pubs.acs.org/est

Article

Microbial Protein out of Thin Air: Fixation of Nitrogen Gas by an Autotrophic Hydrogen-Oxidizing Bacterial Enrichment

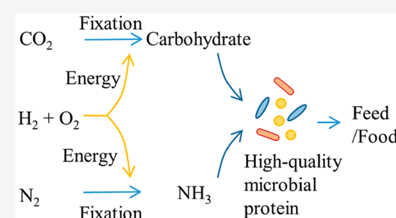
Xiaona Hu, Frederiek-Maarten Kerckhof, Justien Ghesquière, Kristel Bernaerts, Pascal Boeckx, Peter Clauwaert, and Nico Boon*

Cite This: *Environ. Sci. Technol.* 2020, 54, 3609−3617

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: For the production of edible microbial protein (MP), ammonia generated by the Haber-Bosch process or reclaimed ammonia from waste streams is typically considered as the nitrogen source. These processes for ammonia production are highly energy intensive. In this study, the potential for using nitrogen gas (N_2) as a direct nitrogen source for MP production by hydrogen-oxidizing bacteria (HOB) was evaluated. The use of N_2 versus ammonium as nitrogen source during the enrichment process resulted in differentiation of the bacterial community composition of the enrichments. A few previously unknown potential N_2-fixing HOB taxa (i.e., representatives of the genus *Azonexus* and the family *Comamonadaceae*) dominated the enrichments. The biomass yield of a N_2-fixing HOB enrichment was 30−50% lower than that of the ammonium-based HOB enrichment from the same inoculum source. The dried biomass of N_2-fixing HOB had a high protein content (62.0 ± 6.3%) and an essential amino acid profile comparable to MP from ammonium-based HOB. MP from N_2-fixing HOB could potentially be produced in situ without entailing the emissions caused by ammonia production and transportation by conventional means. It could be a promising substitute for N_2-fixing protein-rich soybean because it has 70% higher protein content and double energy conversion efficiency from solar energy to biomass.

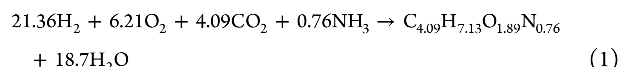


Downloaded via UNIV GENT on May 7, 2020 at 06:56:27 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

1. INTRODUCTION

Food production will need to be increased in response to the growing world population and rising demand for high grade protein per capita.[1,2] Due to the inefficient use of energy-intensive nitrogen fertilizers in contemporary agriculture and main nitrogen losses as manure during plant−livestock conversion,[3,4] further expansion of traditional agricultural practices will exacerbate its environmental damage. Therefore, the interest in microbial protein (MP) production has been renewed and MP could potentially be integrated into the food chain as feed or food.[5−7]

Among various MP producers, such as bacteria, algae, yeast, and fungi, hydrogen-oxidizing bacteria (HOB) are of particular interest because they can grow autotrophically on H_2,[8] which can be produced from renewable energy. The stoichiometry of the autotrophic culture of well-studied HOB, *Cupriavidus necator*, is presented in the following equation:[9]



$$21.36H_2 + 6.21O_2 + 4.09CO_2 + 0.76NH_3 \rightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76} + 18.7H_2O \quad (1)$$

Axenic cultures of HOB can establish a high growth rate (up to 0.42 h^{-1})[9] and both pure cultures and consortia have been studied to produce value-added MP with a protein content of up to 70% of the cell dry weight.[10,11] In addition, the HOB biomass is of high quality, with a protein availability to proteolytic enzymes surpassing that of wheat and an essential amino acid composition similar to that of animal protein.[10,11]

Although the reactive mineral nitrogen uptake efficiency in HOB-based MP production can be close to 100%,[10] the high demand of energy-intensive nitrogen fertilizer (37−45 MJ/kg N as NH_3 via the Haber-Bosch process)[5] for protein synthesis is inevitable. The current technology of ammonia production results in enormous CO_2 emissions (2.9 t CO_2/t NH_3), and the transportation of centrally produced ammonia to end-users leads to additional energy consumption and detrimental gas emissions.[12−14] Direct upgrade of reactive mineral nitrogen into MP from waste streams may have limited applications[15,16] due to the potential influence of pathogens, inhibitors, and hazardous residues in wastes. Additionally, ammonia recovery from wastes by combination of electrolysis and stripping has a high energy requirement (65−76 MJ/kg N),[17−19] which is more energy-demanding than the Haber-Bosch process. To attenuate the dependence of MP production on reactive mineral nitrogen fertilizers, conversion of N_2 to bioavailable nitrogen via biological nitrogen fixation could be an alternative.

