The production of single cell protein from biogas slurry with high ammonia-nitrogen content by screened *Nectaromyces rattus*

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**ABSTRACT** In this study, a novel method was proposed to obtain single cell protein (SCP) in yeast by using biogas slurry as culture medium. The results show that *Nectaromyces rattus* was the most efficient at producing SCP among the 7 different yeasts studied. Acetic acid was a better pH regulator than hydrochloric acid. After culture with the initial NH4+-N concentration 2,000 mg/L, C/N ratio 6:1, the initial pH 5.50 and rotation speed of 200 rpm, a total cell dry weight of 12.58 g/L with 35.96% protein content was obtained. Nineteen amino acids accounted for 46.85% of cell dry weight, and proline content was as high as 12.0% of the cell dry weight. However, sulfur-containing amino acids, including methionine and cystine, were deficient. Further research should focus on the high cell density culture to increase SCP production.

**Key words:** biogas slurry, ammonia nitrogen, single cell protein, *Nectaromyces rattus*

INTRODUCTION

Anaerobic digestion (AD) is one of the main methods used for chicken manure treatment. During the production of biogas, a large amount of biogas slurry containing high ammonia-nitrogen is produced (Sanchez et al., 2006). Biogas slurry acts as a good liquid fertilizer for use in the field. However, when its application exceeds the soil bearing capacity, biogas slurry can cause pollution, at which point it is necessary to treat it as wastewater. At present, traditional physical, chemical, and biological wastewater treatment methods are used to treat biogas slurry with a high concentration of ammonia (Umesh et al., 2017). Biological nitrification and denitrification processes can convert ammonia nitrogen into nitrogen, which is discharged into the atmosphere. This process does not realize the recovery and utilization of nitrogen resources and need high treatment cost. There is an urgent need for an innovative method to utilize the high ammonia-nitrogen in biogas slurry to reduce treatment costs.

Nitrogen is the basic element of organism and the basic component of amino acid, protein and nucleic acid (Fam et al., 2018). If the high ammonia nitrogen in biogas slurry can be converted into protein in one step, it can not only realize the recovery of resources, but also shorten the way of resource utilization compared with fertilizer pathway. In particular, microbial transformation of ammonia nitrogen to synthesize single cell protein (SCP) occurs through a 24-h continuous production process without impacting the environment. SCP is widely used in animal culture as feed protein (Alugongo et al., 2017; Bhatt and Sahoo, 2019). The shortage of protein feed has always been one of the main problems restricting the development of animal husbandry. SCP has great potential in solving the shortage of protein feed (Kand et al., 2018). At present, there are many kinds of microorganisms producing SCP, such as bacteria, yeasts, microalgae and so on (Hülsen et al., 2018; Somda et al., 2018; Al-Mudhafr, 2019). Among them, yeast is the most widely used, and its protein content is 45 to 55%. The yeast is bulky and grows fast (Yang et al., 2017), which can reproduce one generation in 1 to 3 h (Jéssica et al., 2019). In addition, yeast can adjust the balance of microorganisms in animals, improve production performance, regulate immune function and improve disease resistance. Cruz et al. (2019) found that added *Candida utilis* to the diet of weaned piglets to improve the digestive function of piglets while maintaining growth performance. Kim et al. (2000) found that added *Saccharomyces cerevisiae* to the diet of weaned piglets to improve the digestive function of piglets and reduce diarrhea. Chen et al. (2019) found that added 1% hydrolyzed yeast to the diet can promote...
the growth of *Nile tilapia*, enhance immunity and anti-
oxidant capacity.

Before, the team used hydrogen oxidation bacteria to produce SCP (Dou et al., 2019). The results showed that *Paracoccus denitrificans* Y5 could grow autotrophically with H2 as an electron donor, CO2 as a carbon source, and ammonia-nitrogen (NH4\(^+\)-N) as a nitrogen source to produce SCP. However, a maximum cell dry weight (CDW) of only 2.12 g/L was obtained owing to the low gas–liquid mass transfer efficiency. Thus, the purpose of this study was to evaluate the feasibility of SCP production by yeast from biogas slurry, to select high-yield SCP yeast, to optimize the process parameters (or fermentation conditions) of SCP production, and to provide technical support for the use of nutrients in biogas slurry with high ammonia nitrogen.

