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ORIGINAL ARTICLE

Enhancement of Copper Uptake of Yeast Through Systematic Optimization of Medium and the Cultivation Process of *Saccharomyces cerevisiae*

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Abstract

Copper is an essential trace element for living organisms. Copper enriched by yeast of *Saccharomyces cerevisiae* is regarded as the biologically available organic copper supplement with great potentiality for application. However, the lower uptake ratio of copper ions makes the production of copper enriched by yeast uneconomically and environmentally unfriendly. In this study, *S. cerevisiae* Cu-5 with higher copper tolerance and intracellular copper accumulation was obtained by screening of our yeast strains collection. To increase the uptake ratio of copper ions, the medium composition and cultivation conditions for strain Cu-5 were optimized systematically. A medium comprised of glucose, yeast extract, $(NH_4)_2SO_4$, and inorganic salts was determined, then a novel cultivation strategy including pH control at 5.5 and increasing amounts of yeast extract for a higher concentration of copper ion in the medium was developed. The uptake ratios of copper ions were more than 90% after combining 50 to 100 mg/L copper ions with 3.5 to 5.0 g/L yeast extract, which is the highest until now and is conducive to the cost-effective and environmentally friendly production of bioactive copper in yeast-enriched form.

Keywords *Saccharomyces cerevisiae* · Copper enriched by yeast · Combining yeast extract amount with copper ion concentration · Uptake ratio of copper ions

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Introduction

Copper is an essential microelement for living organisms and plays a critical role in metabolic and physiological processes. It is required for the activity of numerous enzymes and electron transport proteins [1, 2]. Copper deficiency impacts the function of a large number of enzymes and increases the incidence of ischemic heart disease, intestinal cancer, and osteoporosis for people and animals [3, 4]. The Food and Nutrition Board estimates that the safe and adequate daily intake of dietary copper is 1.5–3.0 mg for adults [5]. Copper content in diets is generally low compared with the suggested standard [6]. Thus, dietary supplement of copper element is necessary for populations. Moreover, copper element is frequently added to animal feeds as a growth stimulant [7, 8].

Copper can be supplemented in inorganic or organic forms. The low digestibility of inorganic copper leads to large supplemental doses of copper, which may not only increase side effects or cytotoxicity, but also cause environmental pollution due to the excretion of approximately 80% of inorganic copper via feces [9, 10]. Copper in organic form has a higher bioavailability and better resorption in the digestive tract of humans and animals than inorganic copper [11–13]. Furthermore, copper in organic forms is less toxic [14]. Copper enriched in yeast or in the form of gluconate is considered as two kinds of the more biologically available organic copper supplements. The gluconate form of copper is the salt form of copper. Yeast can absorb inorganic copper ions from extracellular solutions and transform them into organic copper forms [15, 16]. Copper ions incorporated into the cells present as complexes with amino acids, proteins, probably lipids, and polysaccharides [2, 17]. Copper-enriched yeast has been proved to be 1.4 times more bioavailable than copper in gluconate form [18].

Both *Saccharomyces cerevisiae* and *Candida utilis* have been used to absorb, transform, and accumulate intracellular organic copper [11, 14, 19]. Copper uptake, intracellular transport, and homeostasis in eukaryotes have been investigated by using *S. cerevisiae* as the model organism [20–25]. *S. cerevisiae* is also the traditional food microorganism and is generally recognized as safe (GRAS), which can be used in pro-biotic, dietary, nutraceuticals, feed additives, and cosmetic products. Therefore, copper-enriched yeast of *S. cerevisiae* is attractive and promising as a suitable organic copper supplement.

Several factors, such as concentration of copper ions, pH, and oxygen level, have been reported to influence the cell growth and bioaccumulation of copper ions in *S. cerevisiae* [11, 15]. Semiaerobic conditions and acidic environments were reported to facilitate the incorporation of copper ions in *S. cerevisiae* [13, 15]. An increase in concentration of copper ions in the medium raised the intracellular copper content, but resulted in a significant reduction in cell growth, leading to the sharp decrease in uptake ratio from 74.2 to 13.0% at concentrations of copper ions ranging from 25 to 300 mg/L [15]. Although 11.74 mg/g of copper amount was detected in yeast cells cultured in the presence of 1.5 g/L copper ions, the uptake ratio was only 2.9% [16], which made 97.1% of the added copper ions remain in the culture broth. Both high uptake ratio of copper ions and high yield of cell biomass are not only beneficial for commercial purposes, but also environment-friendly due to the lowest residual copper ions in the culture broth. Hence, systematic optimization of the cultivation process is necessary for the efficient production of organic copper in yeast enriched form by balancing cell growth and copper accumulation. In this study, a potential copper-enriched *S. cerevisiae* strain was obtained by screening our collection of yeast strains. Medium composition and cultivation process were optimized systematically to maximize the yield of copper-enriched yeast and uptake efficiency of copper ions.

