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# Optimization of culture conditions of screened *Galactomyces candidum* for the production of single cell protein from biogas slurry

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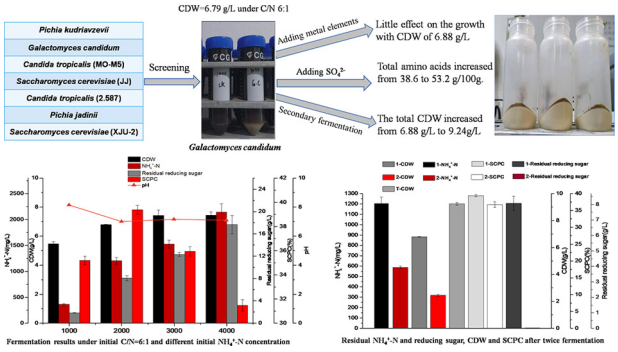
## GRAPHICAL ABSTRACT

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## ABSTRACT

*Background:* The use of single cell protein (SCP) has become a method for alleviating the shortage of protein feed that microorganisms propagate in a suitable culture medium. In this study, SCP was produced by yeast to use the nutrition contained in the biogas slurry of chicken manure.

*Results:* The results showed that *Galactomyces candidum* was the most efficient at producing SCP among the seven yeasts studied. The maximum cell dry weight (CDW) 6.79 g/L and protein content 39.39%, were obtained under the fermentation conditions of initial $NH_4^+$-N concentration of 2000 mg/L and a C/N ratio of 6:1 with acetate as the pH regulator. The total CDW increased to 9.24 g/L after secondary fermentation. Metal elements had a little effect on the growth of *G. candidum*. The addition of sulfur not only promoted the synthesis of sulfur-containing amino acid cysteine but also increased protein content by promoting the synthesis of glutamic acid and glutamine.

*Conclusions:* Future experiments should focus more on achieving high-density cultivation and more efficient utilization of ammonia nitrogen in the biogas slurry.

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## 1. Introduction

High organic content in wastewater is often a major loss of resources, resulting in serious pollution problems [1]. Single cell protein (SCP) production from the wastewater is an attractive

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method for wastewater purification and resource utilization [2]. SCP has many advantages, such as short production time, high protein content in cells, rich in fat, carbohydrates, nucleic acids, vitamins, and minerals, and is rich in some essential amino acids, such as lysine and methionine, which are limited in most animal and plant foods [3,4]. At present, bacteria, yeast, mold, and algae can be used as feed proteins. Yeast has been the most thoroughly researched and is the most widely used microorganism in the market, because of its high tolerance to low pH, high organic matter, and high salt concentration. *Candida*, *Pichia* and *Saccharomyces* have been widely used in high carbohydrate wastewater, such as waste cabbage [5], green salads [6], and tropical fruits [7]. *Galactomyces geotrichum* has the potential to reduce the pollution of silage wastewater, which can produce 9.0 g/L biomass production and its protein content just only 16% [8]. Approximately 23.1–41.4% of the chemical oxygen demand and 15.4–19.2% of the phenolic compounds were removed by *Schwanniomyces etchellsii* and *Candida pararugosa* on olive mill wastewater [2]. What is more, *Candida tropicalis* was able to reduce 68% of chemical oxygen demand and 39% of the total phenols after conditions optimization [9]. *Pichia guilliermondii* can produce biomass in the waste brine of a kimchi factory containing 10% NaCl, and remove about 90% of the biochemical oxygen demand within 24 h [10]. The potential mechanisms of adaptation of various yeast to salt stress include the accumulation of osmotic active compounds (mainly glycerin) to offset the increased external osmotic pressure, and the change of membrane transport systems to extrude $Na^+$ from cells [11].

The above-mentioned SCP production from wastewater mainly uses carbon source including carbohydrate and oil, but there are few studies on the production of SCP using wastewater with high ammonia nitrogen. The biogas slurry is a kind of high ammonia wastewater. In order to use the waste nitrogen source contained in biogas slurry, Dou et al. [12] screened two strains of hydrogen oxidizing bacteria to produce SCP from biogas slurry. Although the removal rate of ammonia nitrogen reached 49.04%, the maximum content of cell dry weight (CDW) was only 2.12 g/L, and it was lack of sulfur-containing amino acids. Yang et al. [13] reported that when photosynthetic bacteria was cultured in biogas slurry with ammonia concentration of 2350 mg/L, the removal rate of ammonia nitrogen could reach 73.3%, while the CDW remained between 0.2 and 0.3 g/L. Moreover, the protein of photosynthetic bacteria also lacked sulfur-containing amino acids.

Therefore, the purpose of this study was to provide yeast strains suitable for high SCP production from biogas slurry with high ammonia, and to optimize the culture conditions, especially for the synthesis of sulfur-containing amino acids, which provides a scientific basis for utilization of nitrogen resource contained in biogas slurry.

## 2. Material and methods

### 2.1. Microorganisms and biogas slurry

All seven yeast strains, including *Pichia kudriavzevii*, *G. candidum*, *Candida tropicalis* (MO-M5), *Saccharomyces cerevisiae* (JJ), *Candida tropicalis* (CGMCC 2.587), *Pichia jadinii*, *and Saccharomyces cerevisiae* (XJU-2), were preserved in the author's laboratory and isolated from distillers' grains, bread flour, and chicken manure fermentation products. The biogas slurry derived from chicken manure was obtained from Shandong Minhe Biology Co. Ltd, Penglai, China and preserved in a refrigerator at −20 °C using a medical gauze filter to remove large particles before use. The characteristics of the biogas slurry are listed in Table 1, with a COD of 16440 mg/L and $NH_4^+$-N concentration of 4560 mg/L.

