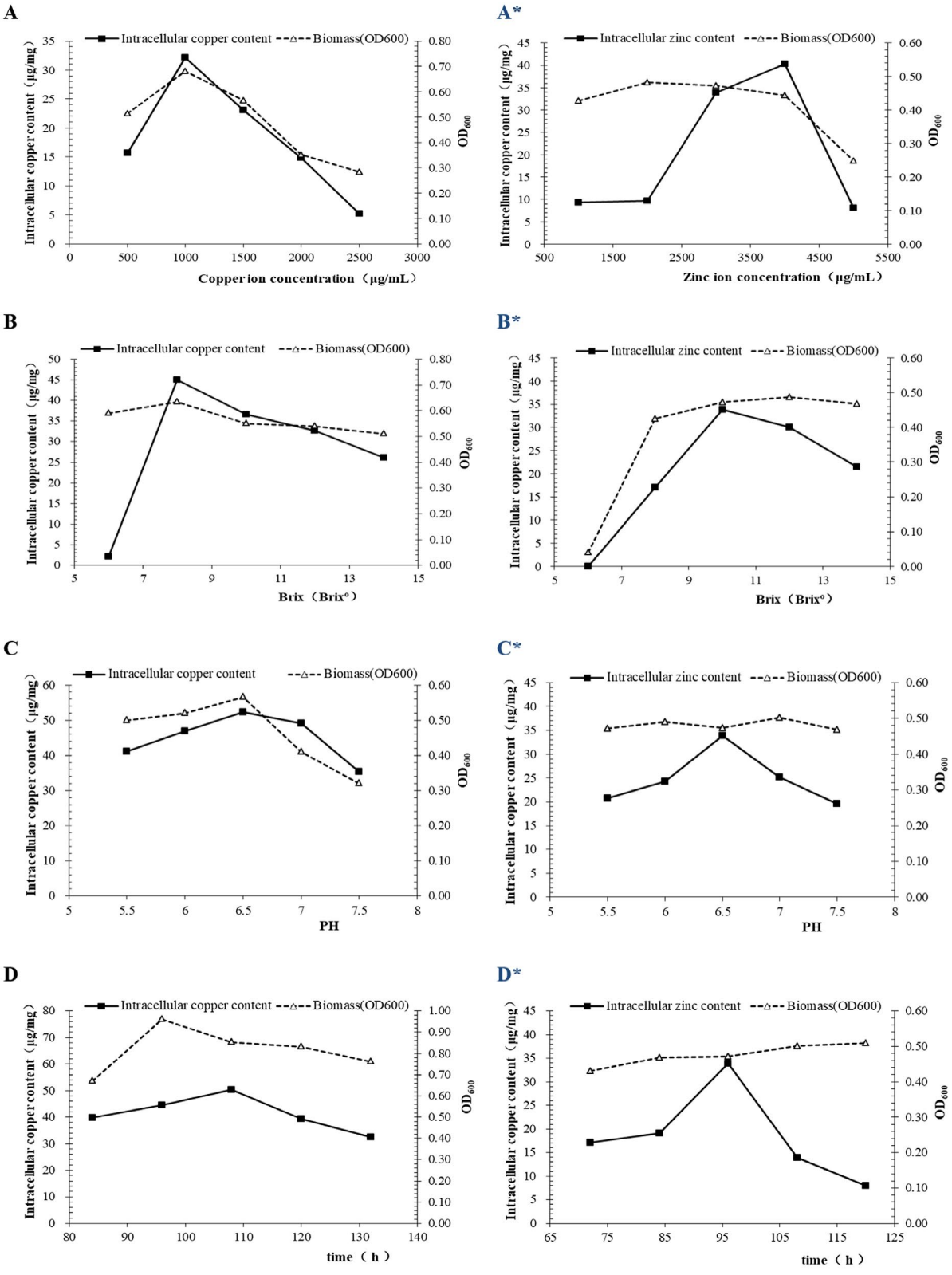

FIGURE 4 Influence of different factors on the growth of and intracellular copper/zinc accumulation in *Saccharomyces cerevisiae*. (a) and (a*) increasing copper/zinc concentrations on the growth of and intracellular accumulation in *S. cerevisiae*; (b) and (b*) increasing sugar content on growth and intracellular copper/zinc content of *S. cerevisiae*; (c) and (c*) different initial pH of culture medium on growth and intracellular copper/zinc content of *S. cerevisiae*; (d) and (d*) increasing fermentation time on growth and intracellular copper/zinc content of *S. cerevisiae*