Materials and Methods

Yeast Strains and Culture Media

More than 100 of *S. cerevisiae* strains maintained in our laboratory were analyzed for their copper tolerance and intracellular copper content (Fig. S1, Table S1), among which strain Cu-5, the same as MT2 derived from *S. cerevisiae* CE25 (CGMCC 2.1418) [26], was screened and used for production of copper-enriched yeast in this study. Yeast cells were usually grown in yeast extract peptone dextrose (YPD) medium containing 10 g/L of yeast extract, 20 g/L of peptone, and 20 g/L of glucose at 30 °C. To explore the influence of carbon source, medium YPM was also used, in which the glucose in YPD was replaced by molasses at reducing sugar concentration of 20 g/L. To select the appropriate nitrogen source, 12 g/L of $(NH_4)_2SO_4$, 6 g/L of urea, or 60 mL/L of corn steep liquor, equivalent to 2.5 g/L of nitrogen, was used respectively to replace the 20 g/L of peptone in YPD.

To determine the influence of micronutrient sources on cell growth and copper accumulation, the inorganic salts mixture (ISM) containing 5 g/L KH_2PO_4, 1 g/L $MgSO_4{\cdot}7H_2O$, 20 mg/L $ZnSO_4{\cdot}7H_2O$, 8 mg/L $FeSO_4{\cdot}7H_2O$, 30 mg/L EDTA (pH 8.0), and 10 mL/L trace element solution was used. The trace element solution comprises 250 mg/L $MnCl_2{\cdot}4H_2O$, 75 mg/L $CoCl_2{\cdot}6H_2O$, 100 mg/L $NaMoO_4{\cdot}2H_2O$, 850 mg/L $CaCl_2$, 250 mg/L H_3BO_3, and 25 mg/L KI.

For fed-batch cultivation, the medium composed of 20 g/L glucose, 12 g/L $(NH_4)_2SO_4$, 3.5 g/L yeast extract, 5 g/L KH_2PO_4, 1 g/L $MgSO_4{\cdot}7H_2O$, 2 mg/L $ZnSO_4{\cdot}7H_2O$, 8 mg/L $FeSO_4{\cdot}7H_2O$, 30 mg/L EDTA, and 10 mL/L trace element solution was used for batch culture. The feeding medium was composed of 200 g/L glucose, 120 g/L $(NH_4)_2SO_4$, 3.5 g/L yeast extract, 5 g/L KH_2PO_4, 1 g/L $MgSO_4{\cdot}7H_2O$, 2 mg/L $ZnSO_4{\cdot}7H_2O$, 8 mg/L $FeSO_4{\cdot}7H_2O$, 30 mg/L EDTA, and 10 mL/L trace element solution. Initial pH value of the medium was adjusted to 5.5 with 0.5 M NaOH solution. $CuCl_2$ was generally added to the media to the final concentration of 50 mg/L of copper ions unless specifically indicated.

Shake-Flask Cultivation

Yeast cells were cultivated in 5 mL YPD at 30 °C and 200 rpm for 18 h. The freshly prepared seed culture was inoculated into 250-mL Erlenmeyer flasks containing 45 mL medium at an inoculation proportion of 10% (v/v). Then, cultivation was conducted at 30 °C and 200 rpm for 28 h.

Fed-Batch Cultivation in Bioreactors

Fed-batch cultivation was performed firstly in a 1-L stirred-tank bioreactor (Multifors; Infors HT, Switzerland) with 0.40 L of initial volume of the medium. For seed culture, individual colony of strain Cu-5 was inoculated into 5 mL YPD and grown at 30 °C and 200 rpm for 16 h, then 5 mL of the culture was transferred to 45 mL YPD in 250-mL Erlenmeyer flask and cultured at 30 °C with shaking at 200 rpm for 18 h. The freshly prepared seed culture (40 mL) was inoculated into 0.40 L medium in a 1-L bioreactor for cultivation. The temperature of the cultivation was controlled at 30 °C. The pH was controlled by the addition of 2.5 M NaOH or 1.2 M HCl. To investigate the effect of pH on cell growth and copper uptake, the pH non-controlled cultures and cultures with pH control at 4.5, 5.5, and 6.0 respectively were compared. Dissolved oxygen (DO) was maintained above 30%

saturation by regulating agitation speed and airflow rate. The temperature, pH, and DO were monitored and recorded automatically during cultivation. Samples were withdrawn at regular intervals to determine the biomass, copper content, and glucose concentration. The glucose and ethanol concentrations were measured using a biosensor (SBA-40C; Institute of Biology Shandong Academy of Sciences, Jinan, China).

After about 7 h of batch cultivation, the glucose concentration in culture decreased to about 1 g/L, and then fed-batch process using the exponential feeding strategy was started. The feeding rate varied according to the following equation [27]:

$$F_s = \left(\frac{\mu}{Y_{xs}} + m_s\right) \cdot \left(\frac{C_{x0} \cdot V_0}{C_{si}}\right) \cdot e^{\mu \cdot t} \quad (1)$$

where F_s is the feeding rate (in L/h), μ is the specific growth rate (in h^{-1}), Y_{XS} is the yield of biomass divided by glucose content (in g cells/g glucose), m_s is the maintenance coefficient (0.024 g glucose/g biomass/h), C_{x0} is the initial biomass concentration (in g/L), V_0 is the initial volume of medium (in L), C_{si} is the glucose concentration in the feeding medium (in g/L), and t is the time after starting the feeding (in h).

