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Autotrophic, Heterotrophic, and Mixotrophic Nitrogen Assimilation for Single-Cell Protein Production by Two Hydrogen-Oxidizing Bacterial Strains

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Abstract To recover a nitrogen resource from high-ammonia-nitrogen wastewater, two amphitrophic hydrogen-oxidizing bacteria (HOB), *Paracoccus denitrificans* Y5 and *P. versutus* D6, capable of nitrogen assimilation for single-cell protein (SCP) production were isolated. These two HOB strains could grow autotrophically with H_2 as an electron donor, O_2 as an electron acceptor, CO_2 as a carbon source, and ammonia nitrogen (NH_4^+-N) as a nitrogen source. The cell molecular formulas of strains Y5 and D6 determined by autotrophic cultivation were $C_{3.33}H_{6.83}O_{2.58}N_{0.77}$ and $C_{2.87}H_{5.34}O_{3.17}N_{0.57}$, respectively. The isolated strains could synchronously remove NH_4^+-N and organic carbon and produce SCP via heterotrophic cultivation. The rates of removal of NH_4^+-N and soluble chemical oxygen demand reached 35.47 and 49.04%, respectively, for Y5 under mixotrophic cultivation conditions with biogas slurry as a substrate. SCP content of strains Y5 and D6 was 67.34–73.73% based on cell dry weight. Compared with soybean meal, the SCP of Y5 contained a variety of amino acids.

Keywords High-ammonia-nitrogen wastewater · Hydrogen-oxidizing bacterium · Nitrogen assimilation · Single-cell protein · Mixotrophy · *Paracoccus*

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Introduction

Nitrogen pollution has become an important environmental problem [1]. High-ammonia-nitrogen wastewater (HAW) is mainly derived from breeding, chemical fertilizers, meat-processing, the petrochemical industry, coking wastewater, and municipal sewage. It is difficult to treat such wastewater owing to the wide range of ammonia nitrogen (NH_4^+-N) concentrations and high chemical oxygen demand (COD). The major treatments of HAW are based on physicochemical and biological technologies. Biological removal of nitrogen has been widely adopted due to its effectiveness and low cost [2]. Biological processes of nitrogen removal include traditional nitrification–denitrification and the emerging technologies of simultaneous nitrification–denitrification [3], short nitrification–gentrification [4], and anaerobic ammonium oxidation [5]. Nonetheless, all these biological processes convert ammonia into nitrogen gas. Nitrogen is a macronutrient used by living organisms for growth and survival [3]. HAW contains organic carbon and micronutrient resources. Theoretically, these nitrogen and carbon resources can be utilized by microorganisms to produce protein.

Single-cell protein (SCP), i.e., dried cells of microorganisms, is a high-value-added product that can serve as a protein supplement in animal feed [6]. For cell growth, hydrogen-oxidizing bacteria (HOB) can heterotrophically utilize NH_4^+-N and organic carbon and/or autotrophically capture NH_4^+-N and CO_2 using H_2 as an electron donor and O_2 as an electron acceptor [7]. There are some advantages of HOB use. First, compared with fungi, algae, and yeast, HOB have high protein content per cell dry weight (CDW) [8, 9]. Second, HOB have a flexible lifestyle: they can alternatively or concomitantly grow in heterotrophic and autotrophic modes [10]. Third, although algae can grow heterotrophically and autotrophically, the growth of HOB is often not limited by light. Fourth, the other cell material of HOB, polyhydroxybutyrate, can serve as a prebiotic [11]. SCP with a prebiotic has greater nutritional value than other microbial proteins. Advantages of the production of SCP from HAW by HOB include the offset running cost of wastewater treatment; lower dependence of SCP production on land, with relieved pressure on agriculture [12]; and capacity for CO_2 fixation, with a reduction in the greenhouse effect.

So far, only a few HOB genera have been reported to produce SCP. These include *Hydrogenomonas* [13], *Alcaligenes* [14], *Aquaspirillum* [15], and *Pseudomonas* [11]. In these bacteria, SCP production is carried out in an autotrophic environment with CO_2 as the carbon source. No report has described both autotrophic and heterotrophic nitrogen assimilation for SCP production and wastewater treatment by HOB. The aims of this study were to (1) isolate HOB capable of utilizing NH_4^+-N, (2) investigate the ability of the isolates to produce SCP under autotrophic and heterotrophic conditions, and (3) to evaluate the feasibility of SCP production from biogas slurry via mixotrophic cultivation of HOB.