HOB comprise diverse genera[20−23] and a few pure cultures are known to perform N_2 fixation while they grow chemolithoautotrophically with H_2, O_2, and CO_2.[23−25] *Xanthobacter*

Received: November 8, 2019
Revised: January 30, 2020
Accepted: February 24, 2020
Published: March 3, 2020

© 2020 American Chemical Society

autotrophicus has been studied for production of ammonia and biofertilizers via its hydrogenotrophic N_2 fixation capacity.[26] However, this group of bacteria are still far from fully explored and exploited. Although hydrogenotrophic N_2 fixation via microorganisms is a promising method to promote the growth of plants sustainably,[27] the value of edible protein assimilated in microbial biomass has, to the authors' knowledge, not been investigated. Little is known about making use of N_2-fixing bacteria in MP production under a minimal nutrient condition.

In this study, the aim was to upgrade N_2, H_2, O_2, and CO_2 into MP by a microbial community without addition of reactive mineral nitrogen fertilizer. Parallel enrichments with either ammonium (NH_4^+) or N_2 as the nitrogen source were performed to investigate the differences in the bacterial communities shaped by the nitrogen sources and uncover the existence of bacteria in the enrichments growing on N_2. In addition, comparative assays using NH_4^+ or N_2 for growth were performed to evaluate N_2 fixation capacity and biomass yield of N_2-fixing HOB. The protein content and essential amino acid composition of N_2-fixing HOB were also analyzed to assess the protein quality.

2. MATERIALS AND METHODS

2.1. Inoculation and Enrichment. Three inocula, including an HOB enrichment (HE),[28] biocathode sludge (BS),[29] and salty sediment (SS) (Supporting Information, SI, S1) were used each in their respective enrichment process by batch incubation at 28 °C. HE was from an HOB chemostat reactor operated for 80 days at 28 °C with ammonium as the nitrogen source. BS was sampled from a microbial fuel cell's cathodic compartment operated at 25 °C and +0.2 V vs Ag/AgCl for 565 days with modified M9 mineral medium free from reactive nitrogen and supplemented with NaCl (18 to 40 mS/cm). SS was sampled from Salines located at Castro Marim, Portugal. The mineral medium H-3[30] was used for chemolithoautotrophic growth of cultures in the "N medium", where reactive mineral nitrogen was provided as NH_4Cl while H-3 medium was modified for the "N-free medium" by omitting NH_4Cl. After 32 days (41 days for SS in N-free medium) of enrichment, the ferric ammonium citrate solution mainly providing iron to the medium was replaced with 0.05 g $FeSO_4 \cdot 7H_2O$ and 0.06 g Na_2EDTA dissolved in 20 mL distilled water to eliminate possible interference of reactive mineral nitrogen from ammonium and bioavailable organic from citrate.

120 mL serum bottles containing 18 mL N (HE-NH_4^+, BS-NH_4^+, and SS-NH_4^+) or N-free (HE-N_2, BS-N_2, and SS-N_2) mineral medium were inoculated (10% v/v) in duplicate. The cultures in N medium served as controls. The bottles were sealed with butyl rubber stoppers, and the headspace was replaced with 2% O_2, 10% H_2, 10% CO_2, and 78% N_2. A low concentration of O_2 was used to alleviate its possible inhibition to N_2 fixation[23] while an H_2/O_2 ratio of 5 was used to provide sufficient energy. CO_2 served as the carbon source of the growth and buffer system together with $NaHCO_3$. N_2 was added to balance the gas composition and to serve as a nitrogen source when ammonium was not available. This initial gas composition was used in all the bottles throughout this entire manuscript. To obtain the specific gas composition, the headspace was first flushed with N_2 and adjusted to around atmospheric pressure (0−4 kPa). Then pure O_2, H_2, and CO_2 were taken from gas sampling bags and injected separately with syringes to the bottles. The bottles were incubated in the dark to avoid phototrophic growth, and pressure changes were recorded to estimate the H_2 oxidation activity. During the first three start-up batches at 120 rpm and 28 °C, 50% of the liquid cultures in these bottles were transferred to fresh media and fresh headspace was made when gas consumption was observed. Then culture transfer and headspace refreshment were repeated once a day as described in SI Table S1. When all the cultures showed active H_2 oxidation, they were incubated in triplicate in 240 mL serum bottles (40 mL mineral medium and 200 mL headspace) from day 25 to 80 (from day 34 to 89 for SS-N_2). To reduce the effect of the anaerobic condition caused by O_2 depletion without losing biomass, the time of shaking was reduced and cultures were transferred every 2 days (SI Table S1).