**MATERIALS AND METHODS**

**Microorganisms and Biogas Slurry**

All 7 yeast strains (*Candida utilis, Saccharomyces cerevisiae, Pichia manshurica, Candida tropicalis, Debaryomyces hansenii, Nectaromyces rattus, Wickerhamomyces anomalus*) were preserved in the author’s laboratory and isolated from vinasse, bread flour, food, and chicken manure. 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 COD 16,440 mg/L and NH4\(^+\)-N concentration 4,560 mg/L.

**Screening of Strains**

After measuring the existing COD and NH4\(^+\)-N concentration in the biogas slurry, it was diluted to NH4\(^+\)-N concentration of 1,000 mg/L. Extra glucose was added to obtain a C/N ratio of 10:1, which not include the existing COD of biogas slurry. Of the mixture, 65 mL was loaded into a 150 mL conical flask with a rubber plug and sterilized (115°C for 30 min) to ensure any foreign bacteria were inactive. The pH of mixture after sterilization was 9.12. Prior to inoculation, in order to adjust the pH of yeast to grow properly, 2 different acids were added: 300 uL of acetic acid to the sterilized liquid and adjust the pH to 7.30, the other is to add 300 uL of HCl to the sterilized liquid, adjust the pH to 3.70. In this case, the probability of acetic acid as carbon source can be ignored. The inoculated amount of yeast was 5%, and the bottle was put into the incubator after inoculation. The culture conditions were 30°C and 160 rpm, and the culture was 4 d. pH, CDW, and single cell protein content (SCPC) based on CDW were determined on the second and fourth day of culture. Three parallel experiments were conducted for each test. Strains with most CDW were used for optimization of subsequent fermentation conditions.

**Optimization of Yeast Fermentation**

**Initial C/N and NH4\(^+\)-N Concentration** Since the ratio of carbon to nitrogen in the biogas slurry (C/N = 1.44) was much lower than the lowest ratios in growing microorganism biomass (C/N = 6:1) (Woertz et al., 2009), two different carbon to nitrogen ratios were set during the experiment: C/N = 10:1 and C/N = 6:1. Under both the conditions of high C/N (10:1) and low C/N (6:1), the selected bacteria were grown in biogas slurry with an initial NH4\(^+\)-N concentration of 1,000, 2,000, 3,000, and 4,000 mg/L, respectively. Three parallels 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 d 2 and 4. CDW was measured on d 4. By comparing the different C/N and initial NH4\(^+\)-N concentrations, 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 initial NH4\(^+\)-N concentration of 2,000 mg/L.

**Rotation Speed** In order to explore the effect of rotation speed on the growth of yeast, the following three rotation speeds were set: 120, 160, and 200 rpm. And the concentration of ammonia nitrogen was 2,000 mg/L, the ratio of carbon to nitrogen was 6:1, the temperature was 30°C and the culture time was 4 d. The CDW of the strain was determined after 4 d of fermentation.

**Concentration of Inorganic Salts** After experiment of rotation speed, the metal elements in the control group and the experimental group were compared. Interestingly, the concentration of Ca2+ decreased from 19.49 mg/L to 0.00 mg/L, the concentration of Cu2+ decreased from 0.62 mg/L to 0.39 mg/L, and the

| 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) | 16440 | Zn (mg/L) | 0.15 |
| NH4\(^+\)-N (mg/L) | 4560 | Ni (mg/L) | 0.36 |
| Reducing sugar (mg/L) | 0.00 | Ca (mg/L) | 44.43 |
| C/N        | 1.44  | Na (mg/L)  | 1094.46 |
| Si (mg/L)  | 59.93 | K (mg/L)   | 2849.88 |
concentration of Fe\(^{2+}\), Mn\(^{2+}\) and Zn\(^{2+}\) decreased significantly. Therefore, it is speculated that these five elements might be the limiting factors (Table 2). In order to optimize the metal elements in the components of the culture system, Ca\(^{2+}\), Mn\(^{2+}\), Fe\(^{2+}\), Zn\(^{2+}\), and Cu\(^{2+}\) were selected to be used as added metal elements in the culture system. The optimization was carried out using orthogonal design software (Design-Expert.V.8) with the L16(45) test (Table 3). Three parallel tests were conducted in each group, and the results were averaged. The culture condition was 30\(^\circ\)C, the culture time was 4 d, and the rotation speed was 200 rpm.