Materials and Methods

Isolation and Identification of HOB

Bacteria and the Culture Medium

Soybean rhizosphere soil, pond water, and biogas slurry of chicken manure (BSCM) were used for the enrichment of HOB. The modified mineral medium (MM) was prepared for HOB

enrichment and isolation. MM was composed of the following (in g/L): 4.0 NH_4Cl, 1 KH_2PO_4, 2 K_2HPO_4, 2 NaCl, 0.01 $CaCl_2$, 0.2 $MgSO_4{\cdot}7H_2O$, 0.01 $FeSO_4{\cdot}7H_2O$, 10 mL of a trace element solution, and 10 mL of a vitamin solution. The trace element solution consisted of (in mg/L) 200 $ZnSO_4{\cdot}7H_2O$, 20.0 $Na_2MoO_4{\cdot}2H_2O$, 1000 $MnSO_4{\cdot}H_2O$, 20.0 $CuCl_2{\cdot}2H_2O$, 200 $CoCl_2{\cdot}6H_2O$, 20.0 $NiCl{\cdot}6H_2O$, 20.0 Na_2SeO_4, and 20.0 Na_2WO_4. The vitamin solution was composed of (in mg/L) 2.0 biotin, 5.0 riboflavin, 10.0 pyridoxine, 2.0 folic acid, 5.0 thiamin, 5.0 nicotinic acid, 5.0 calcium pantothenate, 0.1 cyanocobalamine, 5.0 aminobenzoic acid, and 5.0 thioctic acid. MM was sterilized at 121 °C for 15 min. Solutions of NH_4HCO_3, NH_4OH, and vitamins were sterilized by membrane filtration (0.22-μm pore size) and were added to the medium. Initial pH of the medium was adjusted to 7.0 ± 0.2 with 2-mol/L NaOH and 2-mol/L HCl solutions. Three ammonium salts (NH_4Cl 4.0 g/L, NH_4HCO_3 5.9 g/L, and NH_4OH 2.6 g/L) with the same NH_4^+-N concentration (1046 mg/L) were employed to evaluate the effect of an anion on CDW and SCP production. The characteristics of BSCM are listed in Table 1.

Alcaligenes latus DSM 1122, *Herbaspirillum autotrophicum* DSM 732, *Pseudomonas carboxydohydrogena* DSM 1083, and *Cupriavidus necator* DSM 531, which are all known HOB [14, 16], served as control strains to evaluate the capacity for SCP production. These four strains were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Enrichment, Isolation, and Identification of HOB

Serum bottles and tubes were used for enrichment and isolation. Approximately 5 g of soybean rhizosphere soil, pond water, or BSCM was added separately to 500-mL serum bottles with 200 mL of MM. A gas mixture (H_2:O_2:CO_2 at 7:2:1, *v*/*v*) was injected into each serum bottle

Table 1 Characteristics of BSCM

Parameters	Value	Parameters	Value	Parameters	Value
pH	8.15	Ni (mg/kg)	5.32	Vitamin B6 (μg/100 g)	/
Total reducing sugar (mg/L)	1200	Cu (mg/kg)	4.47	Vitamin B_{11} (μg/L)	/
Volatile fatty acids[a] (mg/L)	153	Mn (mg/kg)	11.84	Ser (μg/100 mL)	< 5.0
Total N (mg/L)	5500	Si (mg/kg)	15.82	Asn (μg/100 mL)	< 5.0
Total K (mg/L)	2400	Fe (mg/kg)	65.22	Glu (μg/100 mL)	265.1
Total P (mg/L)	4310	Co (mg/kg)	0.74	Gln (μg/100 mL)	< 5.0
NH_4^+-N (mg/L)	5271	Na (mg/kg)	530.80	Gly (μg/100 mL)	9.14
NO_3^--N (mg/L)	104	Mg (mg/kg)	25.18	Ala (μg/100 mL)	5.92
COD (mg/L)	20,700	Zn (mg/kg)	48.77	Trp (μg/100 mL)	< 10.0
TS (g/kg)	21.10	Lys (μg/100 mL)	23.77	Val (μg/100 mL)	5.73
VS (g/kg)	10.90	His (μg/100 mL)	< 5.0	Cys (μg/100 mL)	< 5.0
SS (g/kg)	11.30	Arg (μg/100 mL)	< 5.0	Met (μg/100 mL)	< 5.0
VSS (g/kg)	7.70	Pro (μg/100 mL)	53.30	Ile (μg/100 mL)	< 5.0
Electrical conductivity (μS/cm)	42,680	Asp (μg/100 mL)	106.40	Leu (μg/100 mL)	< 5.0
Total coli-forms (MPN/100 mL)	7.9×10^5	Thr (μg/100 mL)	< 5.0	Tyr (μg/100 mL)	< 5.0
Total colony count (CFU/mL)	3.6×10^5	Vitamin B_1 (mg/100 g)	/[b]	Phe (μg/100 mL)	65.79
Humus acid (g/kg)	10.60	Vitamin B_2 (mg/100 g)	/	Total amino acid (μg/100 mL)	535.15

[a] Determined based on acetic acid

[b] The values below the determination limit

[17]. The replacement-and-exhaust method was adopted for the gaseous $H_2/O_2/CO_2$ atmosphere [18]. The gas replacement-and-injection equipment is illustrated in Fig. 1. Enrichment was conducted as described by Bae et al. [19]. Isolation of HOB was carried out by means of serum tubes supplied with the same gas mixture. Determination of the optimal growth temperature and pH of the isolated strains involved the single-variable methodology (30 °C and 7.0).