Headspace composition changes were analyzed to evaluate H_2 oxidation rates on day 5 and day 80 (day 89 for SS-N_2) during the enrichment process. For the batch incubation from day 79 to 80 (day 88 to 89 for SS-N_2), the changes of total nitrogen (TN), soluble total nitrogen (TN_s), total chemical oxygen demand (COD_t), soluble chemical oxygen demand (COD_s) and cell density of the cultures growing on N_2 were measured to compare their biomass growth. DNA samples were taken on day 0, 44, and 80 (day 0, 53, and 89 for SS-N_2) and cell density was measured to obtain absolute abundance in the bacterial communities.

2.2. $^{15}N_2$ Isotope Labeling Experiment. HE-N_2, together with HE-NH_4^+ as control, were used to verify $^{15}N_2$ assimilation into biomass after 80 days' enrichment. 1150 mL gastight glass bottles were used to provide sufficient gaseous nutrients, and all tests were performed in biological triplicate. Inocula from the two enrichments were each diluted 100 times by adding 0.4 mL culture into 39.6 mL medium, and the headspace was prepared as described previously. The bottles were shaken at 120 rpm and 28 °C in the dark. The headspace of $^{15}N_2$ test bottles was amended with $^{15}N_2$ (98% ^{15}N, Buchem B.V., The Netherlands) to reach 1.3% ^{15}N abundance while only regular N_2 was added in the biotic control bottles. The $^{15}N/^{14}N$ isotope ratios of biomass were compared between the test and the biotic control bottles. The test of HE-N_2 was performed in both N and N-free medium to evaluate the influence of reactive mineral nitrogen on N_2-fixing activity and the incubation lasted 72 h. For the test of HE-NH_4^+, only N medium was used and the incubation lasted 48 h. Abiotic control bottles containing 40 mL N or N-free medium and the same headspace were used to monitor the gas equilibration during the test. The observed change in total molar amount of gas in the abiotic controls was 0.29 ± 0.13%, indicating a good condition of gas tightness. At the end of the batch tests, biomass was harvested for isotope analysis.

2.3. Biomass Yield Test. As described in the $^{15}N_2$ isotope labeling experiment, a similar setup was made to monitor biomass growth and nitrogen species concentration changes during the incubation of HE-N_2 in N-free medium and HE-NH_4^+ in N medium, respectively. Due to the limiting liquid volume in each bottle and the need for liquid sampling at an interval of 12 h, multiple replicate bottles were prepared, and three bottles were sacrificed as triplicates for gas and liquid analysis at each time point. The test of HE-N_2 lasted 72 h, and 18 bottles were used whereas the test of HE-NH_4^+ lasted 48 h and 12 bottles were used. TN, TN_s, COD_t, COD_s, total ammonia nitrogen (TAN), cell density, and pH were measured in liquid samples. Headspace composition and pressure were measured to evaluate gas consumption.

https://dx.doi.org/10.1021/acs.est.9b06755
Environ. Sci. Technol. 2020, 54, 3609−3617

Table 1. $\delta^{15}N$ of Biomass Growing with and without Spiked $^{15}N_2$

inoculum	NH_4^+ in medium	spiked with $^{15}N_2$	time (h)	$\delta^{15}N$ (‰)[a]
HE-N_2	no	no	0	−1.16 ± 0.33[b]
	no	no	72	−0.21 ± 0.15
	no	yes	72	2401.73 ± 25.26
	yes	no	72	−13.36 ± 0.76
	yes	yes	72	−13.28 ± 0.81
HE-NH_4^+	yes	no	0	−15.20 ± 0.14
	yes	no	48	−12.64 ± 1.80
	yes	yes	48	−13.36 ± 0.11

[a]For most naturally occurring N containing materials, −30‰ < $\delta^{15}N$ < +30‰. The small difference in observed negative values could be caused by the different $^{15}N/^{14}N$ ratios in natural nitrogen sources. [b]Average ± SD, n = 3.

