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 NH_4^+-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*

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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

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the growth of *Nile tilapia*, enhance immunity and antioxidant 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 H_2 as an electron donor, CO_2 as a carbon source, and ammonia-nitrogen (NH_4^+-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*, D*ebaryomyces 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 NH_4^+-N concentration 4,560 mg/L.

Screening of Strains

After measuring the existing COD and NH_4^+-N concentration in the biogas slurry, it was diluted to NH_4^+-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℃ 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 compared. One is to add 500 uL of 1 mol/l HCl to the sterilized liquid, adjust the pH to 7.30, the other is to add 300 uL of acetic acid to the sterilized liquid and adjust the pH to 5.50. 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℃ 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 NH_4^+-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 NH_4^+-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 NH_4^+-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 NH_4^+-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℃ 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 Ca^{2+} decreased from 19.49 mg/L to 0.00 mg/L, the concentration of Cu^{2+} decreased from 0.62 mg/L to 0.39 mg/L, and the

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)	16440	Zn (mg/L)	0.15
NH_4^+-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

Table 2. The metal element contents after 4 d fermentation with initial NH_4^+-N 2,000 mg/L.

element	CK	*Nectaromyces rattus*	Element	CK	*Nectaromyces rattus*
As	0.1724	0.1720	Mg	13.22	12.93
Ca	19.49	0.00	Mn	0.1870	0.0190
Cd	0.0023	0.0031	Mo	0.1004	0.0701
Co	0.0383	0.0186	Na	480.0	456.8
Cr	0.0345	0.0192	Ni	0.1577	0.0963
Cu	0.6228	0.3893	Pb	0.1870	0.0190
Fe	0.0082	0.0053	Se	0.1037	0.1066
Hg	0.0402	0.0430	Si	26.29	24.99
K	1250	1122	Zn	0.0638	0.0047

Table 3. The orthogonal test factor level of metal elements in *Nectaromyces rattus* strain.

	Factors				
Level	Ca^{2+}(mg/L)	Mn^{2+}(mg/L)	Fe^{2+}(mg/L)	Zn^{2+}(mg/L)	Cu^{2+}(mg/L)
1	0	0	0	0	0
2	10	0.1	0.01	0.05	0.1
3	20	0.2	0.03	0.1	0.2
4	30	0.3	0.05	0.15	0.3

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℃, 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℃. 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^{e}	2.70^{d}	0.32^{e}	0.33^{d}	3.90^{d}	52.89^{a}	2.06^{c}
Saccharomyces cerevisiae	2.96^{c}	2.67^{d}	0.33^{e}	0.35^{d}	4.46^{c}	43.49^{d}	1.94^{c}
Pichia manshurica	2.33^{g}	2.37^{f}	6.63^{c}	6.37^{d}	4.77^{c}	49.77^{b}	2.37^{b}
Candida tropicalis	2.44^{f}	2.26^{g}	0.25^{e}	0.27^{d}	6.65^{a}	45.96^{c}	3.06^{a}
Debaryomyces hansenii	3.35^{b}	2.88^{b}	5.87^{d}	5.30^{c}	6.28^{ab}	30.34^{f}	1.91^{c}
Nectaromyces rattus	2.80^{d}	2.77^{c}	7.56^{b}	7.13^{b}	5.93^{b}	34.16^{e}	2.03^{c}
Wickerhamomyces anomalus	2.63^{e}	2.58^{e}	0.26^{e}	0.31^{d}	4.32^{cd}	45.65^{cd}	1.97^{c}
CK	7.32^{a}	7.35^{a}	24.95^{a}	24.54^{a}	0.80^{e}	0.00^{g}	0.00^{d}
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^{c}	4.24^{e}	2.78^{e}	2.61^{d}	3.18^{e}	53.33^{a}	1.70^{cd}
Saccharomyces cerevisiae	5.46^{b}	7.27^{a}	4.85^{d}	3.23^{c}	3.77^{d}	40.82^{c}	1.54^{d}
Pichia manshurica	4.47^{c}	4.77^{de}	5.27^{d}	2.33^{d}	6.33^{c}	43.09^{c}	2.73^{b}
Candida tropicalis	6.52^{a}	6.09^{bc}	2.73^{e}	2.31^{d}	6.59^{c}	43.21^{c}	2.85^{b}
Debaryomyces hansenii	5.68^{b}	7.36^{a}	10.44^{c}	2.37^{d}	8.30^{b}	23.41^{e}	1.94^{c}
Nectaromyces rattus	5.63^{b}	6.61^{b}	11.93^{b}	5.00^{b}	9.52^{a}	33.88^{d}	3.23^{a}
Wickerhamomyces anomalus	4.88^{c}	4.91^{d}	2.96^{e}	2.64^{d}	4.14^{d}	47.62^{b}	1.97^{c}
CK	6.04^{ab}	5.93^{c}	24.43^{a}	25.02^{a}	0.67^{f}	0.00^{f}	0.00^{e}
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 (HCO_3^-) in the biogas slurry escaped in the form of CO_2 and the NH_4 HCO_3 was changed to form an NH_4Cl 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 the final 6.09 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 NH_4^+-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 NH_4^+-N concentration was 2000 mg/L, after 2 d