Scale-up of fed-batch cultivation was conducted in a 20-L stirred-tank bioreactor (Bio-Flo 4500; New Brunswich Scientific, New Jersey, America) with 8.0 L of the initial volume of the medium. For the preparation of seed culture, an individual colony of strain Cu-5 was inoculated into 8 mL YPD and grown at 30 °C and 200 rpm for 16 h, then 8 mL of the culture was transferred to 72 mL YPD in a 500-mL Erlenmeyer flask and cultivated at 30 °C with shaking at 200 rpm for 18 h. The culture (80 mL) was transferred to 720 mL YPD in 1-L bioreactor. Cultivation was conducted for 18 h at 30 °C with DO maintained above 30% saturation by regulating agitation speed. The freshly prepared seed culture (800 mL) was inoculated into a 20-L bioreactor. Fed-batch phase using the exponential feeding strategy was initiated at 7 h and finished at 25 h. The control of temperature, pH, and DO were the same as the cultivation in 1-L bioreactor.

Determination of Cell Growth and Biomass of Yeast Cells

Cell growth was monitored by measuring OD_{600} with a spectrophotometer (UV1800; Shimadzu, Tokyo, Japan). Biomass was quantified as the grams of dry cell weight (DCW) per liter of culture (g DCW/L). Fifty milliliters of samples was centrifuged at $5000 \times g$ for 5 min (Eppendorf centrifuge 5804R, Germany). The cell pellet was washed three times using deionized water and then dried at 60 °C in a dust-free drying oven to constant weight. The DCW was determined gravimetrically.

Measurement of Intracellular Copper Content

Yeast cells were harvested by centrifugation of samples at $5000 \times g$ for 5 min. The cell pellet was rinsed three times by deionized water and four times with 10 mM citric acid in 0.5% (w/v) NaCl to remove the copper ions adsorbed on the cell surface, then dried at 60 °C in a dust-free drying oven to constant weight. About 0.2 g of dried cells were resuspended in 5 mL HNO_3-$HClO_4$ (4:1, v/v) in the flask covered with a watch glass, and heated on a hot plate at 80 °C for 2 h, then 120 °C for 2 h and 190 °C until no visible white fog in the flask and the remaining liquid being clear and colorless. The digested samples were collected and diluted with ultrapure water (18.2 MΩ cm) to a final volume of 25 mL.

Copper content in the digested solution was quantified by the chemical colorimetry method developed by Snell et al. [28]. The copper content of yeast cells was determined as the amount of copper (mg) per gram DCW (mg/g). The yield of copper enriched by yeast was determined as the amount of bioaccumulated copper per liter of cultivation broth (mg/L), which was calculated as the product of biomass and copper content of yeast cells. Uptake ratio of copper ions was defined as the percentage of the yield of copper enriched by yeast at the end of cultivation to the total amount of copper added into cultivation broth.

Assay of Copper Tolerance of Yeast Strains

Single colony of yeast strains was picked and cultivated in 2 mL YPD medium for 18 h at 30 °C in a rotary shaker at 200 rpm. Yeast cells were harvested by centrifugation at $5000 \times g$ for 5 min, washed twice with sterile water, and resuspended in sterile water to a final cell concentration equivalent to 0.1 of OD_{600}. The cell suspension was serially diluted, and 4 μL of each aliquot (10^{-1}–10^{-3}) was spotted onto YPD plate or YPD plates containing different concentrations of copper ions, and then incubated at 30 °C for 48 h.

Statistical Analysis

All cultivations were conducted at least in triplicates in 250-mL Erlenmeyer flasks or in bioreactor and all determinations were conducted in triplicates. Response surface analysis was conducted using Design-Expert software (Design-Expert 8.0; Stat-Ease Inc., Minneapolis, USA) (Tables S2, S3, S4). All data were analyzed statistically using Data Analysis and Graphing Software (OriginPro 2018, OriginLab Corp. Northampton, America) and presented as mean ± standard deviation (SD). Mean values with $P < 0.05$ were considered significantly different.

Results and Discussion

Screening of *S. cerevisiae* Strains for Copper-Enriched Yeast

To obtain the optimal yeast strain for effective uptake of copper ions, more than 100 *S. cerevisiae* strains collected in our laboratory were compared for their copper tolerance and intracellular copper content (Fig. S1, Table S1). Among these strains, *S. cerevisiae* strain Cu-5 that can be traced back to strain MT2 [26] showed a relatively higher copper tolerance and intracellular copper accumulation (Fig. 1a, b). Hence, *S. cerevisiae* Cu-5 was chosen for further study. The influence of copper concentration on cell growth and copper enrichment was investigated. As shown in Fig. 1c, reduction in cell growth but increase in intracellular copper content were observed with increasing concentration of copper ions in the medium. The highest uptake ratio of copper ions (15.5%) was obtained at 50 mg/L copper ions, which was used in the subsequent investigation.