**Table 1**
Characteristics of biogas slurry derived from chicken manure.

| Parameters | Value | Parameters | Value |
|---|---|---|---|
| pH | 8.35 | Cu(mg/L) | 1.42 |
| Total solid (g/kg) | 17.70 | Mn(mg/L) | 0.43 |
| Volatile solid (g/kg) | 7.20 | Fe(mg/L) | 0.02 |
| Suspended solid (g/kg) | 10.50 | Mg(mg/L) | 30.14 |
| COD (mg/L) | 16,440 | Zn(mg/L) | 0.15 |
| $NH_4^+$-N (mg/L) | 4560 | Ni(mg/L) | 0.36 |
| Reducing sugar (mg/L) | 0 | Ca(mg/L) | 44.43 |
| C/N | 1.44 | Na(mg/L) | 1094.46 |
| Si (mg/L) | 59.93 | K(mg/L) | 2849.88 |

### 2.2. Screening of strains

The method for preparing the biogas slurry culture medium was as follows: the concentration of ammonia nitrogen in the biogas slurry was diluted to 1000 mg/L, and 25 g/L glucose was added so that the ratio of carbon to nitrogen in the biogas slurry was 10:1. After stirring evenly, 65 ml biogas slurry was measured and poured into a 150 ml conical flask. After sealing, the samples were sterilized at 115 °C for 30 min. Before inoculation, the pH of the three bottles of biogas slurry was measured using a pH meter, and the pH of the biogas slurry medium was 9.12. To adjust the pH to encourage the yeast to grow properly, two different acids were compared. For the first acid, 500 μl of 1 mol/L HCl was added to the sterilized liquid, adjusting the pH to 7.30. For the second acid, 300 μl of acetic acid was added to the sterilized liquid, adjusting the pH to 5.50. In this case, the probability of acetic acid serving as a carbon source can be ignored. The inoculated amount of yeast was 5%, and the bottle was placed in the incubator after inoculation. The culture conditions were 30 °C and 160 rpm, and the culture was maintained for 4 d. The pH, cell dry weight (CDW), and single-cell protein content (SCPC) based on CDW were determined on the second and fourth days of culture. Three parallel experiments were conducted for each test.

### 2.3. Optimization of yeast fermentation

#### 2.3.1. Initial C/N and $NH_4^+$-N concentration

The bacteria were tested to different carbon to nitrogen ratios and the tolerance for different concentrations of ammonia nitrogen. Two different carbon–nitrogen ratios, C/N = 10:1 and C/N = 6:1. Under both conditions of high C/N (10:1) and low C/N (6:1), the selected bacteria were grown in a biogas slurry with initial $NH_4^+$-N concentrations of 1000, 2000, 3000, and 4000 mg/L, respectively. Three replicates were made for each test condition, and the culture conditions were as described above. The pH, ammonia–nitrogen, and reducing sugar levels were measured on days 2 and 4. CDW was measured on d 4.

#### 2.3.2. Concentration of metal elements

After the experiment described in Section 2.3.1, it was determined that the fermentation conditions allowing for the most economical and efficient use of nitrogen in biogas slurry were C/N = 6:1 and an initial $NH_4^+$-N concentration of 2000 mg/L. Metal elements in the control and experimental groups were compared. Interestingly, the concentration of $Ca^{2+}$ decreased from 18.46 mg/L to 3.724 mg/L, the concentration of $Mg^{2+}$ decreased from 12.52 mg/L to 4.603 mg/L, the $Cu^{2+}$ decreased from 0.5899 mg/L to 0.0852 mg/L, and the concentration of $Fe^{2+}$, $Mn^{2+}$, $Ni^{2+}$, and $Zn^{2+}$ decreased significantly. Therefore, it is speculated that these seven elements are limiting factors (Table 2). To optimize the metal elements in the components of the culture system, $Ca^{2+}$,

**Table 2**
The metal element contents after 4 d fermentation with initial $NH_4^+$-N 2000 mg/L.

| Element | CK | *Galactomyces candidum* | element | CK | *Galactomyces candidum* |
|---|---|---|---|---|---|
| As | 0.1630 | 0.1560 | Mg | 12.5200 | 4.6030 |
| Ca | 18.4600 | 3.7240 | Mn | 0.1771 | 0.0019 |
| Cd | 0.0022 | 0.0019 | Mo | 0.0951 | 0.0994 |
| Co | 0.0363 | 0.0142 | Na | 454.7 | 463.8 |
| Cr | 0.0327 | 0.0405 | Ni | 0.1494 | 0.0359 |
| Cu | 0.5899 | 0.0852 | Pb | 0.1771 | 0.0019 |
| Fe | 0.0078 | 0.0039 | Se | 0.0982 | 0.1029 |
| Hg | 0.0381 | 0.0301 | Si | 24.90 | 30.48 |
| K | 1184 | 1155 | Zn | 0.0604 | 0.0055 |

**Table 3**
The orthogonal test factor level of metal elements (mg/L).

| Level | Factors | | | | | | |
|---|---|---|---|---|---|---|---|
| | $Ca^{2+}$ | $Mn^{2+}$ | $Fe^{2+}$ | $Zn^{2+}$ | $Cu^{2+}$ | $Mg^{2+}$ | $Ni^{2+}$ |
| 1 | 10 | 0.1 | 0.01 | 0.05 | 0.2 | 3 | 0.05 |
| 2 | 20 | 0.2 | 0.03 | 0.1 | 0.4 | 6 | 0.1 |
| 3 | 30 | 0.3 | 0.05 | 0.15 | 0.6 | 9 | 0.15 |

$Mn^{2+}$, $Fe^{2+}$, $Zn^{2+}$, $Mg^{2+}$, $Ni^{2+}$, and $Cu^{2+}$ were selected as the added metal elements. The optimization was conducted using orthogonal design software (Design-Expert.V.8) with the L16(45) test (Table 3). Three parallel tests were conducted for each group, and the results were averaged. The culture conditions included a temperature of 30 °C, a culture time of 4 d, and a rotation speed of 160 rpm.