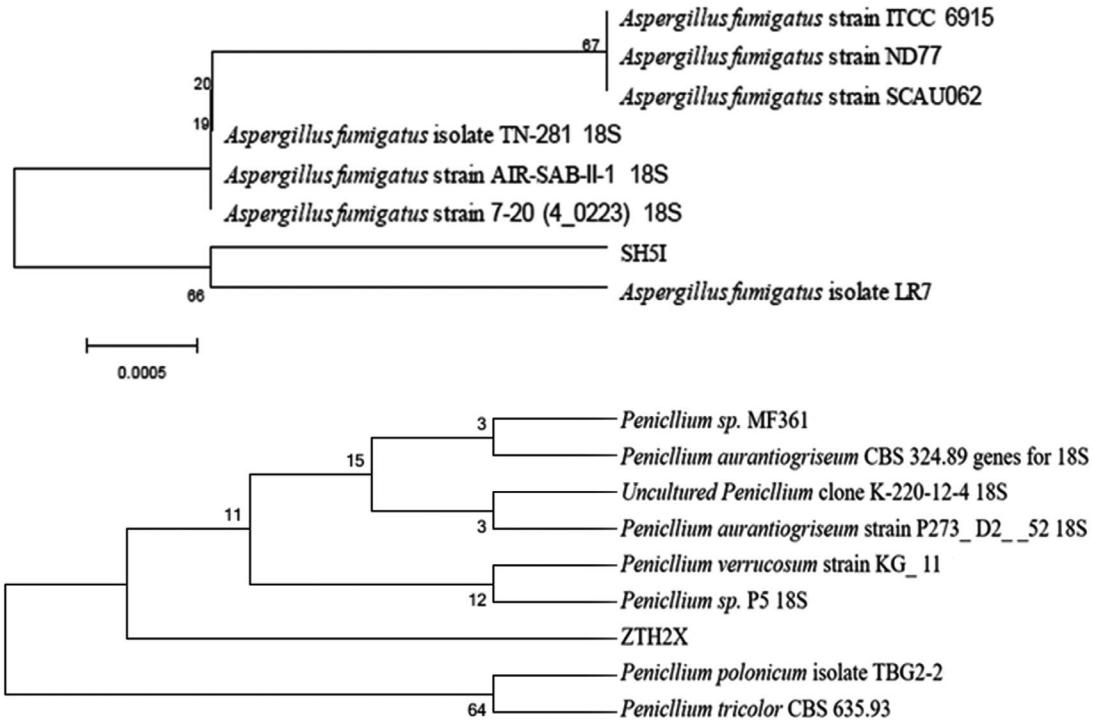

FIGURE 5 Phylogenetic tree based on the 18S srDNA sequence of siderophore-producing strains

TABLE 1 ANOVA of preparations for siderophore-assisted copper and zinc enrichment of *Saccharomyces cerevisiae*

Source	Sum of squares	Degree of freedom	Mean square	*F*	*P*
Copper					
Modified model	1,727.402	6	287.900	28.408	.034
Intercept	9,573.318	1	9,573.318	944.619	.001
A	1,494.663	2	747.332	73.741	.013
B	176.805	2	88.403	8.723	.103
C	55.933	2	27.967	2.760	.266
Pure error	20.269	2	10.135		
Total	11,320.989	9			
Revised total	1,747.671	8			
R^2	0.988				
Zinc					
Modified model	1,199.099	6	199.850	132.304	.008
Intercept	6,320.250	1	6,320.250	4,184.118	0
A′	1,085.515	2	542.758	359.315	.003
B′	86.850	2	43.425	28.748	.034
C′	26.733	2	13.366	8.849	.102
Pure error	3.021	2	1.511		
Total	7,522.370	9			
Revised total	1,202.120	8			
R^2	0.997				

amino acids, B vitamins, digestive enzymes, and other bioactive substances (Kwok & Kosman, 2005). Yeasts' cell wall contains mannose oligosaccharides, which are known to play an important role in animal health (Butt et al., 1984) and hold the potential to be used in the preparation of yeasts enriched with TEs such as Cu, Fe, and Zn (Yuan et al., 2004). In a previously proposed preparation (Sillerová

et al., 2012; Yuan et al., 2004), an initial step would be the domestication of *S. cerevisiae* by gradually increasing the concentration of metal ions in the medium to enhance tolerance and TE enrichment. Other techniques including interspecies protoplast fusion (Fan et al., 2003), ultraviolet radiation, radial distribution of 60Co-γ, N-methyl-N-nitro-N- Nitrosoguanidine (NTG), and UV irradiation mutagenesis (Kuang, 2013) can be used to produce high biomass of TE-enriched yeast, but resulting strains are often unstable and prone to reversion.