Influence of Medium Composition on Yield of Copper Enriched by Yeast

YPD medium was used as the initial medium. The influence of carbon sources, nitrogen sources, and micronutrients on cell growth and intracellular accumulation of copper was

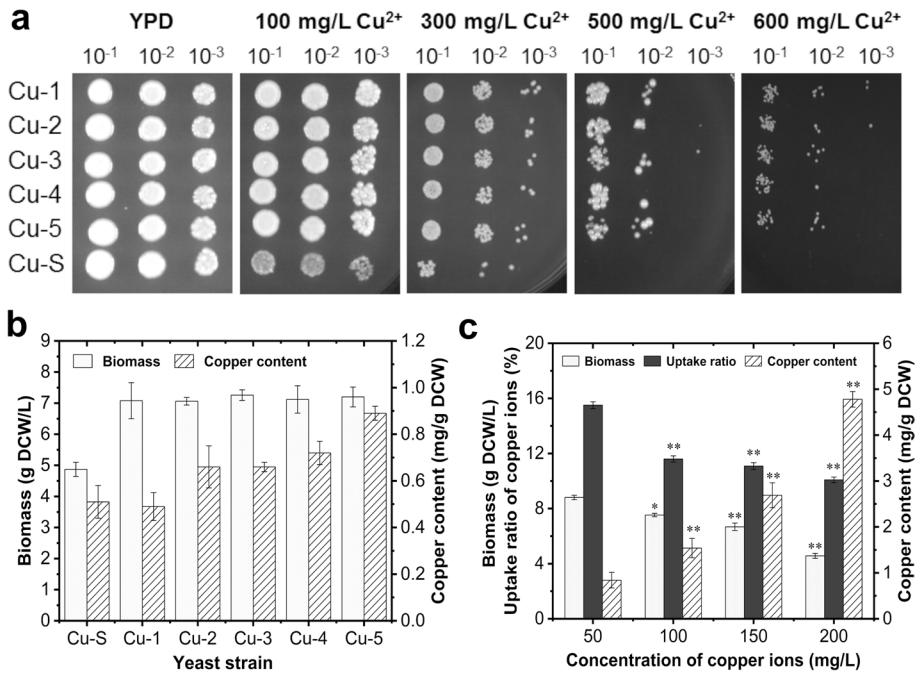


Fig. 1 Copper tolerance and intracellular copper content in different yeast strains. **a** Copper tolerance. **b** Cell growth and intracellular copper content. As a control, the strain numbered 20 in Fig. S1 was used as the strain sensitive to copper ions and named Cu–S. Yeast cells were cultured in YPD medium containing 50 mg/L copper ions at 30 °C for 24 h. **c** Effect of concentration of copper ions on cell growth and intracellular copper content of strain Cu-5. Yeast cells were cultured in YPD media containing different concentrations of copper ions at 30 °C for 28 h. Error bars represent standard deviations ($n=3$). $^{*}P<0.05$, $^{**}P<0.01$

investigated. Glucose and molasses are frequently used as carbon sources in industrial processes. Cell growth and intracellular accumulation of copper of *S. cerevisiae* Cu-5 in YPD medium and YPM medium containing 50 mg/L copper ions were measured respectively. The results indicated that copper content of yeast cells in YPD medium was higher than that in YPM medium, while cell growth in YPD or YPM had no obvious difference (Fig. 2a). Thus, glucose was selected as the carbon source.

To obtain the appropriate and cheap nitrogen source, the peptone in YPD medium was replaced with urea, ammonium sulfate, or corn steep liquor respectively at the same nitrogen level. Although usage of peptone and corn steep liquor as nitrogen sources resulted in higher cell growth than urea or ammonium sulfate, copper content of yeast cells in ammonium sulfate medium was significantly higher than that in the other media (Fig. 2b). In consideration of the economy and yield of copper enriched by yeast, ammonium sulfate was selected as the nitrogen source for the production of copper-enriched yeast.

Copper accumulation in yeast cells cultivated in media containing molasses, peptone, or corn steep liquor was low, which was consistent with the results reported by Mrvcić et al. [11]. Molasses, peptone, and corn steep liquor are all organic substance and complex mixtures. There are some organic ligands in these substances [29], which might interact with copper ions to reduce their availability. Moreover, some ingredients in these substances