The genomic DNA of each bacterial isolate was extracted using the Sangon Biotech Bacteria DNA Kit (Shanghai, China). The DNA fragment containing the 16S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction with universal primers as previously described [20]. The amplicons were sequenced and analyzed by Sangon Biotech. Sequence similarity searches were performed in the GenBank data library via the BLAST program.

SCP Production by HOB

For SCP production, the autotrophic cultivation of the isolated and purchased strains was performed in 250-mL serum bottles using MM and the gas mixture. The 2% (*v*/*v*) inocula were aseptically injected into MM with a sterile syringe. The pressure was maintained at ~ 0.06 MPa. The bottles were shaken at 30 °C and 180 rpm, and the gas consumption was monitored by a pressure gauge every day. When the gauge pressure decreased to zero, the gas mixture was injected again. The CDW and SCP content were measured after gas was no longer consumed. All the experiments were conducted in triplicate.

The heterotrophic cultivation of HOB was performed in a 250-mL triangular flask containing MM and glucose with a breathable plug. Glucose was added to a final concentration of 38.9 g/L to adjust the C/N ratio to a reasonable level (i.e., 15) for microbial growth. The medium was autoclaved at 115 °C for 30 min and inoculated with a 2% HOB seed solution. Cultivation was carried out at 30 °C and 180 rpm. Glucose concentration was measured every day. When the glucose was consumed, CDW and SCP contents were determined. All the experiments were carried out in triplicate.

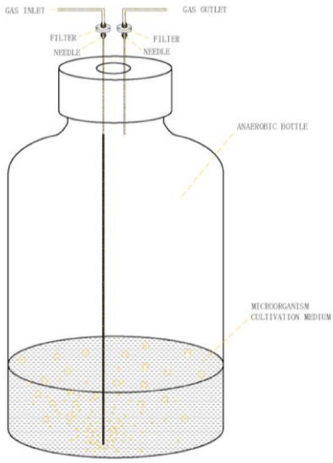
Mixotrophic cultivation was conducted in 500-mL serum bottles using BSCM and the gas mixture. The BSCM was diluted fivefold, and pH was adjusted to 7.0 ± 0.2. The bottles were shaken at 30 °C and 180 rpm. The procedure of pressure monitoring and gas charging was the same as that used for autotrophic cultivation. Soluble chemical oxygen demand (SCOD) and NH_4-N concentration were measured every 4 days. The CDW and SCP contents were determined after there was no gas consumption. All the experiments were conducted in triplicate.

Analytical Methods

Characteristics of original BSCM were analyzed. Total solids (TS), volatile solids (VS), suspended solids (SS), and volatile suspended solids were determined by standard techniques [21]. Total N, total P, total K, NH_4^+-N, NO_3^--N, COD, and SCOD were analyzed on a DR-1900 spectrophotometer (HACH, USA). Electrical conductivity was determined using a CHA-C258 conductivity meter (Shenzhen Ruihao, China). pH was determined with a pHS-3C pH meter (Shanghai Precision & Scientific Instrument Co., Ltd., China). Humus acid was quantified based on the Chinese Standard (HGT3276-1999). Total coliform counts and total colony counts and determination of metallic elements and amino acids followed the National Institute of Measurement and Testing Technology protocols.

The broth of HOB cultures was centrifuged at 10,000 rpm for 3 min for the determination of CDW and SCP content. CDW was measured after water was evaporated at 105 °C for 12 h.

(a)



(b)

(c)

Fig. 1 Experimental setup. A serum bottle for autotrophic and mixotrophic cultivation (**a**). Gas replacement for autotrophic and mixotrophic cultivation (**b**). Triangulation for heterotrophic cultivation (**c**)

Kjeldahl nitrogen quantification was conducted on an Automatic Kjeldahl Apparatus (FOSS 2000). SCP was determined by multiplying the Kjeldahl nitrogen value by a conversion factor of 6.25. Analyses of C, H, N, and S involved a Vario EL element analyzer (Elementar Analysensysteme GmbH, Germany). Glucose consumption was measured by means of an SBA-40E biosensor. The gas mixture ($H_2/O_2/CO_2$) was analyzed on an Agilent 6890N Gas Chromatograph with a thermal conductivity detector and a 2-m stainless-steel column packed with Porapak Q (50/80 mesh) (Agilent Technologies, USA). The amino acid composition was analyzed by Sci-Tech Innovation (China).

Calculations

The average fixation rates of NH_4^+-N by strains Y5 and D6 under autotrophic, heterotrophic, and mixotrophic conditions were calculated as

$$\text{average } NH_4^+\text{-N fixation rate } (\text{mg}/[\text{L} \times \text{day}]) = \frac{NH_4^+\text{-N}_i(\text{mg/L}) - NH_4^+\text{-N}_r(\text{mg/L})}{t\,(d)} \quad (1)$$

where NH_4^+-N_i indicates the initial NH_4^+-N concentration in a medium, NH_4^+-N_r indicates the residual NH_4^+-N in the culture supernatant, and t is the cultivation time.