2.4. Protein Quality Evaluation. A setup including three serially connected 3 L bottles as biological triplicates was supplied with continuous gas (SI S2 and Figure S1) and used to scale up the microbial protein production from HE-N_2 and HE-NH_4^+ respectively. The gas composition remained stable from the inlet to the outlet of the setup, which allowed for the efficient operation of the compact setup without introducing a gas supply difference of individual gas lines. After 8 days' operation, crude protein and essential amino acid content in the dry biomass were measured as described in SI S3.

2.5. Analytical Methods. TN and COD_t were measured on sampled aliquots and TN_s, TAN, and COD_s onfiltered samples (0.45 μm filter, Millipore, Belgium), according to the persulfate method (TN), the open reflux method (COD), and the Nessler method (TAN), respectively.[31] Gas composition was analyzed on a compact gas chromatograph (Global Analyzer Solutions, Breda, The Netherlands), and quantified by gas pressure measurement (UMS INFIELD7, München, Germany). The gas chromatography was equipped with an Rt-Q-bond precolumn and a column for CO_2 analysis, a Molsieve 5A precolumn and a Porabond column for O_2, H_2, and N_2 analysis and a thermal conductivity detector. Cell density was measured with an Accuri C6 benchtop flow cytometer (BD Biosciences, Erembodegem, Belgium) using an established protocol.[32]

For biomass-nitrogen isotope analysis, the cultures were centrifuged (10 min, 7000g), and the supernatant was removed. To eliminate dissolved nitrogen species residues, the pellets were resuspended in a buffer solution (0.23 g/L KH_2PO_4 and 0.29 g/L $Na_2HPO_4{\cdot}2H_2O$) and centrifuged (10 min, 10 000g) for three cycles. The pellets were stored at −20 °C before drying by lyophilization for 24 h. The $^{15}N/^{14}N$ isotope ratios of dry biomass were analyzed by a ANCA-GSL elemental analyzer interfaced with a 20−22 IRMS with SysCon electronics (SerCon, Cheshire, U.K.). The results were expressed in delta values (δ) defined in eq 2, where R_{sample} and $R_{standard}$ represent $^{15}N/^{14}N$ isotope ratios of the sample and standard, respectively. The standard is N_2 in air.

$$\delta^{15}N = [R_{sample}/R_{standard} - 1] \times 10^3 \quad (2)$$

2.6. Bacterial Community Analysis. The culture samples for total DNA extraction were centrifuged (10 min, 10 000g) in 2 mL DNase and RNase free tubes, and the pellets were stored at −20 °C until DNA was extracted by phenol-chloroform extraction.[33] The DNA pellets were dried in air for 20 min and dissolved in 100 μL 1 × TE (10 mM Tris, 1 mM EDTA) and the solutions were stored at −20 °C. Illumina sequencing of the DNA samples was conducted by LGC Genomics GmbH (Berlin, Germany). Method of read assembly and cleanup was largely derived from the MiSeq SOP described by the Schloss lab.[34,35] The raw sequencing data have been submitted to the NCBI SRA under accession number PRJNA580512.

2.7. Statistical Data Analysis. Absolute taxon abundances were calculated as described previously.[36] Graphing and data analysis of community data were performed using R (v 3.6.1). The heat map was made using the NMF package (v 0.21.0).[37] Ecological distance metrics and ordinations were calculated using the vegan package (v 2.5−6),[38] and differential abundance was assessed using a DESeq2 (v 1.24.0)-based workflow.[39,40] The code is publically available through https://github.com/CMET-UGent/HOB_Enrichment. Other graphing was performed using SigmaPlot 13.

2.8. Calculations. The N content of microbial biomass ($TN_{biomass}$) was calculated as the difference of TN and TN_s while COD of biomass ($COD_{biomass}$) was the difference of COD_t and COD_s. Detailed calculations regarding biomass yield are described in SI S4.