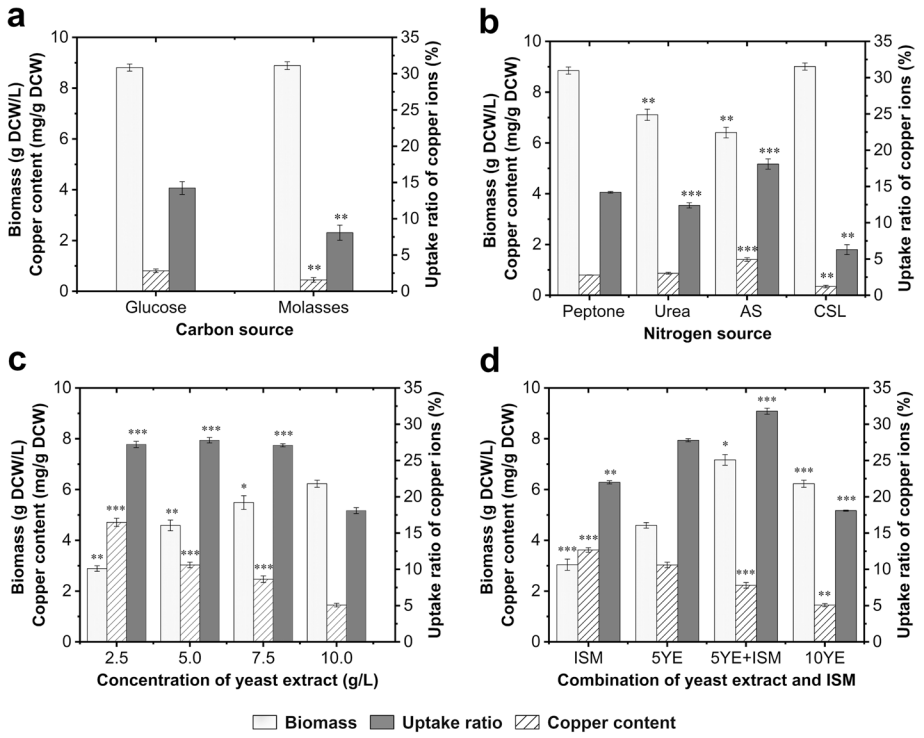


Fig. 2 Effect of medium components on cell growth and intracellular copper content of strain Cu-5. **a** Carbon sources. **b** Nitrogen sources. **c** Concentrations of yeast extract. **d** Combination of yeast extract and inorganic salt mixture. ISM, only inorganic salt mixture; 5YE, only 5 g/L yeast extract; 5YE + ISM, 5 g/L yeast extract and ISM; 10YE, only 10 g/L yeast extract. Yeast cells were cultured in different media containing 50 mg/L copper ions at 30 °C for 28 h. Error bars represent standard deviations ($n = 3$). For significance analysis, results in media with glucose (**a**), peptone (**b**), 10 g/L yeast extract (**c**), and 5 g/L yeast extract (**d**) were used as the control respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

might impair certain functional groups for metal ions binding on the yeast cell wall to inhibit the uptake of copper ions [30].

Yeast extract is also an organic complex substance. Hence, we speculated that the reduction of the amount of yeast extract in the medium might have a vital significance for copper uptake. As shown in Fig. 2c, yeast extract endowed a positive effect on cell growth but presented a negative effect on intracellular copper accumulation. When the concentration of yeast extract in the medium was decreased from 10 to 5 g/L, 25.2% reduction of biomass occurred, while intracellular copper content was approximately doubled, which resulted in a 52.3% increase in the yield of copper enriched by yeast. Yeast extract generally provides micronutrients for yeast cell growth and metabolism. To improve cell growth, some inorganic salts were added to the medium in our investigation. Complete replacement of yeast extract with an inorganic salt mixture (ISM) improved intracellular copper content to some extent, but severely affected cell growth. The combination of 5 g/L of yeast extract and ISM (5YE + ISM) resulted in a 56.2% increase in biomass and 26.7% reduction in the copper content of yeast cells respectively when compared to the medium only containing 5 g/L of yeast extract (5YE), indicating 14.5% increase in uptake ratio of copper ions (Fig. 2d). Moreover, both biomass

and intracellular copper content were increased by 15.1% and 53.1% respectively when compared to the medium containing 10 g/L of yeast extract (10YE). These results suggested that ISM could be used to partially replace yeast extract to maintain yeast cell growth and uptake of copper ions.

Negative effect of ISM addition on intracellular copper accumulation was observed (Fig. 2d). To coordinate the cell growth and copper ion uptake further, each component of ISM was removed and the effects on copper accumulation were analyzed. KH_2PO_4 deficiency in ISM impaired cell growth without obvious influence on intracellular copper content due to the essentiality of phosphorus. The elimination of $MgSO_4$ and $ZnSO_4$ from ISM resulted in an 11.6% and 13.1% reduction of biomass, but 25.7% and 30.2% increase of intracellular copper content respectively (Table 1). These results suggested that magnesium ions and zinc ions might antagonize the incorporation of copper ions into the yeast cells, which was consistent with the previous reports [31, 32]. Both copper and zinc ions have affinity for thiolate ligands. Periplasmic accumulation of copper ions was inhibited by zinc competition to zinc-sulfur clusters for cysteine-rich metal-binding sites [33, 34]. Besides specific transport proteins, ionic copper and zinc could also be transported through unspecific Fet4P [32]. The reduction of copper uptake in yeast cells probably resulted from the competitive binding and transport of zinc ions.