**Secondary Fermentation** After completing the above optimization experiment, the fermentation was conducted at the optimal conditions. 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 left, so the second fermentation was carried out. The pH of the system was adjusted to 6.20 with acetic acid, 5\% *Nectaromyces rattus* was added, and the contents of CDW, SCPC, and ammonia nitrogen were determined after 4 d of culture. Other parameters were consistent with previous tests.

**Analytical Methods**

Characteristics of the biogas slurry and fermentation broth were analyzed. Its pH was determined using a pHS-3C pH meter (Shanghai Precision & Scientific Instrument Co., Ltd., China). Total solids (TS), volatile solids (VS), and suspended solids (SS) were measured using standard methods. The COD was analyzed using a DR-1900 spectrophotometer (HACH, Loveland, CO). 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, Waltham, MA) was used to determine the metallic elements present. The biogas slurry fermentation system was centrifuged at 2,500 rpm for 5 min. The supernatant was used determining 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\(^\circ\)C. In order to eliminate any influence on CDW of impurities in the biogas slurry, the control group weight was subtracted from all CDW values. Kjeldahl nitrogen quantification was conducted on an Automatic Kjeldahl Apparatus (FOSS 2000). The Kjeldahl nitrogen value was multiplied by a conversion factor of 6.25 to obtain the SCPC content. The ammonia-nitrogen concentration was measured using Nessler’s reagent spectrophotometry (Zhu et al., 2019). The amount of reducing sugar used was determined by the 3 to 5 dinitrosalicylic acid method. The amino acid composition was measured by the Sci-Tech Innovation Company (China).

**Statistical Analysis**

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

**RESULTS AND DISCUSSION**

**Screening of Strains and pH Regulator**

As shown in Table 4, when HCl was used to adjust the pH of the culture system, the pH of all strains decreased rapidly from 7.30 to 2.33–3.35 after 2 d. After the next 2 d of culture, the pH and concentration of residual
Table 4. The pH, reducing sugar, CDW, and SCP of cultural system with HCl 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) |
|-------------------------|--------|--------|---------------------------------|---------------------------------|-----------|----------|----------|
| *Candida utilis*        | 2.65\(^b\) | 2.70\(^d\) | 0.32\(^a\)                     | 0.33\(^d\)                      | 3.90\(^d\) | 52.89\(^a\) | 2.06\(^e\) |
| *Saccharomyces cerevisiae* | 2.96\(^e\) | 2.67\(^d\) | 0.33\(^c\)                     | 0.35\(^d\)                      | 4.46\(^d\) | 43.49\(^d\) | 1.94\(^e\) |
| *Pichia manshurica*     | 3.23\(^b\) | 3.27\(^d\) | 0.29\(^c\)                     | 0.37\(^d\)                      | 4.77\(^d\) | 49.77\(^b\) | 2.37\(^b\) |
| *Candida tropicalis*    | 2.44\(^d\) | 2.26\(^d\) | 0.25\(^c\)                     | 0.27\(^d\)                      | 6.65\(^a\) | 45.96\(^d\) | 3.06\(^e\) |
| *Debaryomyces Hansenii* | 3.35\(^a\) | 2.88\(^a\) | 5.87\(^a\)                     | 5.30\(^a\)                      | 6.28\(^b\) | 30.31\(^a\) | 1.91\(^f\) |
| *Nectaromyces rattus*   | 2.80\(^b\) | 2.77\(^c\) | 7.56\(^b\)                     | 7.13\(^c\)                      | 5.93\(^b\) | 34.16\(^a\) | 2.05\(^b\) |
| *Wickerhamomyces anomalus* | 2.67\(^d\) | 2.58\(^d\) | 8.20\(^b\)                     | 0.31\(^d\)                      | 4.82\(^d\) | 45.65\(^d\) | 1.97\(^e\) |
| CK                      | 7.32\(^a\) | 7.35\(^a\) | 24.95\(^a\)                    | 0.80\(^e\)                      | 0.00\(^f\) | 0.00\(^f\) | 0.00\(^f\) |
| SEM                     | 0.012   | 0.006  | 0.064                           | 0.036                           | 0.052     | 0.241     | 0.021    |
| P                       | 0.000   | 0.000  | 0.000                           | 0.000                           | 0.000     | 0.000     | 0.000    |