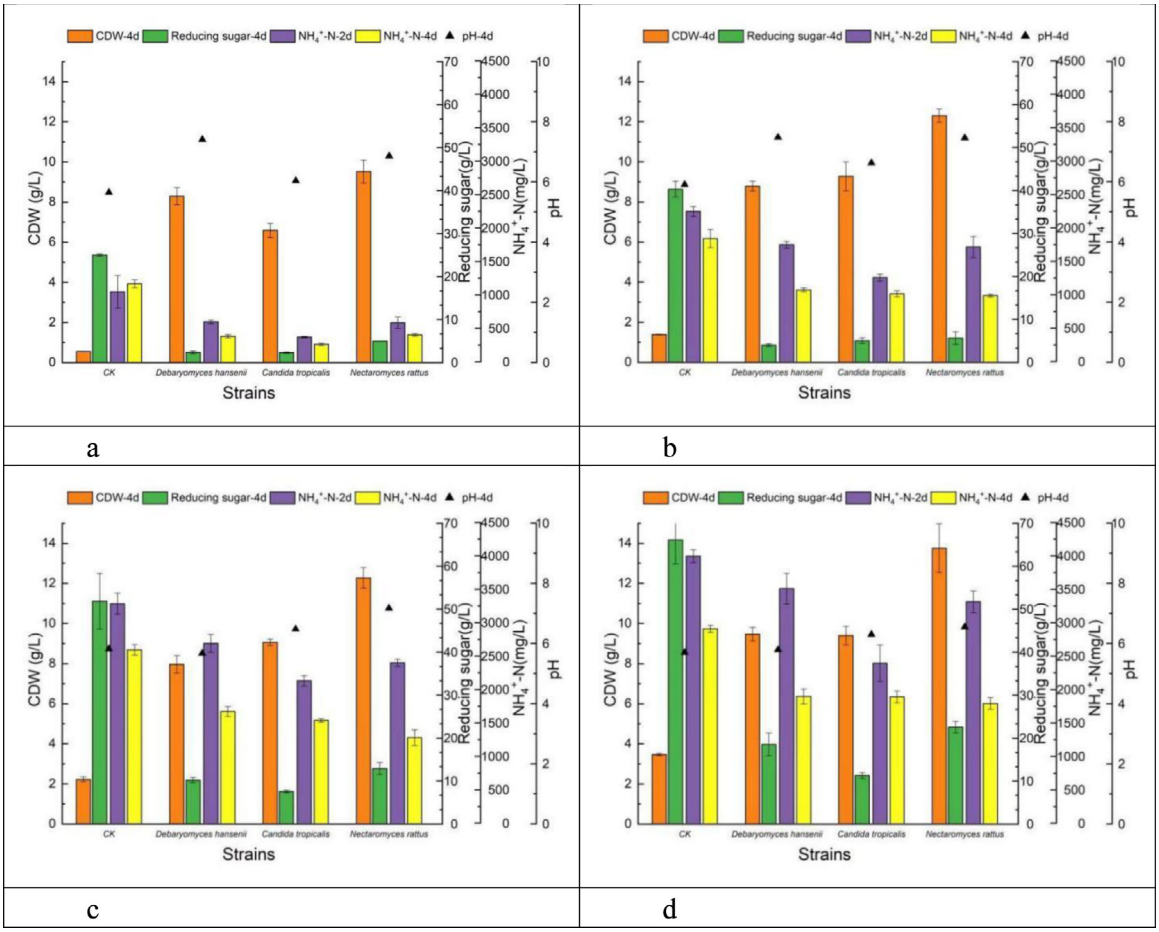

Figure 1. CDW, final pH, residue concentrations of NH_4^+-N and residual reducing sugar of different strains under initial C/N = 10:1 and initial NH_4^+-N concentration of 1,000 mg/L (A) 2,000 mg/L (B), 3,000 mg/L (C), and 4,000 mg/L (D). Abbreviation: CDW, cell dry weight.

of culture, the concentrations of NH_4^+-N used by strains *Debaryomyces hansenii, Candida tropicalis*, and *N. rattus* were 251 mg/L, 743 mg/L, and 284mg/L, respectively. On d 2 to 4 of culture, the concentrations of NH_4^+-N used by strains *Debaryomyces hansenii, Candida tropicalis*, and *N. rattus* was 675 mg/L, 242 mg/L, and 727 mg/L, respectively. Therefore, the strain *Candida tropicalis* can quickly utilize ammonia nitrogen in the early stage of fermentation, and the strain *N. rattus* can quickly use ammonia nitrogen in the later stages of fermentation. After subtracting the CDW in CK, the net CDW of *N. rattus* was highest 10.92 g/L at an ammonia-nitrogen concentration of 2,000 mg/L. The net CDWs of strains *Debaryomyces hansenii* and *Candida tropicalis* were only 7.40 and 7.89 g/L, respectively, after 4 d of culture. The residual reducing sugar content of *N. rattus* was the highest among all at 5.66 g/L. The residual reducing sugar in strains *Debaryomyces hansenii* and *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 *Debaryomyces hansenii, Candida tropicalis*, and *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 *N. rattus* was highest at 8.20 g/L. The net CDW for the strains *Debaryomyces hansenii* and *Candida tropicalis* were only 5.96 and 5.66 g/L, respectively, after four days of culture. The reducing sugar contents of *Debaryomyces hansenii, Candida tropicalis,* and *N. rattus* were 4.12, 4.08, and 3.52 g/L, respectively. In summary, *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 *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