*2.3.3. Secondary fermentation*

Fermentation was conducted under the optimal conditions described above. However, the CDW concentration did not increase after 4 d of culture. After completing the first assay of the CDW, $NH_4^+$-N, and reducing sugar, the centrifugal supernatant was collected. It was found that glucose and ammonia nitrogen were still present, so a second fermentation was carried out. The pH of the system was adjusted to 6.20 with acetic acid, 5% *G. candidum* was added, and the contents of CDW, SCPC and ammonia nitrogen were determined after 4 d of culture. The other parameters were consistent with those of previous tests.

*2.3.4. Adding $K_2SO_4$ for enhancing of SCP production*

Since there is no sulfur in the biogas slurry, a 0.5 ml $K_2SO_4$ solution with a concentration of 45 mg/L was added to 60 ml biogas medium after sterilization with an ammonia nitrogen concentration of 2000 mg/L, and 5% *G. candidum* was inoculated. The culture conditions were 30 °C, the culture time was 4 d, and the rotation speed was 160 rpm. The concentration of $K_2SO_4$ and the composition of amino acids were determined after 4 d of culture.

*2.4. Analytical methods*

The characteristics of the biogas slurry and fermentation broth were analyzed. The pH was determined using a pH meter (Shanghai Precision & Scientific Instrument Co., Ltd., China). Total solids (TS), volatile solids (VS), and suspended solids (SS) were measured using standard methods [14]. The COD was analyzed using a DR-1900 spectrophotometer (HACH, USA) [15]. Analyses of C and N were performed using a Vario EL element analyzer (Elementar Analysensysteme GmbH, Germany). ICP mass spectrometry (ICP-MS, PerkinElmer Nexion 350, USA) was used to determine the presence of metallic elements. The biogas slurry fermentation system was centrifuged at 978 × g for 5 min. The supernatant was used to determine the $NH_4^+$-N and reducing sugar used, and CDW and SCPC were measured from the sediments. The CDW was measured using the weighing method, in which the sediment was dried at 60 °C. The control group weight was subtracted from all CDW values to eliminate any influence of impurities in the biogas slurry. The ammonia nitrogen concentration was measured using Nessler's reagent spectrophotometry [16]. Kjeldahl nitrogen quantification was conducted using an Automatic Kjeldahl Apparatus (FOSS, Denmark). The Kjeldahl nitrogen value was multiplied by a conversion factor of 6.25 to obtain the SCPC content [17]. The amount of reducing sugar used was determined using the 3–5 dinitrosalicylic acid method. The content of $SO_4^{2-}$ was measured according to HACH method 8501 (HACH DR1900, USA). The amino acid composition was measured by the Sci-Tech Innovation Company (China).

*2.5. Statistical analysis*

The experimental data were evaluated using one-way analysis of variance (ANOVA), and Duncan's multiple comparison test was used to detect differences using SPSS (version 19.0; IBM Corp., Armonk, NY, USA) for Windows.

## 3. Results and discussion

*3.1. Screening of strains and pH regulator*

As shown in Table 4, when 1 mol/L HCl was used to adjust the pH of biogas slurry, the pH of biogas slurry decreased rapidly from 7.32 to 2.30–2.67 after 2 d of fermentation. After 4 d of fermentation, the pH of each experimental group decreased slightly. The CDW content of *Candida tropicalis* (2.587) was the highest at 6.73 g/L. The CDW content of *G. candidum* and *Candida tropicalis* (MO-M5) was 5.01 g/L and 4.94 g/L, respectively. Except for CK, the residual reducing sugar content of *G. candidum* was the highest, at 8.07 g/L, and the content of residual reducing sugar in the other experimental groups was less than 0.35.

As shown in Table 5, when acetic acid was used to adjust the pH of the culture system, after 2 d of fermentation, the pH of *Pichia kudriavzevii*, *Candida tropicalis* (MO-M5), *Saccharomyces cerevisiae*, *Candida tropicalis* (2.587), and *Saccharomyces cerevisiae* (XJU-2) increased, while the pH of *G. candidum* and *Pichia jadinii* decreased. After 4 d of fermentation, the contents of CDW and residual reducing sugar in *G. candidum* were the highest at 8.90 g/L and 4.70 g/L, respectively. The CDW of *Candida tropicalis* (MO-M5) and *Candida tropicalis* (2.587) was 6.66 g/L and 6.55 g/L, respectively. Except