In previous studies conducted by our group, it has been found that these microorganisms existed in certain Fe-rich environments (Zhang et al., 2014b) and transport Fe using siderophores. In the present study, siderophores obtained from Fe-resistant strains isolated previously from Fe-rich environments were shown to promote growth of Cu- and Zn-enriched *S. cerevisiae* (Figure 1); however, the siderophores TZT-SH2B had instead a detrimental effect on the growth of *S. cerevisiae*. Among siderophores with growth-promoting effects, the addition of siderophores SH5I and ZTH2X to Wort liquid medium led to the highest Cu/Zn conversion rate and biomass of *S. cerevisiae*, which was further used in the preparation of siderophore-mediated Cu and Zn enrichment of *S. cerevisiae*. Overall, the findings revealed herein confirmed that the addition of siderophores can enhance tolerance of *S. cerevisiae* to high concentrations of Cu and Zn by 1.5 and 3 times, respectively, which is conducive to the transformation and enrichment of Cu and Zn in *S. cerevisiae*. To the best of our knowledge, this is the first report showing that siderophores assist *S. cerevisiae* in promoting conversion of Cu and Zn.

Furthermore, our results suggested tolerance limit of *S. cerevisiae* to Cu and Zn was 600 and 900 μg/ml, and higher concentrations of such TEs in the medium inhibited the growth of *S. cerevisiae*. However, after the addition of siderophores to Wort solid medium, *S. cerevisiae* grew under 1,500 μg/ml of Cu or 4,000 μg/ml of Zn (Figure 2), thus confirming that the tolerance of *S. cerevisiae* to Cu and Zn was greatly improved during growth in the presence of siderophores. The findings presented herein revealed that tolerance of *S. cerevisiae* to high levels of Cu and Zn in the medium was remarkably increased by the addition of siderophores. Thus, these findings provide a novel way to obtain copper, zinc, and other organic TEs using siderophores.

Previous studies have shown that Zn present in yeast was more biologically available in healthy male volunteers than Zn found in gluconate salts (Tompkins et al., 2007), and could also alleviate cognitive impairments caused by Zn deficiency in rats. Moreover, Zn-enriched yeast also significantly increased seric lysozyme and complement activity and total immunoglobulin in rainbow trout (Gharekhani et al., 2015). As a Zn supplement, Zn-enriched yeast has been widely used as the raw material of medicines, functional foods, and other food products such as dairy products, biscuits, beverages, and flour (Gharekhani et al., 2015; Zhang et al., 2019; Zhang, Zhang, et al., 2014). Moreover, Cu-enriched yeast advantageously eliminates metal toxicity (Manzoni et al.) and can be potentially used as a supplement of this important microelement in feed and/or food nutrition in natural form. In China, Cu-enriched yeast is approved by the Chinese government (Announcement No. 1126 of Ministry of Agriculture, 2008) for use in animal feed to promote growth.

Siderophores are produced by different microorganisms including bacteria, fungi, and actinomycetes (Rajkumar et al., 2010; Wang et al., 2014), and more than 500 siderophores have been isolated from different microorganisms so far. Pócsi et al. reported that Aspergillus oryzae were used to produce a siderophore (deferriferrichrysin) and the result showed that fungal siderophores can function as protective agents of low-density lipoprotein oxidation and are promising anti-atherosclerotic metabolites in functional food (Pócsi et al., 2008). In another study by Emri et al., the siderophore coprogen produced by Penicillium nalgiovense, and the results suggested that the siderophores can be developed siderophore-rich food additives or functional foods to increase the siderophore uptake in people prone to cardiovascular diseases. Moreover, it should be noted that the above-mentioned siderophores did not observe obvious cytotoxicity (Emri et al., 2013). Although different species of the genus Aspergillus and Penicillium are used for the production of mold-fermented foods, these fungi can secrete toxic metabolites (mycotoxins), and can even produce spores that cause diseases, such as allergies and asthma, especially to human beings (Dupont et al., 2017; Larsen & Knechel, 1997; Sheikh-Ali et al., 2014). In this study, the siderophores TZT-SH5I and TZT-ZTH2X were produced by *Aspergillus* sp. and *Penicillium* sp., respectively. Therefore, considering the possible future research in this field, further research is needed to determine or eliminate the possible risks from mycotoxins or spores produced by these fungi.

The present study provides a convenient and feasible strategy for the preparation of yeast-based organic trace element enriched supplements. The findings presented herein provide a reference for future use in the research and development of novel ways to obtain organic TEs.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Xiao-ying Fan: Formal analysis; Validation; Writing-original draft; Writing-review & editing. **Zi-yu Liu:** Formal analysis; Investigation; Methodology; Validation. **Zhi-peng Jia:** Formal analysis; Methodology; Validation. **Ya-ru Wei:** Formal analysis; Methodology; Validation. **Dong-dong Xie:** Formal analysis; Validation. **Ji Zhang:** Project administration; Supervision. **Bei Wang:** Formal analysis; Supervision; Writing-original draft. **Xin-guo Zhang:**

Conceptualization; Formal analysis; Funding acquisition; Project administration; Resources; Supervision; Writing-original draft; Writing-review & editing.

DATA AVAILABILITY STATEMENT

Data available in article Supporting Information.

ORCID

Xin-guo Zhang https://orcid.org/0000-0002-7266-2471

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