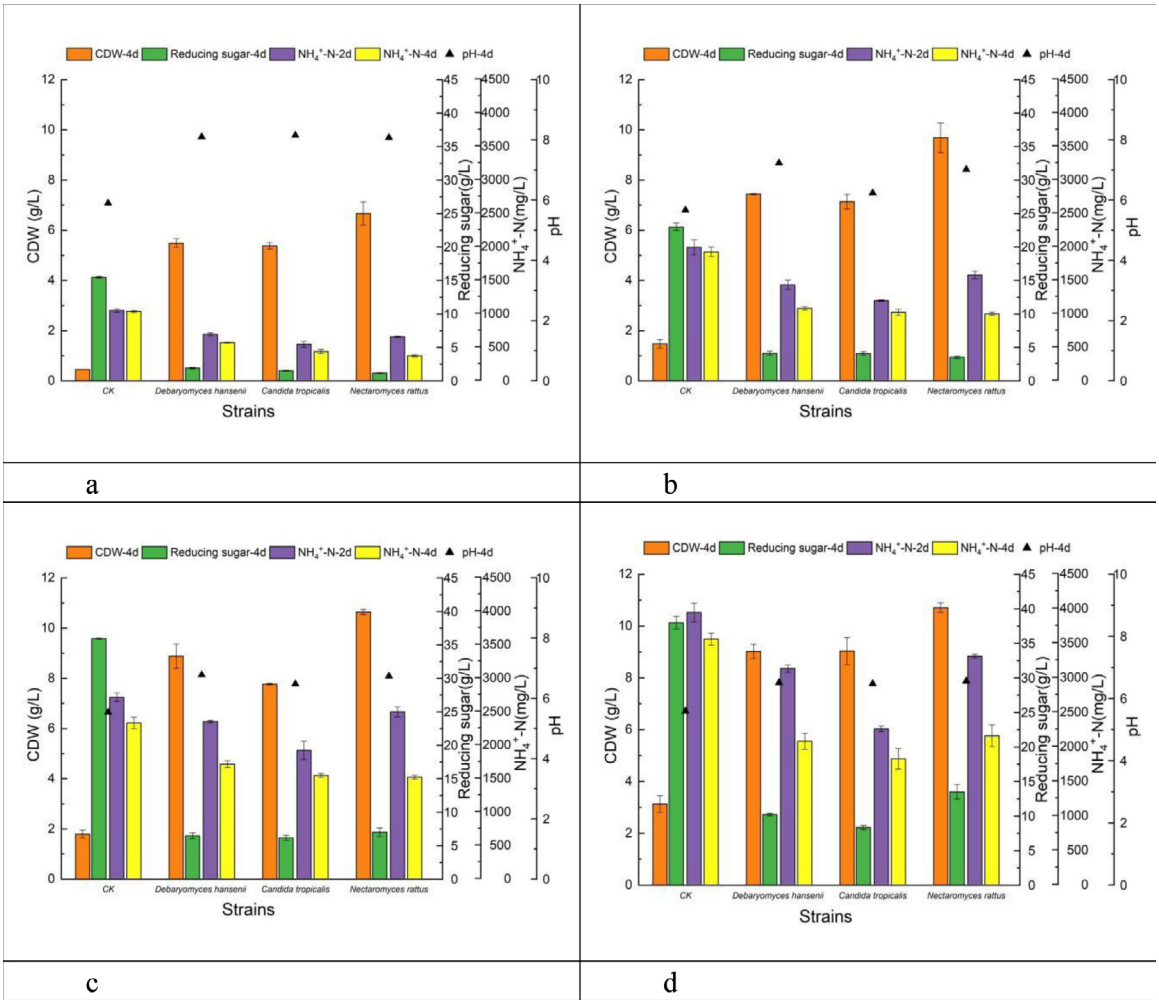

Figure 2. CDW, final pH, residue concentrations of NH4+-N and residual reducing sugar of different strains under initial C/N = 6:1 and initial NH_4^+-N concentration of 1,000 mg/L (A), 2,000 mg/L, (B), 3,000 mg/L (C), and 4,000 mg/L (D). Abbreviation: CDW, cell dry weight.

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 NH_4^+-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, Ca^{2+}, an extracellular enzyme stabilizer and protease cofactor, can participate in the formation of bacterial spores and fungal spores. Mg^{2+} 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: Ca^{2+}>Cu^{2+}>Fe^{2+}>Zn^{2+}>Mn^{2+}. The elements were added as follows: 10 mg/L of Ca^{2+}, 0.1 mg/L of Mn^{2+}, 0.03 mg/L of Fe^{2+}, 0.05 mg/L of Zn^{2+}, and 0.1 mg/L of Cu^{2+} 0.1 mg/L. The results of Iida et al. (1990) show that the addition of Ca^{2+} clearly influences the cell cycle of yeast and might regulate the