**Table 4**
The pH, reducing sugar and CDW of cultural system with HCl as pH regulator.

| Strains | pH-2d | pH-4d | Residual reducing sugar-2 d (g/L) | Residual reducing sugar-4 d (g/L) | CDW (g/L) | SCPC (%) | CP (g/L) | PY (g/g) |
|---|---|---|---|---|---|---|---|---|
| *Pichia kudriavzevii* | 2.41$^{e}$ | 2.37$^{d}$ | 0.31$^{c}$ | 0.30$^{c}$ | 4.43$^{c}$ | 50.86$^{a}$ | 2.25$^{b}$ | 0.093$^{c}$ |
| *Galactomyces candidum* | 2.62$^{c}$ | 2.53$^{c}$ | 8.48$^{b}$ | 8.07$^{b}$ | 5.01$^{b}$ | 33.63$^{e}$ | 1.68$^{d}$ | 0.104$^{b}$ |
| *Candida tropicalis (MO-M5)* | 2.35$^{e}$ | 2.25$^{e}$ | 0.24$^{c}$ | 0.25$^{c}$ | 4.94$^{b}$ | 46.37$^{b}$ | 2.29$^{b}$ | 0.094$^{c}$ |
| *Saccharomyces cerevisiae (JJ)* | 2.76$^{b}$ | 2.64$^{b}$ | 0.26$^{c}$ | 0.30$^{c}$ | 3.90$^{d}$ | 41.41$^{d}$ | 1.61$^{d}$ | 0.067$^{d}$ |
| *Candida tropicalis (2.587)* | 2.38$^{e}$ | 2.15$^{f}$ | 0.23$^{c}$ | 0.25$^{c}$ | 6.73$^{a}$ | 44.14$^{c}$ | 2.97$^{a}$ | 0.122$^{a}$ |
| *Pichia jadinii* | 2.50$^{d}$ | 2.36$^{d}$ | 0.30$^{c}$ | 0.28$^{c}$ | 3.81$^{d}$ | 49.95$^{a}$ | 1.90$^{c}$ | 0.078$^{d}$ |
| *Saccharomyces cerevisiae (XJU-2)* | 2.61$^{c}$ | 2.59$^{bc}$ | 0.32$^{c}$ | 0.32$^{c}$ | 3.54$^{d}$ | 45.73$^{bc}$ | 1.62$^{d}$ | 0.067$^{d}$ |
| CK | 7.32$^{a}$ | 7.35$^{a}$ | 24.95$^{a}$ | 24.54$^{a}$ | 0.00$^{e}$ | 0.00f | 0.00$^{e}$ | 0.000$^{e}$ |
| SEM | 0.010 | 0.009 | 0.198 | 0.142 | 0.040 | 0.786 | 0.069 | 0.047 |
| P | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

CDW, cell dry weight; SCPC, single cell protein content based on CDW; CP, concentration of protein; PY, Protein produced per gram of used glucose; Means within the same column with different letters differ significantly from each other ($P < 0.05$). SEM, standard error of the mean.

**Table 5**
The pH, reducing sugar and CDW of cultural system with acetic acid as pH regulator.

| Strains | pH-2d | pH-4d | Residual reducing sugar-2d (g/L) | Residual reducing sugar-4d (g/L) | CDW (g/L) | SCPC (%) | CP (g/L) | PY (g/g) |
|---|---|---|---|---|---|---|---|---|
| *Pichia kudriavzevii* | 6.65$^{c}$ | 6.02$^{cd}$ | 3.15$^{c}$ | 2.65$^{c}$ | 6.44$^{b}$ | 36.41$^{f}$ | 2.59$^{c}$ | 0.116$^{d}$ |
| *Galactomyces candidum* | 5.94$^{d}$ | 6.82$^{b}$ | 9.59$^{b}$ | 4.70$^{b}$ | 8.90$^{a}$ | 33.10$^{g}$ | 3.16$^{b}$ | 0.156$^{a}$ |
| *Candida tropicalis (MO-M5)* | 6.71$^{bc}$ | 6.33$^{bc}$ | 2.70$^{c}$ | 2.30$^{c}$ | 6.66$^{b}$ | 42.58$^{d}$ | 3.12$^{b}$ | 0.137$^{c}$ |
| *Saccharomyces cerevisiae (JJ)* | 7.22$^{ab}$ | 7.68$^{a}$ | 2.68$^{c}$ | 2.028$^{c}$ | 3.24$^{d}$ | 51.02$^{b}$ | 1.99$^{d}$ | 0.088$^{e}$ |
| *Candida tropicalis (2.587)* | 6.59$^{c}$ | 6.10$^{cd}$ | 2.77$^{c}$ | 2.44$^{c}$ | 6.55$^{b}$ | 45.19$^{c}$ | 3.26$^{a}$ | 0.145$^{b}$ |
| *Pichia jadinii* | 4.87$^{e}$ | 5.56$^{d}$ | 2.93$^{c}$ | 2.58$^{c}$ | 5.08$^{c}$ | 53.30$^{a}$ | 3.06$^{b}$ | 0.137$^{c}$ |
| *Saccharomyces cerevisiae (XJU-2)* | 7.47$^{a}$ | 7.83$^{a}$ | 2.49$^{c}$ | 2.22$^{c}$ | 3.35$^{d}$ | 41.26$^{e}$ | 1.66$^{d}$ | 0.073$^{e}$ |
| *CK* | 6.04$^{d}$ | 5.93$^{cd}$ | 24.32$^{a}$ | 24.32$^{a}$ | 0.00$^{e}$ | 0.00$^{h}$ | 0.00$^{e}$ | 0.000$^{f}$ |
| *SEM* | 0.061 | 0.060 | 0.242 | 0.312 | 0.059 | 0.520 | 0.049 | 0.063 |
| *P* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

CDW, cell dry weight; SCPC, single cell protein content based on CDW; CP, concentration of protein; PY, protein yield (Protein produced per gram of used glucose); Means within the same column with different letters differ significantly from each other ($P < 0.05$). SEM, standard error of the mean.