3. RESULTS

3.1. Enriching Hydrogen-Oxidizing Communities. The enrichment process was started with an HOB enrichment (HE), biocathode sludge (BS), or salty sediment (SS) as inocula, with or without reactive mineral nitrogen. The enrichments growing on NH_4^+ were used as a benchmark for those growing on N_2 regarding their H_2 oxidation activity. During the enrichment, the N_2-enrichments (HE-N_2, BS-N_2, and SS-N_2) showed H_2 oxidation activity, i.e., gas consumption rate, similar to that observed in the NH_4^+-enrichments (HE-NH_4^+, BS-NH_4^+, and SS-NH_4^+) (SI Figures S2 and S3a). SS-N_2 and SS-NH_4^+ needed a longer start-up time of 21 days and 14 days, respectively, compared to the 4 days of start-up period for HE-N_2, BS-N_2, HE-NH_4^+, and BS-NH_4^+ (SI Figure S2). From day 31 (day 40 for SS-N_2), gas consumption rates of N_2- and NH_4^+-enrichments during incubation with shaking were stable at 2.4 ± 0.3 and 3.2 ± 0.4 mmol/(L·h), respectively (SI Figure S2). After 80 to 89 days of enrichment, biomass accumulated in all the enrichments. Cell density in the N_2-enrichments increased from (4.31 ± 5.14) × 10^7 to (4.57 ± 0.90) × 10^8 cells/mL, whereas their end cell density was 42 ± 9% lower compared to the NH_4^+-enrichments from the same inoculum source (SI Figure S3b).

The potential N_2-fixing activity was observed by an increase of 4.3 ± 0.7 mg/L in TN during a batch incubation of the three N_2-enrichments on day 80 (day 89 for SS-N_2) (SI Table S2). In addition, after the 40 h batch incubation, the biomass concentrations of the N_2-enrichments in terms of cell density, $TN_{biomass}$ and $COD_{biomass}$ increased by 104 ± 26%, 112 ± 20%, and 191 ± 49%, respectively (SI Table S2).

https://dx.doi.org/10.1021/acs.est.9b06755
Environ. Sci. Technol. 2020, 54, 3609−3617