A response surface method was further used to determine the optimal levels of yeast extract, $MgSO_4$ and $ZnSO_4$ in cultivation medium due to their significant influences on cell growth and uptake of copper ions, which were 3.5 g/L of yeast extract, 1 g/L of $MgSO_4{\cdot}7H_2O$, and 2 mg/L of $ZnSO_4{\cdot}7H_2O$ respectively (Tables S2, S3, S4). Under this culture condition, yield of copper enriched by yeast was 19.97 mg/L, which was very close to the value predicted by the model (19.52 mg/L). Hence, the optimized cultivation medium was determined through systematic optimization, which contains 20 g/L glucose, 12 g/L $(NH_4)_2SO_4$, 3.5 g/L yeast extract, 5 g/L KH_2PO_4, 1 g/L $MgSO_4{\cdot}7H_2O$, 2 mg/L $ZnSO_4{\cdot}7H_2O$, 8 mg/L $FeSO_4{\cdot}7H_2O$, 30 mg/L EDTA, and 10 mL/L trace element solution. The uptake ratio of copper ions reached 39.9%, which was 2.8-fold of that when using the initial YPD medium (Table 2). To better assess the copper accumulating level of strain Cu-5 and evaluate the contribution of yeast strain and the culture condition, the typical laboratory strain S288C was used as the control. No obvious difference in cell growth was observed between the two strains, while strain Cu-5 accumulated 2.5-fold or 3.8-fold higher copper ions than strain S288C in YPD medium or in the optimal medium containing

Table 1 Influence of elimination of each inorganic salt from ISM on cell growth and copper enrichment

Elimination of inorganic salt	Biomass (g/L)	Copper content (mg/g)	Uptake ratio of copper ions (%)
NE[a]	7.67 ± 0.24	2.22 ± 0.26	34.06 ± 1.57
$MgSO_4{\cdot}7H_2O$	6.78 ± 0.69	2.79 ± 0.14	37.84 ± 2.94
$ZnSO_4{\cdot}7H_2O$	6.67 ± 0.07	2.89 ± 0.04	38.56 ± 0.51
$FeSO_4{\cdot}7H_2O$	7.64 ± 0.26	2.22 ± 0.07	33.96 ± 1.10
KH_2PO_4	4.79 ± 0.29	2.13 ± 0.27	20.38 ± 0.99
EDTA	7.39 ± 0.14	2.38 ± 0.17	35.14 ± 0.98
Trace elements	7.20 ± 0.25	2.28 ± 0.05	32.84 ± 0.30

[a]*NE*, no elimination of any inorganic salt from ISM

Values are means of three replications ± standard deviation

Table 2 Cell growth and intracellular copper content of different yeast strains in YPD and the optimized medium

Strain	YPD		Optimized medium	
	S288C	Cu-5	S288C	Cu-5
Biomass (g/L)	8.65 ± 0.12	8.63 ± 0.25	5.84 ± 0.15	5.79 ± 0.54
Copper content (mg/g)	0.24 ± 0.04	0.83 ± 0.04	0.72 ± 0.06	3.45 ± 0.15
Uptake ratio of copper ions (%)	4.15	14.32	8.45	39.90

Values are means of three replications ± standard deviation

50 mg/L copper ions respectively (Table 2). These results indicated that the improved copper accumulation in yeast cells depended on both the strain performance and culture conditions such as medium composition. The replacement of peptone and yeast extract in cultivation medium with NH_4SO_4 and inorganic salts makes the production of copper enriched by yeast more economical.

Effect of pH on Production of Copper Enriched by Yeast

Fed-batch cultivation was conducted in the aforementioned medium in a 1-L bioreactor supplemented with 50 mg/L of copper ions. To investigate the influence of pH on yield of copper enriched by yeast, the pH non-controlled culture, and cultures with pH control at 4.5, 5.5, and 6.0 respectively during the cultivation process were compared. When pH was controlled at 5.5, cell growth of strain Cu-5 was significantly improved, but intracellular copper content decreased by 21.9% and 26.1% respectively compared to those at pH 4.5 and non-controlled pH (Fig. 3a). In pH non-controlled culture, yeast cells accumulated the highest copper ions (2.45 mg/g DCW), in which pH values dropped from the initial 5.0 to 3.5 at the end of cultivation. Acidic environment has been reported to facilitate the incorporation of copper in *S. cerevisiae* [13, 15]. However, cell growth was affected greatly by pH in the presence of metal ions [15, 35]. It is evident that the optimal pH should be determined by overall consideration of cell growth and incorporation of copper ions. Shift