The NH_4^+-N conversion and utilization efficiency by Y5 and D6 were calculated as

$$NH_4^+\text{-N conversion efficiency } (\%) = \frac{NH_4^+\text{-N}_i(\text{mg/L}) - NH_4^+\text{-N}_r(\text{mg/L})}{NH_4^+\text{-N}_i(\text{mg/L})} \quad (2)$$

$$NH_4^+\text{-N utilization efficiency } (\%) = \frac{\text{CDW-N (mg/L)}}{NH_4^+\text{-N}_i(\text{mg/L}) - NH_4^+\text{-N}_r(\text{mg/L})} \quad (3)$$

In Eq. (3), CDW-N was determined according to the Kjeldahl method.

The CDW yield from the different gases and NH_4^+-N in the autotrophic condition was calculated as

$$Y_{\text{CDW/gas}}\left(\frac{\text{gCDW}}{\text{g gas}}\right) = \frac{\text{CDW(g/L)} \times \text{Liquid volume(L)}}{\text{Gas uptake(g)}} \quad (4)$$

$$Y_{\text{CDW/NH4+-N}}\left(\frac{\text{gCDW}}{\text{g } NH_4^+\text{-N}}\right) = \frac{\text{CDW(g/L)} \times \text{Liquid volume(L)}}{NH_4^+\text{-N}_i(\text{g/L}) - NH_4^+\text{-N}_r(\text{g/L})} \quad (5)$$

The carbon and nitrogen balance in the autotrophic condition was calculated as

$$Y_C\left(\frac{\text{gCell-C}}{\text{g}CO_2\text{-C}}\right) = \frac{\text{CDW(g/L)} \times C_{\text{cell}}(\%) \times \text{Liquid volume(L)}}{CO_2\text{uptake(mol)} \times 12(\text{gC/mol})} \quad (6)$$

$$Y_N\left(\frac{\text{gCell-N}}{\text{g}NH_4^+\text{-N}}\right) = \frac{\text{CDW(g/L)} \times N_{\text{cell}}(\%)}{NH_4^+\text{-N}_i(\text{g/L}) - NH_4^+\text{-N}_r(\text{g/L})} \quad (7)$$

In Eqs. (6) and (7), C_{cell} and N_{cell} indicate the cell carbon and nitrogen content determined by elemental analysis.

Results and Discussion

Isolation, Identification, and Optimization

Two strains capable of growth on CO_2 and NH_4Cl as the sole inorganic carbon and nitrogen source, respectively, were isolated. According to their 16S rRNA gene sequences, these strains were identified as *Paracoccus denitrificans* Y5 and *P. versutus* D6, respectively. The effects of temperature and pH on cell growth are depicted in Fig. 2. The optimal growth temperature and pH of Y5 and D6 were 30 °C and 7.0, respectively. Figure 3 illustrates the CDW yield and SCP content of strain Y5 obtained in the three-ammonium-salt medium. NH_4HCO_3 was the most suitable nitrogen source for SCP production because of its neutrality. It is known that HCO_3^- is alkalescent, which enabled the adjustment and maintenance of the soda acid balance in the culture. The average fixation rate of NH_4^+-N and volumetric productivity of Y5 and D6 with NH_4HCO_3 as a nitrogen source were slightly higher than the values obtained on NH_4Cl (Table 2). Thus, NH_4HCO_3, which simulated the NH_4^+-N of BSCM, was favorable for producing SCP from biogas slurry containing a high NH_4^+-N concentration and carbon dioxide derived from biogas utilization as substrates.

Autotrophic SCP Production by HOB

Autotrophic SCP production was carried out in a shake serum bottle with CO_2 as the sole carbon source. This test was completed after 5 days, when no further gas consumption was evident. The profiles of CDW and SCP content of different HOB are presented in Fig. 4. All