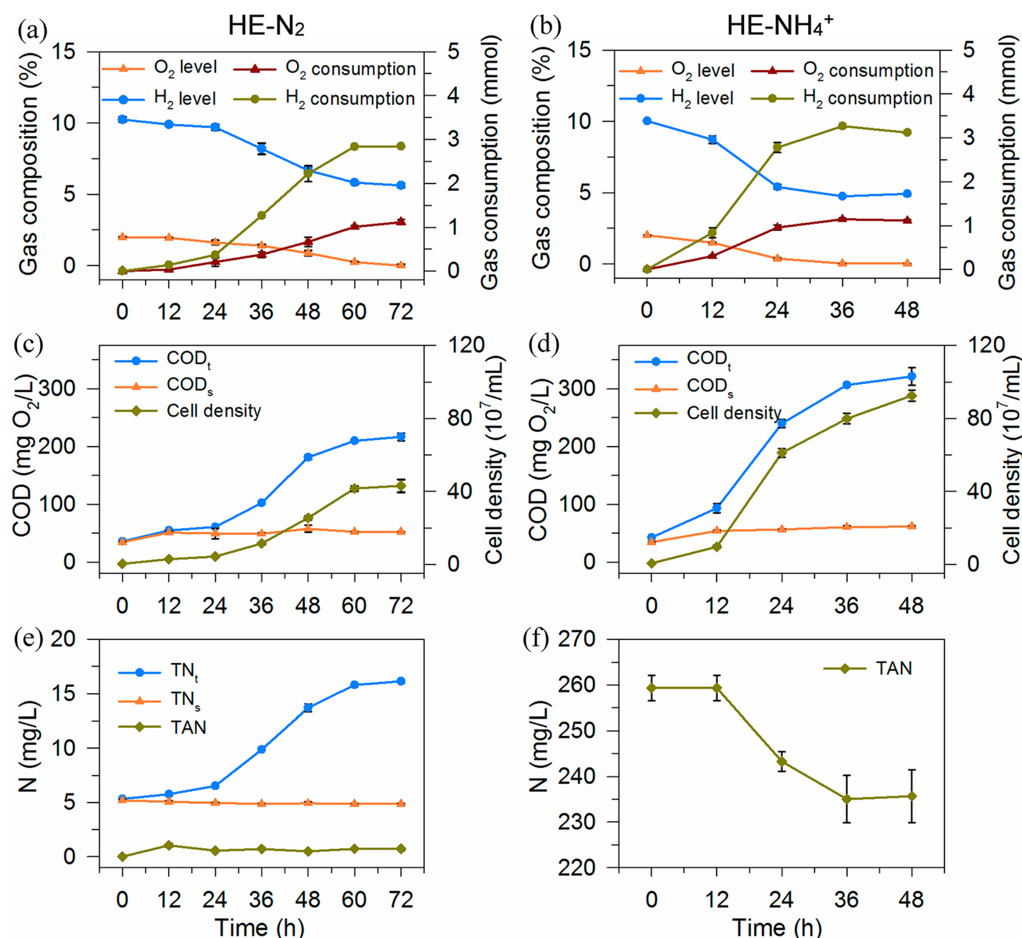


Figure 1. Change of (a) O_2 and H_2; (c) COD_t, COD_s, and cell density; and (e) TN_t, TN_s, and TAN during the biomass yield test of HE-N_2. Change of (b) O_2 and H_2; (d) COD_t, COD_s, and cell density; and (f) TAN during the biomass yield test of HE-NH_4^+. Average values are presented and error bars represent standard deviations. Values were calculated on biological triplicates.

3.2. Confirmation of Biological Nitrogen Fixation by $^{15}N_2$ Incorporation. HE-N_2 and its control HE-NH_4^+ were chosen among these active enrichments as representatives for further study of N_2 fixation and biomass growth. In the medium free of mineral reactive nitrogen, incubation of HE-N_2 with spiked $^{15}N_2$ resulted in an increase of $\delta^{15}N$ in biomass compared to the biomass grown with only regular N_2 (Table 1). This demonstrates that N_2 was assimilated into the microbial biomass. On the contrary, the presence of NH_4^+ in medium stopped $^{15}N_2$ incorporation into cells of HE-N_2. The HE-NH_4^+ incubated in N medium did not show N_2-fixing capacity either.

3.3. Biomass Growth of HE-N_2 and HE-NH_4^+. Biomass was the main product while relatively low amounts of soluble organic compounds (49 ± 7 mg COD_s/L for HE-N_2 and 53 ± 11 mg COD_s/L for HE-NH_4^+) were released into the liquid during the growth of both HE-N_2 and HE-NH_4^+ in the batch activity tests (Figure 1c, d). In addition, N_2 was mainly assimilated into the biomass of HE-N_2 rather than soluble nitrogen-containing compounds in the medium as evidenced by the increasing TN concentration and stable TN_s concentration (Figure 1e), whereas growth of the HE-NH_4^+ cultures resulted in TAN consumption of 23.6 ± 6.5 mg/L. The cell density and COD_t increased gradually along with H_2 and O_2 consumption, and the biomass growth rate was positively correlated to the H_2 oxidation rate (Figure 1). The low O_2 concentration (<0.26 ± 0.04%) in the headspace limited further H_2 oxidation and cell growth of HE-N_2 after 60 h (Figure 1a) while O_2 limitation (<0.03 ± 0.02%) also occurred in the HE-NH_4^+ after 36 h (Figure 1b).

The use of N_2 as the nitrogen source resulted in a lower biomass growth of HE-N_2 when similar amounts of O_2 and H_2 were consumed by HE-N_2 (1.12 ± 0.06 mmol O_2, 2.85 ± 0.03 mmol H_2) and HE-NH_4^+ (1.12 ± 0.02 mmol O_2, 3.12 ± 0.01 mmol H_2) in the batch tests (Figure 1a, b). The biomass yield obtained with HE-N_2 in terms of cell number was half that with HE-NH_4^+, and the yield in terms of $COD_{biomass}$ was almost 70% of that with HE-NH_4^+ (SI Table S3). Furthermore, the energy demand for N_2 assimilation into biomass by HE-N_2 was approximately twice that needed for the same amount of NH_4^+ assimilation by HE-NH_4^+ (SI Table S3).