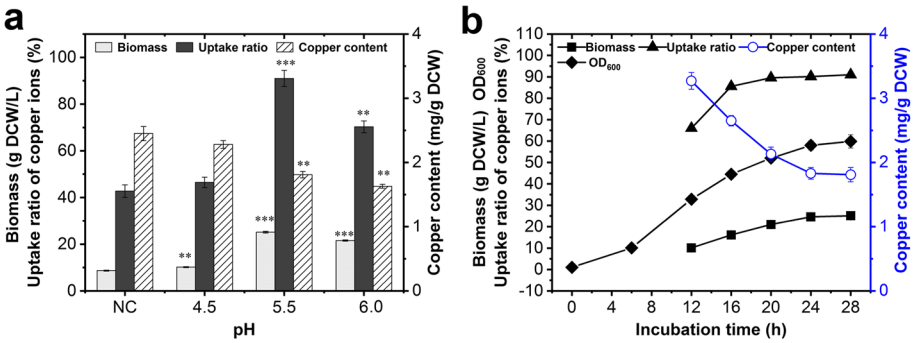


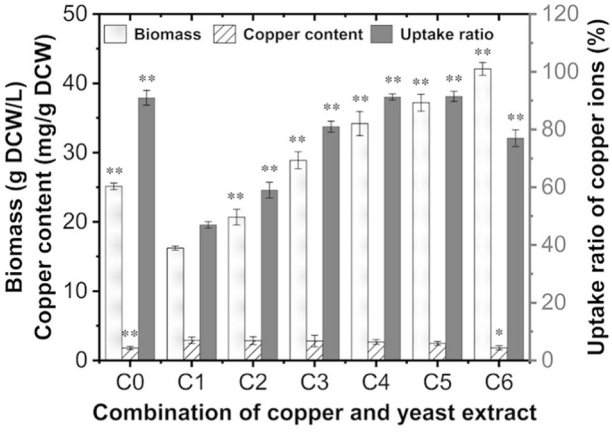
Fig. 3 Effect of pH on cell growth and intracellular copper content in the fed-batch cultivation. **a** Effect of pH control. **b** Cultivation profiles of strain Cu-5 in fed-batch cultivation with pH control at 5.5. The concentration of copper ions was 50 mg/L. Error bars represent standard deviations ($n = 3$). For significance analysis, results under the condition without control of pH (NC) were used as control. $^{**}P < 0.01$, $^{***}P < 0.001$

of pH from 4.5 to 5.5 made the increase rate of biomass much higher than the decrease rate of intracellular copper content, leading to the highest yield of copper enriched by yeast (45.49 mg/L) at pH 5.5 with an uptake ratio of 91% (Fig. 3). Both the cell growth and copper content at pH 6.0 decreased by 14.2% and 9.9% respectively when compared to those at pH 5.5 (Fig. 3a). Sarais et al. found the presence of microcrystals formed in medium with pH 5.5 or above [36], which affects the solubility of copper ions. Therefore, the culture pH was controlled at 5.5 in further investigation.

The copper content in yeast cells decreased from 12 to 28 h (Fig. 3b), which is consistent with the observation from Jasna et al. [11]. They found that the highest amount of copper incorporation under dynamic conditions was obtained between 5 and 6 h of culture, then it decreased until the end of cultivation. The high physiological and metabolic activities of yeast cells, as well as the high copper concentration in a medium at the early growth stage, might be conducive to the uptake of copper ions by yeast cells. Thus, a higher copper content in yeast cells may be possible by increasing copper ions in cultivation medium.

The Comprehensive Effect of Copper Ions and Yeast Extract

To obtain a higher level of intracellular copper accumulation, the concentration of copper ions in cultivation medium was raised from 50 to 100 mg/L. The copper content of cells was found to be increased by 57.5% (2.85 mg/g), but the biomass fell sharply from 25.13 to 16.11 mg/L (Fig. 4). The inhibitory effect of increased copper ions on cell growth resulted in a significant drop in uptake ratio to 50.5%. To improve yeast cell growth in the presence of 100 mg/L copper ions, the concentration of yeast extract in the medium was increased from 3.5 to 6.0 g/L in a stepwise manner due to its positive effect on cell growth. The highest uptake ratio of copper ions (91.5%) was obtained under a condition with 5.0 mg/L of yeast extract, in which a double amount of biomass was obtained when compared to 3.5 mg/L of yeast extract, while the intracellular copper content was slightly affected (Fig. 4). This indicated that the addition of yeast extract perhaps relieved the inhibitory effect on cell growth at a higher concentration of copper ions. Hence, a fed-batch



	C0	C1	C2	C3	C4	C5	C6
Copper ions (mg/L)	50	100	100	100	100	100	100
Yeast extract (g/L)	3.5	3.5	4.0	4.5	5.0	5.5	6.0

Fig. 4 Comprehensive effect of copper ions and yeast extract on cell growth and intracellular copper content. Fed-batch cultivation was conducted in 1-L bioreactor at pH 5.5 with combination of 50 mg/L copper ions and 3.5 g/L yeast extract (C0), 100 mg/L copper ions and 3.5 g/L yeast extract (C1), 4.0 g/L yeast extract (C2), 4.5 g/L yeast extract (C3), 5.0 g/L yeast extract (C4), 5.5 g/L yeast extract (C5), or 6.0 g/L yeast extract (C6), respectively. Error bars represent standard deviations ($n = 3$). For significance analysis, results of C1 were used as control. $^{**}P < 0.01$

cultivation strategy was established for efficient production of copper-enriched yeast by using *S. cerevisiae* Cu-5, in which the pH was controlled at 5.5, and the amount of yeast extract was increased to cope with a higher concentration of copper ion in medium.