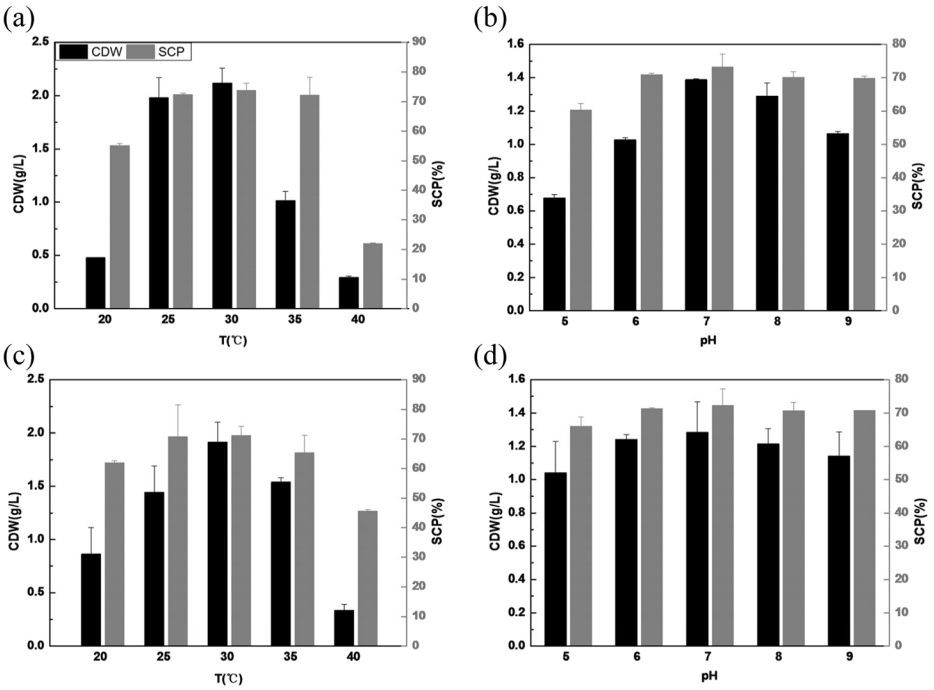


Fig. 2 Optimal growth temperature (**a**) and pH (**b**) of isolated strain Y5 and optimal growth temperature (**c**) and pH (**d**) of isolated strain D6 after autotrophic cultivation for 5 days

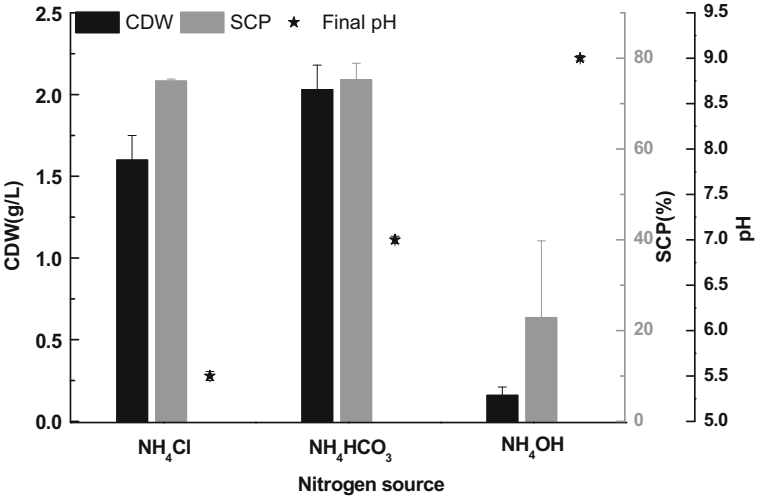


Fig. 3 CDW, SCP production, and final pH of strain Y5's culture supernatant containing different ammonium salts after autotrophic cultivation for 5 days

the strains were able to grow autotrophically. The maximum CDW production of 2.12 g/L and SCP content of 73.73% were obtained with strain Y5. *C. necator* DSM 531 has been widely studied concerning autotrophic production of SCP [22, 23]. SCP content of Y5 was much higher than that of DSM 531 (67%), algae (40–60%), yeast (45–55%), fungi (30–45%), and soybean (45%) [9]. The elemental composition of Y5 was as follows (major components only): 40.00% C, 10.84% N, 6.83% H, and 1.00% S, with a cell molecular formula of $C_{3.33}H_{6.83}O_{2.58}N_{0.77}$. The stoichiometry of strain Y5 is roughly indicated by Formula (8). The elemental composition of D6 (major components only) was 34.42% C, 7.99% N, 5.34% H, and 1.58% S, with a cell molecular formula of $C_{2.87}H_{5.34}O_{3.17}N_{0.57}$. The stoichiometry of D6 is indicated by Formula (9):

$$21.51H_2 + 7.59O_2 + 3.33CO_2 + 0.77NH_3 \rightarrow C_{3.33}H_{6.83}O_{2.58}N_{0.77} + 19.25H_2O \quad (8)$$

$$17.98H_2 + 6.80O_2 + 2.87CO_2 + 0.57NH_3 \rightarrow C_{2.87}H_{5.34}O_{3.17}N_{0.57} + 16.16H_2O \quad (9)$$

The low conversion efficiency resulted in a high concentration of residual NH_4^+-N (Table 2). The cause of the low conversion efficiency may be that the HOB growth was limited by the partial vacuum caused by gas consumption and discontinuous gas feeding. This situation was confirmed on *Hydrogenomonas eutropha* [24]. Cohen [25] has also found that HOB grow better under steady gas pressure near atmospheric pressure with continuous gas feeding. The continuous bioreactor culture system will be used in future research. As demonstrated in Table 3, the CDW yield per carbon dioxide in strain Y5 was higher than the maximum value reported in a sequencing batch reactor and continuous reactor [26]. The rates of utilization of carbon and nitrogen by Y5 were greater than the rates manifested by D6. On the other hand, the utilization rates of carbon and nitrogen were not 100%. This state of affairs may be associated with produced secondary metabolites including amino acids and volatile fatty acids.