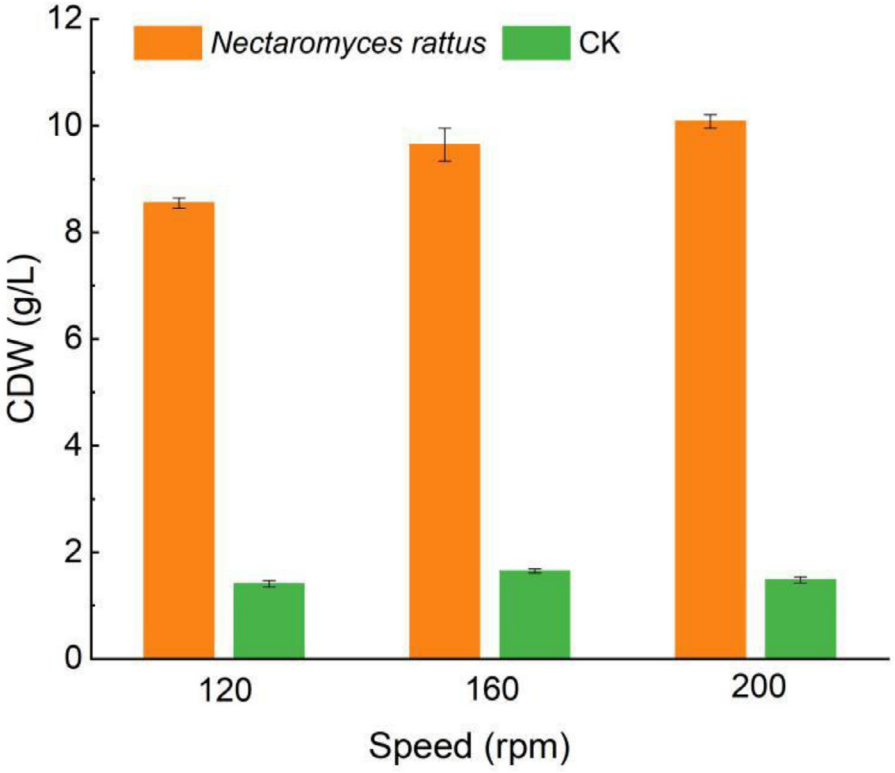

Figure 3. The growth of *Nectaromyces rattus* at different rotation speeds.

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

	Factors					
Treatment group	Ca^{2+}	Mn^{2+}	Fe^{2+}	Zn^{2+}	Cu^{2+}	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.

level of cyclic adenosine monophosphate. Gao et al. (2012) reported that when the soy molasses medium was supplemented with $CaCl_2$ (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 $ZnSO_4 \bullet 7H_2O$ (0.05 g/L) had no notable effect. However, Ogejo et al. (2009) reported that excessive Ca^{2+}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 NH_4^+-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 NH_4^+-N concentration