Abbreviations: CDW, cell dry weight; CP, concentration of protein; SCPC, single cell protein content based on CDW; SEM, standard error of the mean. Means within the same column with different letters differ significantly from each other (P < 0.05).

Table 5. The pH, reducing sugar, CDW, and SCP 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) |
|-------------------------|--------|--------|---------------------------------|---------------------------------|-----------|----------|----------|
| *Candida utilis*        | 4.39\(^e\) | 4.24\(^d\) | 2.78\(^a\)                     | 2.61\(^d\)                      | 3.18\(^a\) | 53.33\(^a\) | 1.79\(^d\) |
| *Saccharomyces cerevisiae* | 5.46\(^b\) | 7.27\(^a\) | 4.85\(^a\)                     | 3.23\(^c\)                      | 3.77\(^d\) | 40.82\(^d\) | 1.54\(^f\) |
| *Pichia manshurica*     | 4.47\(^c\) | 4.77\(^b\) | 5.27\(^a\)                     | 2.33\(^d\)                      | 6.33\(^a\) | 43.09\(^d\) | 2.73\(^b\) |
| *Candida tropicalis*    | 6.52\(^a\) | 6.09\(^b\) | 2.73\(^c\)                     | 2.31\(^d\)                      | 6.59\(^a\) | 43.21\(^d\) | 2.85\(^b\) |
| *Debaryomyces Hansenii* | 5.68\(^a\) | 7.36\(^a\) | 10.44\(^a\)                    | 2.37\(^d\)                      | 8.30\(^a\) | 23.41\(^a\) | 1.94\(^f\) |
| *Nectaromyces rattus*   | 5.63\(^a\) | 6.61\(^b\) | 11.93\(^a\)                    | 5.00\(^b\)                      | 9.52\(^a\) | 33.88\(^d\) | 3.23\(^a\) |
| *Wickerhamomyces anomalus* | 4.88\(^b\) | 4.91\(^d\) | 2.96\(^c\)                     | 2.64\(^d\)                      | 4.14\(^d\) | 47.62\(^d\) | 1.97\(^b\) |
| CK                      | 6.04\(^b\) | 5.93\(^d\) | 24.43\(^a\)                    | 25.02\(^a\)                     | 0.67\(^e\) | 0.06\(^f\) | 0.00\(^f\) |
| SEM                     | 0.063   | 0.071  | 0.174                           | 0.057                           | 0.060     | 0.336     | 0.041    |
| P                       | 0.000   | 0.000  | 0.000                           | 0.000                           | 0.000     | 0.000     | 0.000    |

Abbreviations: CDW, cell dry weight; CP, concentration of protein; SCPC, single cell protein content based on CDW; SEM, standard error of the mean. Means within the same column with different letters differ significantly from each other (P < 0.05).

Reducing sugar did not change significantly. This indicated that the added bacteria did not consume the carbon source during d 2 to 4. The final CDW of each strain ranged from 3.90 g/L to 6.65 g/L, and the CDW contents for fermentation by *Candida tropicalis*, *Debaryomyces Hansenii*, and *Nectaromyces rattus* were 6.65 g/L, 6.28 g/L and 5.93 g/L, respectively.