Table 2 Nitrogen assimilation of strains Y5 and D6 under autotrophic, heterotrophic, and mixotrophic conditions

Strains	NH_4^+-N_i (mg/L)	NH_4^+-N_r (mg/L)	CDW-N[a] (mg/L)	Average fixation rate of NH_4^+-N (mg/[L·day])	Conversion efficiency of NH_4^+-N (%)	Utilization efficiency of NH_4^+-N (%)	Volumetric productivity (mg CDW/[L day])	SCP content (%)
Autotrophic cultivation								
Y5[b]	1046	817.7 ± 4.0	145.8	45.6	21.83	63.86	286	63.65
Y5	1046	722.1 ± 6.2	250.1	64.8	30.97	77.22	424	73.73
D6[b]	1046	877.5 ± 20.0	136.6	42.1	16.11	81.07	271	63.05
D6	1046	727.9 ± 27.0	218.8	63.6	30.41	68.78	384	71.22
Heterotrophic cultivation								
Y5	1255	55.9 ± 16.0	596.9	239.8[c]	95.55[c]	49.78[c]	1034	72.21
D6	1255	95.6 ± 14.0	508.2	231.9[c]	92.38[c]	43.83[c]	898	70.83
Mixotrophic cultivation								
Y5	1060	684 ± 2.1	356.4	47.0[d]	35.47	94.79	400	69.58
D6	1060	688 ± 8.2	355.3	46.5[d]	35.09	95.51	413	67.34

[a] CDW-N was determined according to the Kjeldahl method

[b] The results were obtained with NH_4Cl as a nitrogen source; other results were obtained with NH_4HCO_3 as the nitrogen source

[c] The values are meaningless due to the volatilization of ammonia under the heterotrophic condition with breathable plug

[d] The mixotrophic cultivation period was the first 8 days

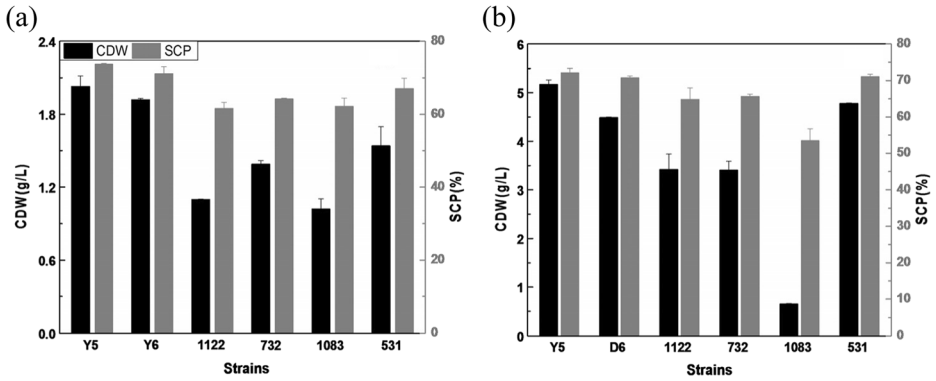


Fig. 4 Comparison of CDW and SCP content among isolated Y5, D6, and the four HOB strains available from DSMZ after autotrophic (**a**) and heterotrophic (**b**) cultivation for 5 days with NH_4HCO_3 as the nitrogen source

Heterotrophic SCP Production by HOB

The main soluble organic carbon was reduced sugar instead of volatile fatty acids and amino acids (Table 1). Hence, glucose, which simulated the carbon source of BSCM, was added into MM for heterotrophic SCP production. After 5 days, the glucose was completely consumed. The CDW and SCP contents are shown in Fig. 4b. The maximal CDW (5.17 g/L) and SCP content (72.2%) were also obtained with strain Y5. The results also revealed that the CDW and SCP content of the six strains under heterotrophic conditions were substantially higher than those under autotrophic conditions, except for *P. carboxydohydrogena* DSM 1083. The volumetric productivity of heterotrophic cultivation in terms of CDW was higher than that for autotrophic cultivation (Table 2). This result is similar to that on the HOB growth on organic substrates in the air atmosphere; this number was twofold higher than that under autotrophic conditions [15]. The reason was the restricted supply of a carbon source owing to the slower gas-liquid mass transfer rate during autotrophic cultivation [17].

For heterotrophic cultivation, although the conversion efficiency of NH_4^+-N was 92.38–95.55%, only 43.83–49.78% of NH_4^+-N was converted to CDW. This situation resulted from the volatilization of ammonia during shaking cultivation. Therefore, the values of average fixation rate, conversion efficiency, and utilization efficiency of NH_4^+-N were meaningless.