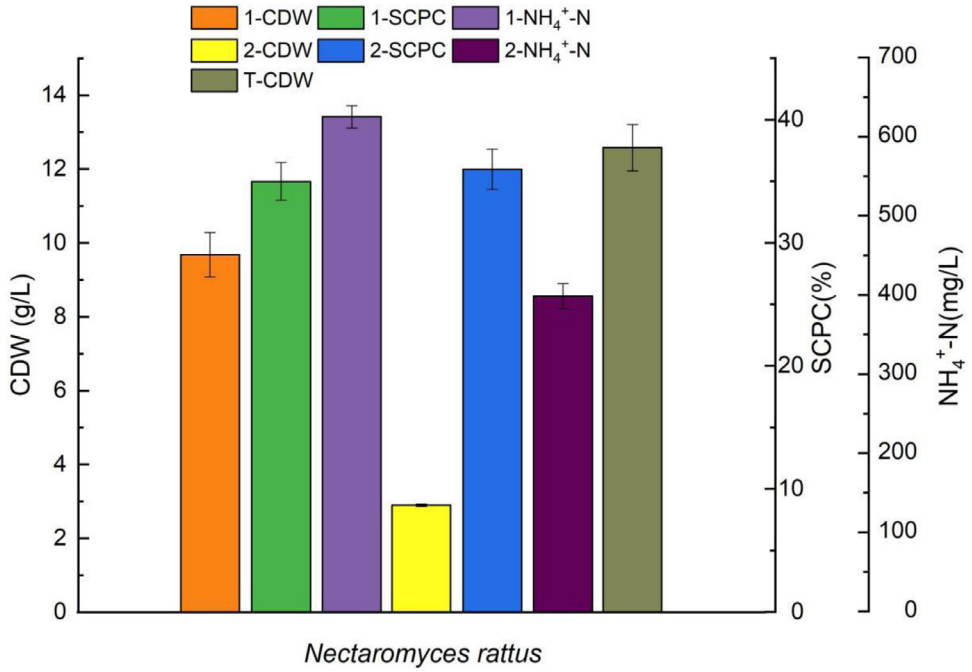

Figure 4. Residual NH_4^+-N, CDW and SCPC after twice fermentation. 1 represent first fermentation, 2 represent secondary fermentation, T-CDW represent total cell dry weight. Abbreviations: CDW, cell dry weight; SCPC, single cell protein content based on CDW.

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 *Blastobotrys 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 H_2S 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: NH_4^+-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.

REFERENCES

Al-Mudhafr, A. W. 2019. Microbiological sources and nutritional value of single cell protein (SCP). Int. J. Res. Appl. Sci. Biotechnol. 6:1–3.