Scale-up of the Developed Fed-Batch Cultivation Strategy

Fed-batch cultivation was conducted in a 20-L bioreactor using the exponential feeding cultivation strategy with pH-controlled at 5.5, in which 100 mg/L of copper ions was coupled with 5.0 g/L of yeast extract in cultivation medium (Fig. 5a). Similar profiles of cell growth and uptake of copper ions to those in the 1-L bioreactor were observed. After 28 h of cultivation, 34.45 g/L of biomass was obtained, and the intracellular copper content was 2.81 mg/g (Fig. 5b). The uptake ratio of copper ions reached 96.8%, reflecting the effectiveness of the optimized medium and cultivation process.

Conclusion

Copper enriched by *S. cerevisiae* is regarded as a biologically available organic copper supplement, which has extensive application potential in pharmaceutical area, healthcare area, food and feed additives fields. In this study, in order to increase the uptake ratio of copper ions by *S. cerevisiae* effectively, the medium and fed-batch cultivation processes were optimized systematically. A fed-batch cultivation strategy was developed with pH control at 5.5 and by increasing amounts of yeast extract for a higher concentration of a copper ion in a medium. The uptake ratios of copper ions were all greater than 90% after combining 50 mg/L and 100 mg/L of copper ions with 3.5 g/L and 5.0 g/L of yeast extract, respectively. Scale-up of cultivation to 20-L bioreactor confirmed the effectiveness of the optimized fed-batch cultivation process. The uptake efficiency of copper ions obtained in this study, which was much higher than that in other reports [11, 13, 15, 16], is conducive to the cost-effective and environmentally friendly production of bioactive copper in yeast-enriched form. Moreover, the optimized fed-batch cultivation strategy hints the potentiality for other bioactive mineral element manufacturers.

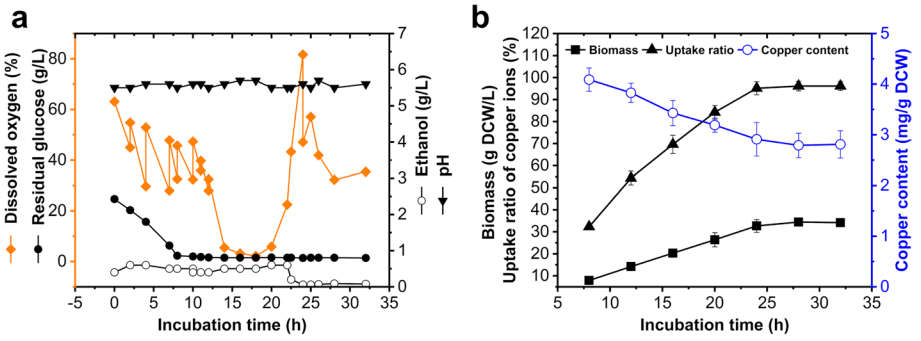


Fig. 5 Production of copper-enriched yeast by scale-up of the developed fed-batch cultivation strategy. **a** Time course profiles of dissolved oxygen, residual glucose, ethanol level, and pH. **b** Cell growth and intracellular accumulation of copper. Cultivation was conducted in a 20-L bioreactor, in which 5.0 g/L yeast extract was combined with 100 mg/L copper ions. Error bars represent standard deviations ($n = 3$)

In this study, glucose concentration was used as the main parameter for feeding rate control. However, the concentration of nitrogen source is also an important factor for cell growth and uptake of copper ions. Furthermore, to maintain cell growth and physiological activity, dissolved oxygen (DO) was set at more than 30% saturation by regulating agitation speed and airflow rate in the experiment design. However, DO was below 10% during 13–20 h in the actual scale-up of cultivation process (Fig. 5a), even when agitation speed and airflow rate were set to the maximum. The sharp decrease of DO resulted from the rapid growth of yeast cells during 13–20 h (Fig. 5b), in which consumption of oxygen exceeded the supply of oxygen. The low DO might have reverse effects on cell growth and physiological activity. Hence, optimization of ammonium concentration and improvement of oxygen supply during rapid growth period should be conducted in future studies for much more effective production of bioactive copper in yeast-enriched form.

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Author Contribution Xue-Na Guo designed and performed the experiments, processed and interpreted the data, and prepared the manuscript. Xiao-Xian He participated in designing and performing experiments, processing and interpreting data. Li-Bin Zhang, Yan-Fei Cheng, and Xiu-Mei Bai performed the experiments. Zhao-Yue Wang processed and interpreted the data. Xiu-Ping He supervised the experiments and revised the manuscript.

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Data Availability The authors declare that the data and materials are transparent.

Code Availability Not applicable.

Declarations

Ethics Approval This article does not contain any studies involving humans and animals.

Conflict of Interest The authors declare no competing interests.

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