for *G. candidum*, the contents of reducing sugars in the other groups were 2.28–2.65 g/L. The single cell protein content (SCPC) of *Pichia jadinii* was the highest at 53.30%, while the concentration of protein (CP) of *Candida tropicalis* (2.587) was the highest at 3.26 g/L. Based on the above, the protein yield (PY, protein produced per gram of glucose used) for different strains was compared. The highest PY of 0.156 g – was obtained by *G. candidum*. *G. candidum* is considered to be the most potent strain for SCP production. The strains screened for the different raw materials were different. Moeini et al. [18] screened 11 strains of yeast strains for SCP production from whey wastewater and found that *Kluyveromyces Lactis* had the strongest ability to produce SCP by studying the physiological and biochemical characteristics of 11 strains and SCP production capacity, in which CDW can reach up to 11.79 g/L. Arous et al. [10] screened 11 yeast strains from olive mill wastewater; among them, the strain *Candida pararugosa* BM24 can obtain the maximum CDW, reaching 21.68 g/L. The CDW obtained in this study was lower than that reported, which may be due to the inhibition of high concentrations of ammonia or the lack of nutritional elements.

### 3.2. Selection of initial C/N and initial $NH_4^+$-N concentration

Nitrogen is the primary nutrient for yeast growth and should be adjusted to provide a C/N ratio from 6:1 to 10:1 to favor high protein content [10]. Two different carbon–nitrogen ratios were designed to increase the yield of single cells, namely C/N = 10:1 and C/N = 6:1. As shown in Fig. 1, when C/N = 10:1, with an initial $NH_4^+$-N concentration of 1000 mg/L, the CDW was 8.90 g/L, and the remaining reducing sugar was 4.70 g/L. When the concentration of $NH_4^+$-N was 2000 mg/L, the CDW was 9.90 g/L, the remaining reducing sugar was 7.86 g/L, and the SCPC increased from 33.09% to 36.42%. When the concentration of ammonia nitrogen exceeded 2000 mg/L, the CDW content did not increase significantly. This may be due to the inhibition of high ammonia nitrogen. A high concentration of ammonia causes a significant reduction in microbial activity, and the primary route is due to the high permeability of $NH_4^+$ -N to bacterial cell membranes [19]. When $NH_4^+$ enters the cell membrane, the cell must use a potassium ($K^+$) pump to maintain the intracellular pH, which consumes energy in the proton balance. This increases the demand for energy maintenance and may lead to the inhibition of specific enzyme reactions [20].

As shown in Fig. 2, when C/N = 6:1, with the concentration of $NH_4^+$-N is 1000 mg/L, CDW is 5.92 g/L, and the remaining reducing sugar concentration is 4.70 g/L; when the concentration of $NH_4^+$-N is 2000 mg/L, CDW is 6.79 g/L, the remaining reducing sugar concentration is 8.07 g/L, and the SCPC achieves a maximum of 39.39%. When the ammonia nitrogen concentration exceeded 2000 mg/L, the CDW content did not increase significantly, but the SCPC content significantly decreased. By comparing the CDW and reducing sugar used by *G. candidum* under the conditions of C/N = 10:1 and C/N = 6:1, with an initial $NH_4^+$-N concentration of 2000 mg/L, it was found that when C/N = 10:1, 32.45 g of glucose was used to produce 9.90 g CDW. The corresponding biomass yield was 0.31 g CDW/g glucose. At C/N = 6:1, 14.94 g glucose was used to produce 6.79 g. The corresponding biomass yield was 0.45 g CDW/g glucose. To improve the production performance of SCP, *G. candidum* was selected for further optimization of fermentation conditions under initial conditions of C/N = 6:1 and $NH_4^+$-N concentration of 2000 mg/L. This result was consistent with Arous et al. [10], in which the optimal C/N ratio ranged from 6:1 to 8:1, and a maximum biomass production of 15.11 and 21.68 g/L was achieved for *Schwanniomyces etchellsii* M2 and *Candida pararugosa* BM24, respectively. When the optimal C/N ratio was 3:1, the crude