As shown in Table 5, when acetic acid was used to adjust the pH of the culture system, the pH of all strains changed from 5.50 to 4.39–6.52 after 2 d of culture. After the next 2 d of culture, the pH of all strains changed to 4.27 to 7.36. The reducing sugar continued to be consumed in the next 2 d of culture by *S. cerevisiae*, *Pichia manshurica*, *Debaryomyces Hansenii*, and *Nectaromyces rattus*. The final CDW for *N. rattus* fermentation was 9.53 g/L, which was significantly higher than the values of 8.30 g/L and 6.59 g/L obtained for the *Debaryomyces Hansenii* and *C. tropicalis* strains, respectively. The content of residual reducing sugar and CP of strain *N. rattus* were 5.00 g/L and 3.23 g/L, respectively. The CDW, residual reducing sugar, and concentration of protein (CP) for *N. rattus* were significantly higher than those for other strains. This indicates that the strain *N. rattus* had the highest efficiency of carbon utilization for SCP production.

Environmental pH is closely related to yeast activity. It not only affects the charge of the cell membrane, but also changes the state of some compounds entering the cell, thus promoting or inhibiting cell growth (Boer et al., 2010). It is widely known that the pH range most suitable for yeast growth is 5.0 to 6.0. When a large amount of HCl was used to adjust the pH, all alkaline bicarbonate groups (HCO3\(^-\)) in the biogas slurry escaped in the form of CO2 and the NH4 HCO3 was changed to form an NH4Cl solution system. The culture system would soon become acidic with a pH of 2.0 to 3.0 because of the consumption of ammonia. This is not conducive to cell growth. When organic acetate was used as a pH regulator, the pH of the system increased from 5.5 to 7.36 for the strains *Candida tropicalis*, *Debaryomyces Hansenii*, *N. rattus*, and *Saccharomyces cerevisiae* due to the yeast’s consumption of acetic acid as a carbon source. For *Candida utilis*, *Pichia manshurica*, and *Wickerhamomyces anomalus*, the pH of the system decreased from 5.5 to the final 4.24 to 4.91. This could be due to acid metabolites being produced during cell growth.

**Selection of Initial C/N and Initial NH4\(^+\)-N Concentration**

Nitrogen sources are mainly used by yeast to synthesize various amino acids and bases in cells, and then to synthesize cell components such as proteins and nucleic acids (Nasseri et al., 2011). As shown in Figure 1, when the initial C/N ratio was 10:1 and the initial NH4\(^+\)-N concentration was 2000 mg/L, after 2 d
of culture, the concentrations of NH$_4^+$-N used by strains \textit{Debaryomyces hansenii}, \textit{Candida tropicalis}, and \textit{N. rattus} were 251 mg/L, 743 mg/L, and 284 mg/L, respectively. On d 2 to 4 of culture, the concentrations of NH$_4^+$-N used by strains \textit{Debaryomyces hansenii}, \textit{Candida tropicalis}, and \textit{N. rattus} was 675 mg/L, 242 mg/L, and 727 mg/L, respectively. Therefore, the strain \textit{Candida tropicalis} can quickly utilize ammonia nitrogen in the early stage of fermentation, and the strain \textit{N. rattus} can quickly use ammonia nitrogen in the later stages of fermentation. After subtracting the CDW in CK, the net CDW of \textit{N. rattus} was highest at 10.92 g/L at an ammonia-nitrogen concentration of 2,000 mg/L. The net CDWs of strains \textit{Debaryomyces hansenii} and \textit{Candida tropicalis} were only 7.40 and 7.89 g/L, respectively, after 4 d of culture. The residual reducing sugar content of \textit{N. rattus} was the highest among all at 5.66 g/L. The residual reducing sugar in strains \textit{Debaryomyces hansenii} and \textit{Candida tropicalis} were only 4.02 g/L and 5.08 g/L, respectively. When the concentration of ammonia nitrogen increased from 2,000 to 3,000 mg/L, or even 4,000 mg/L, the CDW of the three strains did not increase significantly.