Alugongo, G. M., J. X. Xiao, Y. H. Chung, S. Z. Dong, S. L. Li, I. Yoon, Z. H. Wu, and Z. J. Cao. 2017. Effects of saccharomyces cerevisiae fermentation products on dairy calves: performance and health. J. Dairy Sci 100:1189–1199.

Bhatt, R. S, and A. Sahoo. 2019. Effect of adding formaldehyde treated protein alone and with Saccharomyces cerevisiae in diet on plane of nutrition, growth performance, rumen fermentation and microbial protein synthesis of nisher lambs. Small Ruminant Res 171:42–48.

Boer, V. M., C. A. Crutchfield, P. H. Bradley, D. Botstein, and J. D. Rabinowitz. 2010. Growth-limiting intracellular metabolites in yeast growing under diverse nutrient limitations. Mol. Biol. Cell 21:198–211.

Chen, X. Q., W. Zhao, S. W. Xie, J. J. Xie, Z. H. Zhang, L. X. Tian, Y. J. Liu, and J. L. Niu. 2019. Effects of dietary hydrolyzed yeast (Rhodotorula mucilaginosa) on growth performance, immune response, antioxidant capacity and histomorphology of juvenile Nile tilapia (Oreochromis niloticus). Fish Shellfish Immun. 90:30–39.

Christgen, S. L., and D. F. Becker. 2017. Role of proline in pathogen and host interactions. Antioxid. Redox Sign 30:683–709.

Coimbra, J. M., K. C. dos Reis, R. F. Schwan, and C. F. Silva. 2021. Effect of the strategy of molasses supplementation in vinasse to high SCP production and rose flavor compound. Waste Biomass. Valori 12:359–369.

Cruz, A., I. M. Hkensen, A. Skugor, L. Mydland, and M. Verland. 2019. Candida utilis yeast as a protein source for weaned piglets: effects on growth performance and digestive function. Livestock Sci. 226:31–39.

Dou, J., Y. Huang, H. Ren, Z. Li, Q. Cao, X. Liu, and D. Li. 2019. Autotrophic, heterotrophic, and mixotrophic nitrogen assimilation for single-cell protein production by two hydrogen-oxidizing bacterial strains. Appl. Biochem. Biotech. 187:338–351.

Fam, R. R., K. C. Hiong, C. Y. Choo, W. P. Wong, S. F. Chew, and Y. K. Ip. 2018. Molecular characterization of a novel algal glutamine synthetase (GS) and an algal glutamate synthase (GOGAT) from the colorful outer mantle of the giant clam, Tridacna squamosa, and the putative GS-GOGAT cycle in its symbiotic zooxanthellae. Gene 656:40–52.

Fendt, S. M., and U. Sauer. 2010. Transcriptional regulation of respiration in yeast metabolizing differently repressive carbon substrates. BMC Syst. Biol. 4:12.

Gao, Y., D. Li, and Y. Liu. 2012. Production of single cell protein from soy molasses using Candida tropicalis. Ann. Microbiol. 62:1165–1172.

Ghaly, A. E., and M. A. Kamal. 2004. Submerged yeast fermentation of acid cheese whey for protein production and pollution potential reduction. Water Res. 38:631–644.

Hülsen, T., K. Hsieh, Y. Lu, S. Tait, and D. J. Batstone. 2018. Simultaneous treatment and single cell protein production from agri-industrial wastewaters using purple phototrophic bacteria or microalgae—a comparison. Bioresour. Technol. 254:214–223.

Iida, H., S. Sakagachi, Y. Yagawa, and Y. Anraku. 1990. Cell cycle control by Ca2+ in Saccharomyces cerevisiae. J. Biol. Chem. 265:21216–21222.

Jéssica, F. S., V. C. Eliana, A. M. Ernesto, R. C. L. Souza, and E. A. Rodrigues. 2019. Treatment of sugarcane vinasse from cachaça production for the obtainment of Candida utilis CCT 3469 biomass. Biochem. Eng. J. 148:131–137.