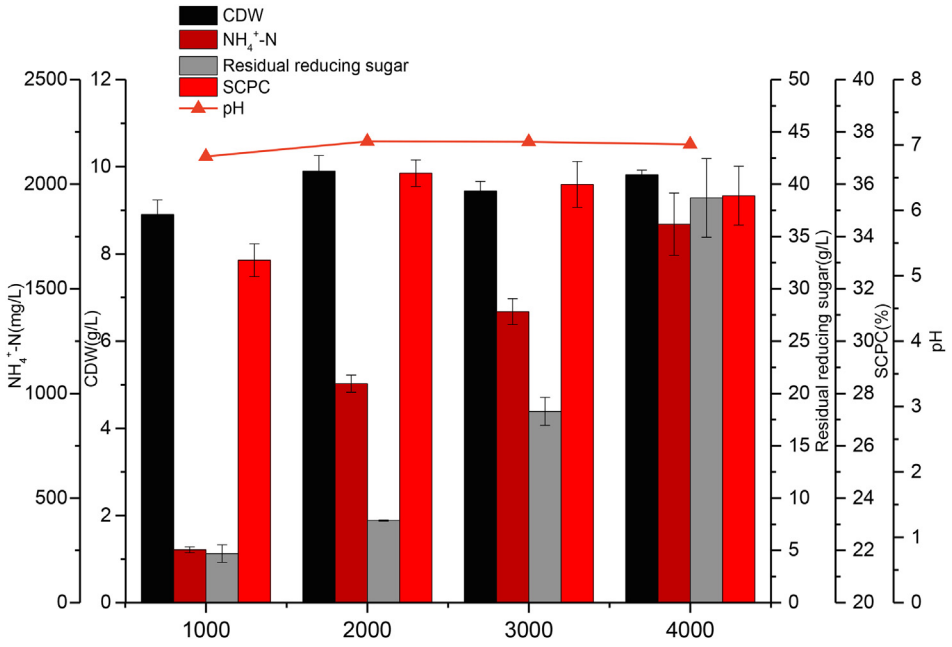


**Fig. 1.** CDW, final pH, residue concentrations of $NH_4^+$-N and residual reducing sugar of *Galactomyces candidum* after 4 d of fermentation were determined under initial C/N = 10:1 and initial $NH_4^+$-N concentration of 1000 mg/L (a), 2000 mg/L (b), 3000 mg/L (c) and 4000 mg/L (d).

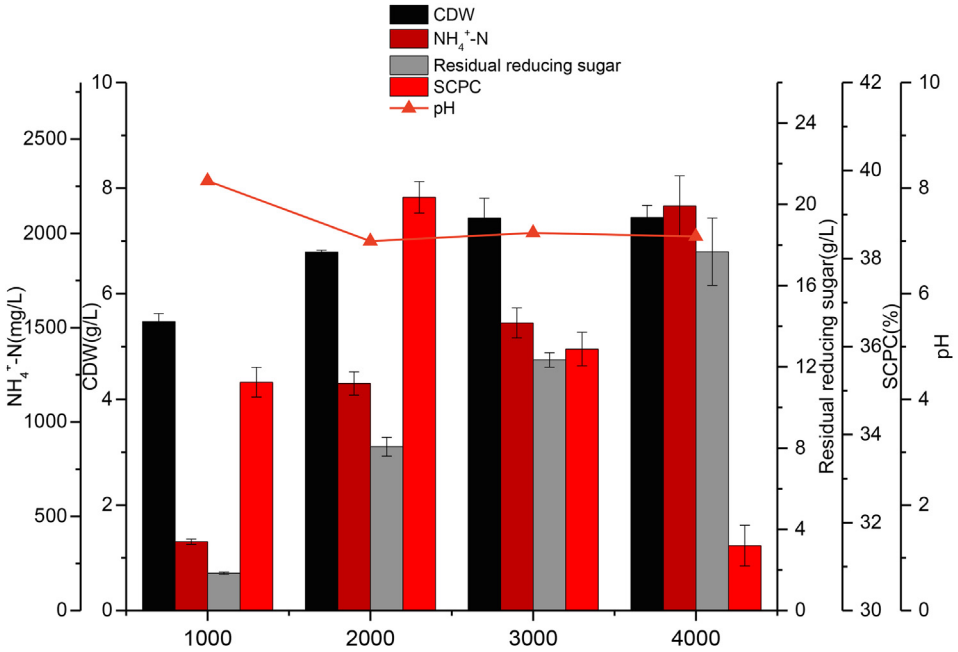


**Fig. 2.** CDW, final pH, residue concentrations of $NH_4$ + -N and residual reducing sugar of *Galactomyces candidum* after 4 d of fermentation were determined under initial C/N = 6:1 and initial $NH_4^+$-N concentration of 1000 mg/L (a), 2000 mg/L (b), 3000 mg/L (c) and 4000 mg/L (d).

protein content of *Fusarium venenatum* was 47.34% [21]. However, when the optimal C/N ratio was 5.5:1, 70.02% crude protein in the dry product was produced and the maximum biomass was 4 g/L [22]. So the optimal C/N ratio can make the microorganism obtain the maximum or the most economical biomass yield. Additionally, the C/N ratio is an important parameter that affects lipid production. Liu et al. [23] found that as the C/N ratio increased from 40 to 60, the biomass increased from 16.8 g/L to 18.3 g/L. However, as the C/N ratio further increased, the biomass gradually decreased, and the fat content of the bacteria continued to increase.

### 3.3. Optimization of metal elements

Yeasts require a range of metals for optimal growth, metabolism, and fermentation performance. Yeast-derived feed typically contains 4%–10% minerals (on a dry matter basis), including some trace elements and macro elements, such as P, Na, Cu, Mn, Zn, and Fe [24]. The requirement for metal ions varies so widely with the different strains that it is necessary to adjust the composition of the medium to avoid the inhibitory effects of others [25]. According to the results of metal element optimization, as shown in