As shown in Figure 2, when the initial C/N ratio was 6:1 and the initial NH$_4^+$-N concentration was 2,000 mg/L, after four days of culture, the concentrations of NH$_4^+$-N used by the strains \textit{Debaryomyces hansenii}, \textit{Candida tropicalis}, and \textit{N. rattus} were 926 mg/L, 985 mg/L, and 1,011 mg/L, respectively. After subtracting the CDW in CK, the net CDW obtained with \textit{N. rattus} was highest at 8.20 g/L. The net CDW for the strains \textit{Debaryomyces hansenii} and \textit{Candida tropicalis} were only 5.96 and 5.66 g/L, respectively, after four days of culture. The reducing sugar contents of \textit{Debaryomyces hansenii}, \textit{Candida tropicalis}, and \textit{N. rattus} were 4.12, 4.08, and 3.52 g/L, respectively. In summary, \textit{N. rattus} can produce the highest concentration of SCP by utilizing the nitrogen in the initial NH$_4^+$-N 2,000 mg/L biogas slurry.

By comparing the CDW and reducing sugar used by \textit{N. rattus} under the conditions of C/N = 10:1 and C/N = 6:1, with an initial NH$_4^+$-N concentration of 2,000 mg/L, it was found that when C/N = 10:1, 36.34 g of glucose was used to produce 10.92 g CDW. The corresponding biomass yield was 0.30 g CDW/g glucose. At C/N = 6:1, 17.48 g glucose was used to produce 8.20 g. The corresponding biomass yield was 0.47 g CDW/g glucose. It is possible that under a high C/N ratio, the yeast strains convert glucose into CO$_2$ through respiration instead of synthesizing cell biomass. Fendt and Sauer (2010) showed that glucose does not contribute much to the energy production of cells in the respiratory

Figure 1. CDW, final pH, residue concentrations of NH$_4^+$-N and residual reducing sugar of different strains under initial C/N = 10:1 and initial NH$_4^+$-N concentration of 1,000 mg/L (A) 2,000 mg/L (B), 3,000 mg/L (C), and 4,000 mg/L (D). Abbreviation: CDW, cell dry weight.
system. The strain producing the highest levels of CDW, *N. rattus* was selected for further optimization of fermentation conditions under initial conditions of C/N = 6:1 and NH₄⁺-N concentration of 2,000 mg/L.

**Rotation Speed**

Oxygen is crucial to the growth rate and metabolic pathway of yeast. With sufficient oxygen, the yeast grows rapidly, carries out aerobic respiration, and produces a large cell biomass. Under limited oxygen or no oxygen, yeast mainly carries out the fermentation process (Madeo et al., 1999). Increasing the rotation speed can effectively increase the dissolved oxygen in the liquid medium (Thongchul and Yang, 2006). As shown in Figure 3, when the speed increased from 120 to 200 rpm, the CDW of *N. rattus* gradually increases from 8.55 g/L to 10.08 g/L. This indicates that increased oxygen supply is conducive to the growth of *N. rattus*. This result is consistent with that of Ghaly and Kamal (2004), who showed that with an increase in dissolved oxygen concentration, the yeast number increases, but when the growth of yeast enters a decay period, the cell number decreases with time, and at this point an increase in dissolved oxygen does not lead to an increase in yeast number.

**Optimization of Inorganic Salts**

Inorganic salts are indispensable for microbial activity (Coimbra et al., 2021). Their main functions are to: constitute bacterial components, act as components of enzyme active groups, and maintain enzyme activity. For example, Ca²⁺, an extracellular enzyme stabilizer and protease cofactor, can participate in the formation of bacterial spores and fungal spores. Mg²⁺ is the active center component of many enzymes (hexose phosphorylase, isocitrate dehydrogenase, etc.) and can regulate osmotic pressure, pH, and redox potential. According to the results of metal elements optimization, as shown in Table 6, the order of influence of these five elements was as follows: Ca²⁺ > Cu²⁺ > Fe²⁺ > Zn²⁺ > Mn²⁺. The elements were added as follows: 10 mg/L of Ca²⁺, 0.1 mg/L of Mn²⁺, 0.03 mg/L of Fe²⁺, 0.05 mg/L of Zn²⁺, and 0.1 mg/L of Cu²⁺. The results of Iida et al. (1990) show that the addition of Ca²⁺ clearly influences the cell cycle of yeast and might regulate the
level of cyclic adenosine monophosphate. Gao et al. (2012) reported that when the soy molasses medium was supplemented with CaCl₂ (0.05 g/L), the total protein increased from 4.58 to 5.60 g/L, and CDW from 8.62 to 9.95 g/L. In contrast, the addition of ZnSO₄·7H₂O (0.05 g/L) had no notable effect. However, Ogejo et al. (2009) reported that excessive Ca²⁺ can lead to the precipitation of carbonate and phosphate, and lead to the fouling of reactors and bacterial cells.