Kand, D., I. B. Raharjo, J. Castro-Montoya, and U. Dickhoefer. 2018. The effects of rumen nitrogen balance on in vitro rumen fermentation and microbial protein synthesis vary with dietary carbohydrate and nitrogen sources. Anim. Feed Sci. Tech. 241:184–197.

Kim, J. D., Y. Hyun, K. S. Sohn, T. J. Kim, H. Woo, and I. K. Han. 2000. Effects of mannanoligosaccharide and protein levels on growth performance and immune status in pigs weaned at 21 days of age. Korean J. Anim. Sci 42:489–498.

Lo, S. N., and J. R. Moreau. 1986. Mixed-culture microbial protein from waste sulfite pulping liquor II: its production on pilot-plant scale and use in animal feed. Can. J. Chem. Eng 64:639–646.

Madeo, F., E. Fröhlich, M. Ligr, M. Grey, S. J. Sigrist, D. H. Wolf, and K. U. Fröhlich. 1999. Oxygen stress: a regulator of apoptosis in yeast. J. Cell Biol 145:757–767.

Nasseri, A. T., S. Rasoul-Amini, M. H. Morowvat, and Y. Ghasemi. 2011. Single cell protein: production and process. Am. J. Food Technol. 6:103–116.

Ogejo, J. A., Z. Wen, J. Ignosh, E. Bendfeldt, and E. R. Collins. 2009. Biomethane Technology. Virginia Cooperative Extension Publication, Blacksburg, VA.

Ohlsson, J. A., M. Olstorpe, V. Passoth, and L. L. Su-lin. 2019. Yeast single cell protein production from a biogas co-digestion substrate. BioRxiv 766345.

Pires, J. F., G. M. Ferreira, K. C. Reis, R. F. Schwan, and C. F. Silva. 2016. Mixed yeasts inocula for simultaneous production of SCP and treatment of vinasse to reduce soil and freshwater pollution. J. Eviron. Manag 182:455–463.

Sakomura, N. K., R. D. Ekmay, S. J. Mei, and C. N. Coon. 2015. Lysine, methionine, phenylalanine, arginine, valine, isoleucine, leucine, and threonine maintenance requirements of broiler breeders. Poult. Sci. 94:2715–2721.

Sanchez, J. B., J. M. Quiroga, and M. D. Coello. 2006. Use of microbial activity parameters for determination of a biosolid stability index. Bioresour. Technol 97:562–568.

Somda, M. K., M. Nikiema, I. Keita, I. Mogmenga, S. H. S. Kouhounde, and Y. Dabire. 2018. Production of single cell protein (scp) and essentials amino acids from candida utilis fmj12 by solid state fermentation using mango waste supplemented with nitrogen sources. Afr. J. Biotechnol. 17:1.

Spalvins, K., K. Ivanovs, and *Blumberga.. Single cell protein production from waste biomass: review of various agricultural by-products*. Agron. Res 16(Suppl. 2):1493–1508.

Thongchul, N., and S. T. Yang. 2006. Controlling biofilm growth and lactic acid production by Rhizopus oryzae in a rotating fibrous bed bioreactor: effects of dissolved oxygen, rotational speed, and urea concentration. J. Chin. Inst. Eng 37:49–61.

Umesh, M., K. Priyanka, B. Thazeem, and K. Preethi. 2017. Production of single cell protein and polyhydroxyalkanoate from Carica papaya waste. Arab. J. Sci. Eng 42:2361–2369.

Woertz, I., A. Feffer, T. Lundquist, and Y. Nelson. 2009. Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock. J. Environ. Eng 135:1115–1122.

Yang, A., G. Zhang, F. Meng, P. Lu, X. Wang, and M. Peng. 2017. Enhancing protein to extremely high content in photosynthetic bacteria during biogas slurry treatment. Bioresour. Technol 245:1277–1281.

Zheng, Y., T. Li, X. Yu, P. D. Bates, T. Dong, and S. Chen. 2013. High-density fed-batch culture of a thermotolerant microalga chlorella sorokiniana for biofuel production. Appl. Energy 108:281–287.

Zhu, X., Q. Cao, Y. Chen, X. Sun, X. Liu, and D. Li. 2019. Effects of mixing and sodium formate on thermophilic in-situ biogas upgrading by H2 addition. J. Clean Prod. 216:373–381.