**Table 6**
The results of orthogonal test for the optimization of metal elements.

| Treatment | Factors | | | | | | | CDW (g/L) |
|---|---|---|---|---|---|---|---|---|
| | Ca2+ | Cu2+ | Fe2+ | $Mg^{2+}$ | $Mn^{2+}$ | $Ni^{2+}$ | $Zn^{2+}$ | |
| 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 6.30 |
| 2 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 6.00 |
| 3 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 6.06 |
| 4 | 2 | 1 | 1 | 2 | 2 | 3 | 3 | 5.94 |
| 5 | 2 | 2 | 2 | 3 | 3 | 1 | 1 | 6.05 |
| 6 | 2 | 3 | 3 | 1 | 1 | 2 | 2 | 6.17 |
| 7 | 3 | 1 | 2 | 1 | 3 | 2 | 3 | 6.29 |
| 8 | 3 | 2 | 3 | 2 | 1 | 3 | 1 | 6.06 |
| 9 | 3 | 3 | 1 | 3 | 2 | 1 | 2 | 5.85 |
| 10 | 1 | 1 | 3 | 3 | 2 | 2 | 1 | 6.21 |
| 11 | 1 | 2 | 1 | 1 | 3 | 3 | 2 | 6.03 |
| 12 | 1 | 3 | 2 | 2 | 1 | 1 | 3 | 6.28 |
| 13 | 2 | 1 | 2 | 3 | 1 | 3 | 2 | 6.53 |
| 14 | 2 | 2 | 3 | 1 | 2 | 1 | 3 | 6.59 |
| 15 | 2 | 3 | 1 | 2 | 3 | 2 | 1 | 6.88 |
| 16 | 3 | 1 | 3 | 2 | 3 | 1 | 2 | 6.59 |
| 17 | 3 | 2 | 1 | 3 | 1 | 2 | 3 | 6.50 |
| 18 | 3 | 3 | 2 | 1 | 2 | 3 | 1 | 6.62 |
| Mean 1 | 6.147 | 6.310 | 6.250 | 6.377 | 6.307 | 6.277 | 6.253 | |
| Mean 2 | 6.360 | 6.205 | 6.295 | 6.292 | 6.202 | 6.342 | 6.195 | |
| Mean 3 | 6.318 | 6.310 | 6.280 | 6.200 | 6.311 | 6.207 | 6.277 | |
| Range | 0.213 | 0.105 | 0.045 | 0.133 | 0.115 | 0.135 | 0.158 | |

Table 6, the order of influence of these metal elements was as follows: $Ca^{2+} > Zn^{2+} > Ni^{2+} > Mg^{2+} > Mn^{2+} > Cu^{2+} > Fe^{2+}$. The elements were added as follows: 20 mg/L of $Ca^{2+}$, 3 mg/L of $Mg^{2+}$, 0.3 mg/L of $Mn^{2+}$, $Fe^{2+}$ of 0.03 mg/L, 0.05 mg/L of $Zn^{2+}$, 0.2 mg/L of $Cu^{2+}$, 0.1 mg/L of $Ni^{2+}$, which produced the most CDW at 6.88 g/L. While 6.79 g/L was obtained in the above fermentation without the addition of metal elements. This reveals that the optimization of metal elements had little effect on the growth of *G. candidum*. This result is not consistent with those of Pradeep and Pradeep [26], who found that after adding $K^+$, $Mg^{2+}$, $Zn^{2+}$, $Fe^{2+}$, and $Cu^{2+}$, the biomass and pigment production increased significantly and among which $K^+$ produced the highest biomass production. Additionally, studies have found that metal ions can not only participate in the formation of cell membranes but also affect the activity of lipase, which in turn affects the accumulation of oil. Chen et al. [27] added different kinds of inorganic salts to the corncob acid hydrolysate and found that adding an appropriate amount of inorganic salts can significantly stimulate the growth of the strain *Trichosporon cutaneum* CH002 and the synthesis of oil.

### 3.4. Secondary fermentation

After the first fermentation, the $NH_4^+$-N concentration decreased from 2000 to 1204 mg/L. The residual concentration of reducing sugars was 8.07 g/L. A reason for these outcomes might be the limitation of high cell density. The supernatant obtained after

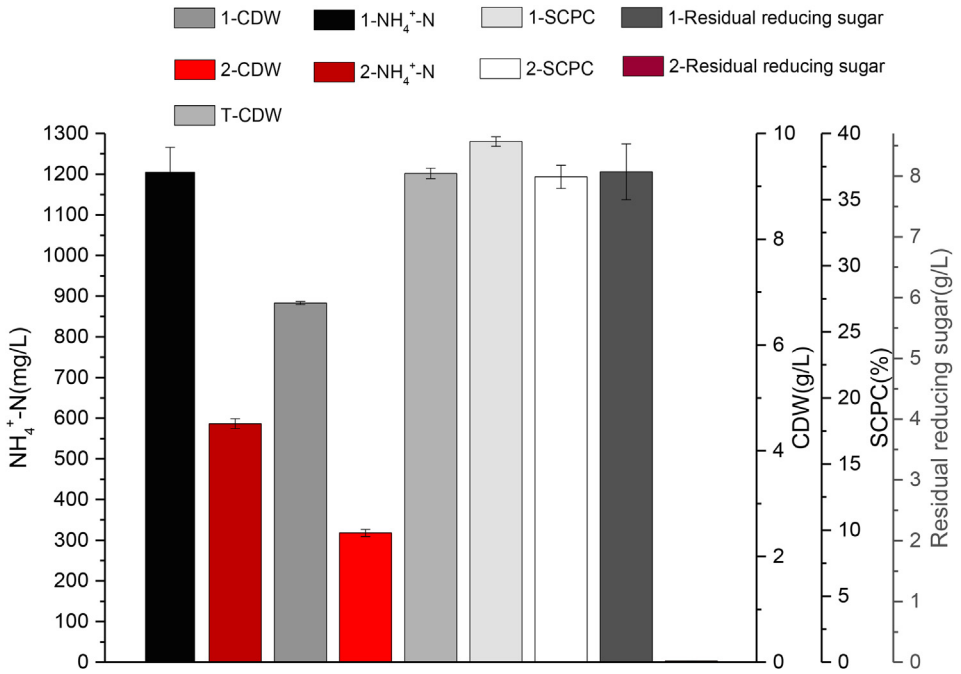


**Fig. 3.** Residual $NH_4^+$-N, CDW and SCPC after twice fermentation. 1 represent first fermentation, 2 represent secondary fermentation, T-CDW represent total cell dry weight.

centrifugation was used as a second fermentation to address this possibility. As shown in Fig. 3, the $NH_4^+$-N concentration decreased from 1204 mg/L to 586 mg/L, and residual reducing sugars decreased from 8.07 g/L to 0.02 g/L after secondary fermentation. After secondary fermentation, the total CDW reached 9.24 g/L, with the secondary fermentation accounting for 26.5% of the total CDW. Even though the consumption of $NH_4^+$-N and reducing sugars in secondary fermentation was similar to that of the first fermentation, the CDW of 2.45 g/L from secondary fermentation was much lower than that of 6.79 g/L from the first fermentation. A possible reason for this was that ammonia was only assembled into amino acids rather than protein.