Mixotrophic SCP Production by HOB

The COD/TN ratio of BSCM was 3.76, with the corresponding C/N ratio of 1.41, which is much lower than the required value for SCP production (Table 1). Therefore, mixotrophic SCP

Table 3 Carbon and nitrogen balance analysis of strains Y5 and D6 in the autotrophic condition with NH_4HCO_3 as a nitrogen source

Strains	C_{cell} (%)	N_{cell} (%)	$Y_{CDW/CO2}$ (g CDW/g CO_2)	$Y_{CDW/H2}$ (g CDW/g H_2)	$Y_{CDW/O2}$ (g CDW/g O_2)	$Y_{CDW/NH4+-N}$ (g CDW/g NH_4^+-N)	Y_C (g cell C/g CO_2-C)	Y_N (g cell N/g NH_4^+-N)
Y5	40.00	10.84	0.35	1.11	0.24	0.33	0.52	0.71
D6	34.42	7.99	0.30	0.95	0.21	0.30	0.38	0.48

production was carried out with the gas mixture and BSCM as substrates. The mixotrophic cultivation was terminated when there was no further ammonia consumption. Unfortunately, none of the six strains could grow with the sterilized original BSCM without dilution. This result may be explained as follows: the HOB could not adapt to a new environment containing high concentrations of metal ions and other complex salts. Hence, BSCM was diluted fivefold with water. All six strains grew in the diluted and sterilized BSCM. As demonstrated in Fig. 5a, the NH_4^+-N concentration and SCOD of the strains both declined. Taking this one step further, the drop of SCOD was greater in the first 8 days than in the last 8 days. There was no gas consumption during the first 3 days, indicating that heterotrophic metabolism occurred

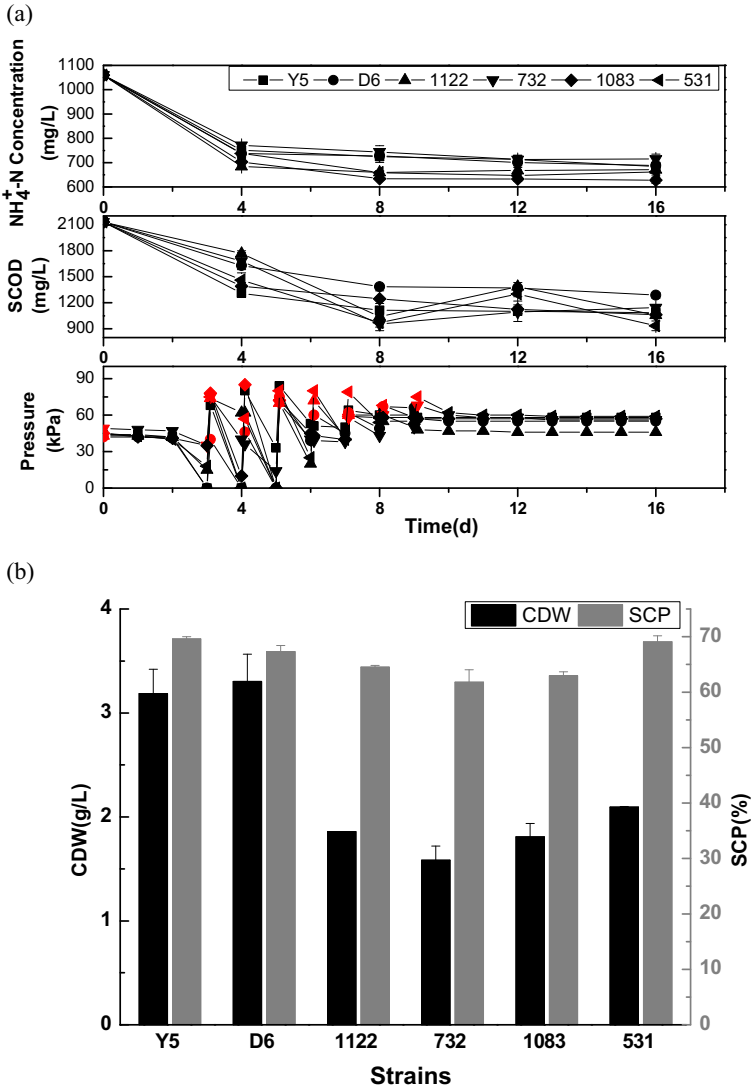


Fig. 5 Concentration of NH_4^+-N and SCOD, and gas pressure (**a**) and CDW and SCP content (**b**) of six strains after mixotrophic cultivation for 16 days with diluted BSCM as a substrate. Red symbols indicate the pressure after gas injection

preferentially during the mixotrophic cultivation. The results supported the hypothesis that key enzymes for autotrophic metabolism form during heterotrophic growth [23]. The remaining difficult-to-degrade organic matter could not be used by HOB. During the latter 8 days of cultivation, the NH_4^+-N concentrations of strains Y5 and D6 decreased slowly, and the amount of SCOD and gas consumption hardly decreased. The results showed that the cultivation period of 8 days was suitable for mixotrophic SCP production from BSCM. The rates of removal of NH_4^+-N and SCOD reached 35.47 and 49.04% for strain Y5. The CDW and SCP content of Y5 was 3.19 g/L and 69.6%, respectively, and was 3.3 g/L and 67.3%, respectively, for strain D6 (Fig. 5b). This result showed that it is possible that strain Y5 synchronously removed NH_4^+-N and SCOD and produced SCP. The order of volumetric productivity for strains Y5 and D6 was heterotrophic > mixotrophic > autotrophic cultivation.