### Secondary Fermentation

After the first fermentation, the NH₄⁺-N concentration decreased from the 2,000 mg/L to 625 mg/L. The residual concentration of reducing sugars was 3.52 g/L. A reason for these outcomes might be caused by a limitation to high cell density. To address this, the supernatant after centrifugation was fermented for a second time. As shown in Figure 4, the NH₄⁺-N concentration

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**Figure 3.** The growth of *Nectaromyces rattus* at different rotation speeds.

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

| Treatment group | Ca²⁺ | Mn²⁺ | Fe²⁺ | Zn²⁺ | Cu²⁺ | CDW (g/L) |
|-----------------|------|------|------|------|------|-----------|
| 1               | 1    | 1    | 1    | 1    | 1    | 9.24      |
| 2               | 1    | 2    | 2    | 2    | 2    | 10.42     |
| 3               | 1    | 3    | 3    | 3    | 3    | 10.36     |
| 4               | 1    | 4    | 4    | 4    | 4    | 9.13      |
| 5               | 2    | 1    | 2    | 3    | 4    | 9.39      |
| 6               | 2    | 2    | 1    | 4    | 3    | 9.75      |
| 7               | 2    | 3    | 4    | 1    | 2    | 9.99      |
| 8               | 2    | 4    | 3    | 2    | 1    | 10.06     |
| 9               | 3    | 1    | 3    | 4    | 2    | 9.14      |
| 10              | 3    | 2    | 4    | 3    | 1    | 8.4       |
| 11              | 3    | 3    | 1    | 2    | 4    | 8.49      |
| 12              | 3    | 4    | 2    | 1    | 3    | 8.99      |
| 13              | 4    | 1    | 4    | 2    | 3    | 9.57      |
| 14              | 4    | 2    | 3    | 1    | 4    | 9.83      |
| 15              | 4    | 3    | 2    | 4    | 1    | 9.18      |
| 16              | 4    | 4    | 1    | 3    | 2    | 9.89      |

Mean 1          | 9.787 | 9.335 | 9.343 | 9.512 | 9.220     
Mean 2          | 9.798 | 9.600 | 9.495 | 9.635 | 9.860     
Mean 3          | 8.755 | 9.505 | 9.848 | 9.510 | 9.668     
Mean 4          | 9.617 | 9.518 | 9.273 | 9.300 | 9.210     
Range           | 1.043 | 0.265 | 0.575 | 0.335 | 0.650   

Abbreviation: CDW, cell dry weight.
decreased from 625 mg/L to 398 mg/L, and residual reducing sugars decreased from 3.52 g/L to 0.02 g/L after secondary fermentation. After twice fermentation, the total CDW reached 12.58 g/L, with the second fermentation accounting for 23.05% of the total CDW. After the second fermentation, the culture entered a stage of carbon source limitation. The production of SCP depends not only on the type of carbon source, but also on the concentration of carbon source in culture (Spalvins et al., 2018). Zheng et al. (2013) reported conducting fed-batch cultivation to maintain the glucose concentration at a proper range for algal growth, and therefore to achieve a much higher cell density. In addition, the limitation of high cell density can be removed by carrying out multiple fermentation rounds to realize the full potential of ammonia nitrogen. Of course, other reasons such as dissolved oxygen limitation cannot be ruled out. Therefore, besides fed-batch cultivation, high-density inhibition problems need to be solved. The dissolved oxygen concentration should be increased in order to achieve high-density fermentation for SCP production.