### 3.5. Amino acids composition of SCP

The amino acid composition of SCP from *G. candidum* was determined without the addition of $K_2SO_4$ (Table 7). Protein composition analysis showed that there are abundant essential amino acids in *G. candidum* such as threonine, valine, methionine, isoleucine, leucine, phenylalanine, and lysine, as well as some nonessential amino acids, such as asparagine, glutamic acid, glutamine, arginine, and proline, and the glutamic acid content was the highest (6.122 g/100 g). However, the sulfur-containing amino acid cysteine did not appear in the amino acid composition. A possible reason for this is the lack of sulfur in the biogas slurry because sulfur was transformed into biogas in the form of $H_2S$ during anaerobic digestion. The growth and protein synthesis of *G. candidum* requires sulfur. Therefore, the effects of sulfur on the growth of *G. candidum* were investigated. Notably, after adding $K_2SO_4$, 0.436 g/100 g of the sulfur-containing amino acid cysteine appeared in the amino acid composition of the protein produced by *G. candidum*. Methionine content increased slightly. This may be because $SO_4^{2-}$ is absorbed and reduced to sulfur-containing organic matter (R-SH) through assimilation-type sulfate reduction [28]. The content of amino acids increased from 35.584 g/100 g to 53.153 g/100 g, and the content of CDW did not increase significantly. Additionally, the $K_2SO_4$ content decreased from 45 to 29 mg/L. This indicates that sulfur not only promotes the synthesis of sulfur-containing amino acids but also promotes the synthesis of other biomass, especially glutamic acid and glutamine. The content of glutamate increased from 6.122 g/100 g to 18.114 g/100 g, and the content of glutamine increased from 0.628 g/100 g to 5.398 g/100 g. Nitrogen metabolism in yeast is very complex [29]. There are two primary pathways for nitrogen metabolism-ammonia assimilation: (1) the glutamate dehydrogenase (GDH) pathway first transfers α-ketoglutarate into the cytoplasm through the three transporters Odc1p, Odc2p, and Yhm2p located in the inner mitochondrial membrane of yeast [30,31]. Then, after $NH_4^+$ in the participation of GDH to generate glutamate, the generated glutamate can react with another molecule of α-ketoglutarate to form glutamine. The two formed amino acids, glutamate and glutamine, account for 85% and 15% of total cell nitrogen, respectively [32]. (2) The glutamine synthetase-glutamate synthase (GS/GOGAT) pathway, that is, glutamate and $NH_4^+$ generate glutamine through GOGAT, while glutamine and α-ketoglutarate can generate glutamate through GS [33,34], and finally react to generate two glutamate molecules. The increase in glutamate and glutamine may be caused by the enhancement of the intermediate compound product acetyl-CoA with the addition of sulfur. Acetyl-CoA production promotes the formation of α-ketoglutarate A-ketoglutarate and $NH_4^+$ can form glutamic acids via NADPH reduction. Glutamic acid forms glutamine via a second pathway.

**Table 7**
Amino acid composition of the product SCP (g/100 g).

| Amino acids | No adding $K_2SO_4$ | Adding $K_2SO_4$ |
|---|---|---|
| Essential amino acids | | |
| Threonine | 2.108 | 1.005 |
| Valine | 1.436 | 1.262 |
| Methionine | 0.185 | 0.203 |
| Isoleucine | 1.299 | 1.127 |
| Leucine | 1.903 | 1.611 |
| Phenylalanine | 1.293 | 0.873 |
| Lysine | 1.930 | 1.012 |
| Nonessential amino acids | | |
| Aspartic acid | 1.123 | 0.630 |
| Serine | 1.163 | 1.248 |
| Asparagine | 2.329 | 1.483 |
| Glutamate | 6.122 | 18.114 |
| Glutamine | 0.628 | 5.398 |
| Glycine | 1.368 | 1.154 |
| Cysteine | / | 0.436 |
| Tyrosine | 1.215 | 0.920 |
| γ-aminobutyric acid | 6.046 | 4.423 |
| Histidine | 0.814 | 0.696 |
| Arginine | 1.684 | 2.394 |
| Proline | 1.133 | 2.421 |
| Total | 38.584 | 53.153 |

## 4. Conclusions

*G. candidum* was the most efficient at producing SCP among the seven yeasts studied. The maximum CDW of 6.79 g/L and SCPC of 39.39% were obtained at the fermentation conditions of initial $NH_4^+$-N concentration of 2000 mg/L and C/N ratio of 6:1. Secondary fermentation can increase the cell yield, and the CDW content increased from 6.79 to 9.24 g/L. Metal elements have little effect on the growth of *G. candidum*. However, sulfur has a greater impact on the growth of *G. candidum*, and its addition not only promotes the synthesis of sulfur-containing amino acid cysteine but also increases the protein content by promoting the synthesis of glutamic acid and glutamine.

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## Conflict of interest

The authors declare that they have no competing interests.

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