On the basis of the data above, a possible process scheme for SCP production from HAW by HOB is proposed in Fig. 6.

Amino Acid Profiles

The amino acid composition of SCP from strains Y5 and D6 was measured and compared with soybean meal as reported by Lo and Moreau [27]. The profiling (Table 4) indicated that strains Y5 and D6 contained a variety of amino acids, whose concentrations exceeded those of soybean meal. Proline, aspartic acid, glutamic acid, and serine, which were not present in soybean meal, were evident in the SCP of strains Y5 and D6. The arginine content of Y5 was relatively higher than that of soybean meal. Arginine is one of the important amino acid additives in fish feed. The total content of methionine and cystine in strain Y5 was similar to that reported for microbial protein [26]. Methionine and cystine are two amino acids that are essential for poultry and pigs [27]. Concentrations of threonine, valine, alanine, and glycine were higher than those in soybean meal.

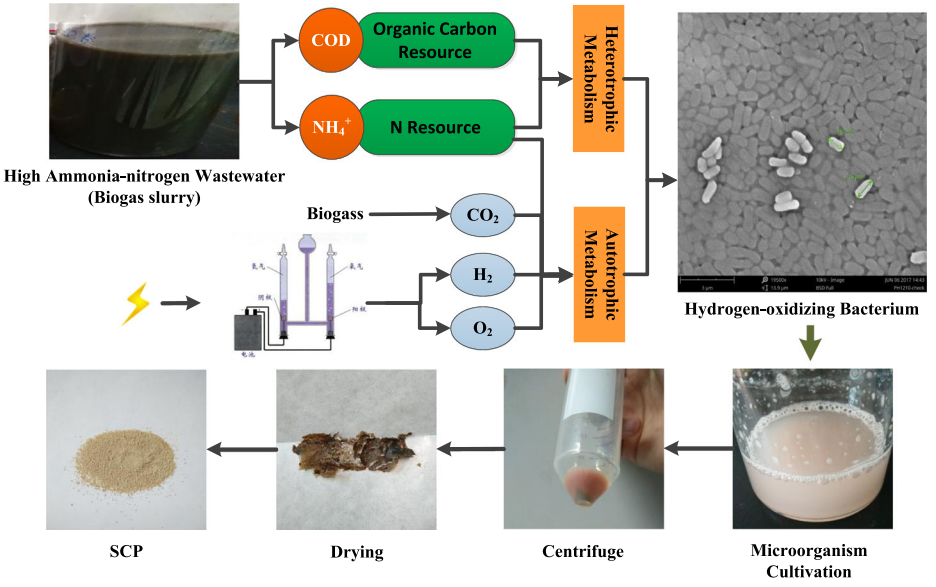


Fig. 6 The process scheme for SCP production from HAW by HOB

Table 4 Amino acid composition of strains Y5 and D6 in the autotrophic condition as compared to soybean meal (g per 100 g of CDW)

Amino acid		Y5	D6	Soybean meal
Essential	Arginine	4.04	2.99	3.80
	Histidine	1.16	0.81	1.30
	Isoleucine	2.22	1.66	2.57
	Leucine	3.85	2.78	3.80
	Lysine	2.54	1.65	3.20
	Methionine	0.91	0.90	0.72
	Phenylalanine	2.07	1.44	2.60
	Proline	2.07	1.70	0
	Threonine	2.64	1.94	1.97
	Valine	3.26	2.46	2.70
Nonessential	Alanine	3.49	2.52	2.56
	Aspartic acid	7.22	4.50	0
	Cysteine	0.95	0.35	0.74
	Glutamic acid	5.80	3.92	0
	Glycine	3.05	2.09	2.43
	Serine	1.79	1.27	0
	Tyrosine	2.00	1.40	1.95

Conclusions

Isolated strains *P. denitrificans* Y5 and *P. versutus* D6 produce SCP under autotrophic, heterotrophic, and mixotrophic conditions. The optimal growth temperature and pH of Y5 and D6 are 30 °C and 7.0, respectively. The cell molecular formulae of strain Y5 ($C_{3.33}H_{6.83}O_{2.58}N_{0.77}$) and D6 ($C_{2.87}H_{5.34}O_{3.17}N_{0.57}$) were determined during autotrophic cultivation. The order of volumetric productivity for Y5 and D6 was heterotrophic > mixotrophic > autotrophic cultivation. SCP contents of strain Y5 and D6 were 67.34–73.73% based on CDW with NH_4HCO_3 as a nitrogen source. The SCP contains proline, aspartic acid, glutamic acid, and serine, which are not present in soybean meal.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that there are no conflicts of interest.

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