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

| Amino acids            | Nectaromyces rattus | Blastobotrys adeninivorans |
|------------------------|---------------------|----------------------------|
| Essential amino acids  |                     |                            |
| Threonine              | 1.959               | 1.390                      |
| Valine                 | 2.263               | 1.550                      |
| Methionine             | 0.270               | 0.417                      |
| Isoleucine             | 1.796               | 1.100                      |
| Leucine                | 2.656               | 1.840                      |
| Phenylalanine          | 1.760               | 1.090                      |
| Lysine                 | 2.197               | 1.74                       |
| Nonessential amino acids |                   |                            |
| Aspartic acid          | 2.398               | 2.420                      |
| Threonine              | 1.959               | 1.390                      |
| Serine                 | 1.953               | 1.440                      |
| Asparagine             | 1.808               | /                          |
| Glutamic acid          | 0.954               | 4.080                      |
| Glutamine              | 0.365               | /                          |
| Glycine                | 1.868               | 1.280                      |
| Cysteine               | /                   | 0.275                      |
| Tyrosine               | 1.476               | 1.000                      |
| γ-aminobutyric acid    | 5.529               | /                          |
| Histidine              | 0.507               | 0.572                      |
| Arginine               | 0.723               | 1.360                      |
| Proline                | 12.004              | 1.440                      |
| Total                  | 46.848              | 22.719                     |

Abbreviation: SCP, single cell protein.

### Amino Acids Composition of SCP

The amino acid composition of SCP from *N. rattus* was determined and compared with the highest product yielding species (*Blastobotrys adeninivorans*) as reported by Ohlsson et al. (2019). The profiling in Table 7 indicates that the SCP from the strain *N. rattus* contains a variety of amino acids, whose concentrations exceed that from the strain *Blastobotrys adeninivorans*. Asparagine, γ-aminobutyric acid, and glutamine, which were not present in the SCP from *B. adeninivorans*, were evident in that from *N. rattus*. *N. rattus* SCP has a series of essential amino acids: threonine, valine, methionine, isoleucine, leucine, phenylalanine, and lysine. The content of essential amino acids in *N. rattus* SCP is 3.77% higher than that of *B. adeninivorans* and similar to that of *Saccharomyces cerevisiae* strain, CCMA 0137 (Pires et al., 2016). These amino acids are especially important for the maintenance and tissue protein hyperplasia of broilers (Sakomura et al., 2015). The proline
content of *N. rattus* SCP was relatively high compared to that of *Blastobotrephy adeninivorans*. Proline has complex roles in a variety of biological processes, including cell signaling, stress protection, and energy production (Christgen and Becker, 2017). Arginine is an important amino acid additive in fish feed. Sulfur-containing amino acids, including methionine and cystine, are essential for poultry and pigs (Lo and Moreau, 1986). However, cystine was not detected in any of the samples in the study, and the methionine content was exceptionally low. One possible reason for this is the lack of sulfur in the biogas slurry due to the fact that the sulfur was transformed into biogas in the form of H₂S during anaerobic digestion. In the future application, extra sulfur element is recommended to be added to biogas slurry.

**CONCLUSIONS**

This is the first time that *N. rattus* was used to assimilate ammonia nitrogen in chicken manure biogas slurry to synthesize SCP, which is a potential feed ingredient for aquaculture and other animal production industries. *N. rattus* can tolerate chicken manure biogas slurry with high ammonia-nitrogen content of 4,000 mg/L. Acetate was a better pH regulator than HCl. *N. rattus* could produce CDW of 12.58 g/L after twice fermentation under initial conditions of: NH₄⁺-N concentration of 2,000 mg/L, C/N ratio of 6:1, pH of 5.50, and a rotation speed of 200 rpm. The protein content of CDW was 35.96%. However, the product was deficient in sulfur-containing amino acids.

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**DISCLOSURES**

The authors declare that they have no competing interests.

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