Interestingly, the protein content of dry biomass from HE-N_2 and HE-NH_4^+ reached similar levels, which was 62.0 ± 6.3% and 71.8 ± 0.8%, respectively, and they had similar essential amino acid composition (SI Table S4). In the crude protein of HE-N_2 biomass, all essential amino acids were present and constituted around half of the protein weight. Moreover, its crude protein was rich in Lysine (7.3 ± 1.2%) and Methionine (3.1 ± 0.4%) which are normally deficient in plant-based proteins.

3.4. Bacterial Community. *3.4.1. Bacterial Community Structure of the Enrichments.* The bacterial community

structures of the enrichments were clearly different from that of their original inocula (SI Figure S4), and samples of these enrichments were divided into two main clusters according to nitrogen sources by principal coordinates analysis (PCoA) (Figure 2). Cluster 1 contained all the N_2-enrichments while

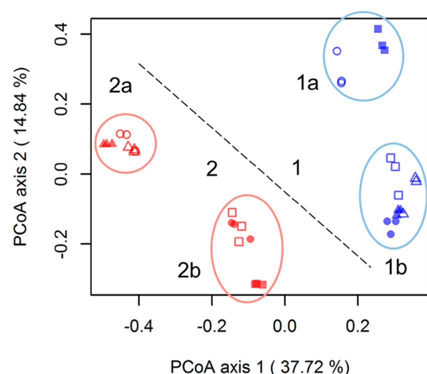


Figure 2. Principal coordinates analysis (PCoA) plot based on abundance-based Jaccard distances showing the bacterial community similarity among the enrichments (in triplicates) in N-free and N medium. Red and blue symbols represent the NH_4^+-enrichments and N_2-enrichments, respectively. Open and filled symbols represent enrichments of day 44 (day 53 for SS-N_2) and day 80 (day 89 for SS-N_2) respectively. Squares, circles, and triangles represent the enrichments originated from BS, HE, and SS respectively.

cluster 2 contained all the NH_4^+-enrichments. Furthermore, these two main clusters were each characterized by two subclusters which were related to the inoculum source and enrichment length. For the N_2-enrichments in cluster 1, SS-N_2 maintained stable bacterial community structures in subcluster 1b over the course of the enriching process while HE-N_2 and BS-N_2 communities showed migration between subcluster 1a and 1b. In cluster 2, bacterial community structures of SS-NH_4^+ and BS-NH_4^+ were stably maintained in subcluster 2a and 2b, respectively, while that of HE-NH_4^+ experienced variation from 2a to 2b during the enrichment process.

3.4.2. Bacterial Community Composition. The N_2-enrichments and NH_4^+-enrichments shared some dominant genera, whereas these genera showed a high degree of variation of absolute abundances (Figure 3). Specific taxa, such as *Azonexus*, *Xanthobacter*, and unclassified *Comamonadaceae*, were frequently observed in most of the communities after the enriching process under the atmosphere of $H_2/O_2/N_2/CO_2$ although different nitrogen and inoculum sources were used. These three taxa were also observed to be differentially abundant taxa between the N_2-enrichments and NH_4^+-enrichments (SI Figure S5). Regarding OTU2, unclassified *Comamonadaceae*, the NH_4^+-enrichments obtained higher abundances of $(2.66 \pm 1.00) \times 10^8$ cells/mL compared to $(2.30 \pm 1.46) \times 10^7$ cells/mL in the N_2-enrichments (Figures 3 and SI S5). As a less abundant taxon, *Xanthobacter* was more abundant in the N_2-enrichments than in the NH_4^+-enrichments originating from the same inocula (Figures 3 and SI S5).

The structures of the dominant taxa inside the two groups of communities were different. For the N_2-enrichments, *Azonexus* with abundances of $(1.95 \pm 0.75) \times 10^8$ cells/mL always dominated over the unclassified *Comamonadaceae* with abundances of $(2.30 \pm 1.46) \times 10^7$ cells/mL. In contrast to the N_2-enrichments, the structures of *Azonexus* and the unclassified *Comamonadaceae* in the NH_4^+-enrichments were

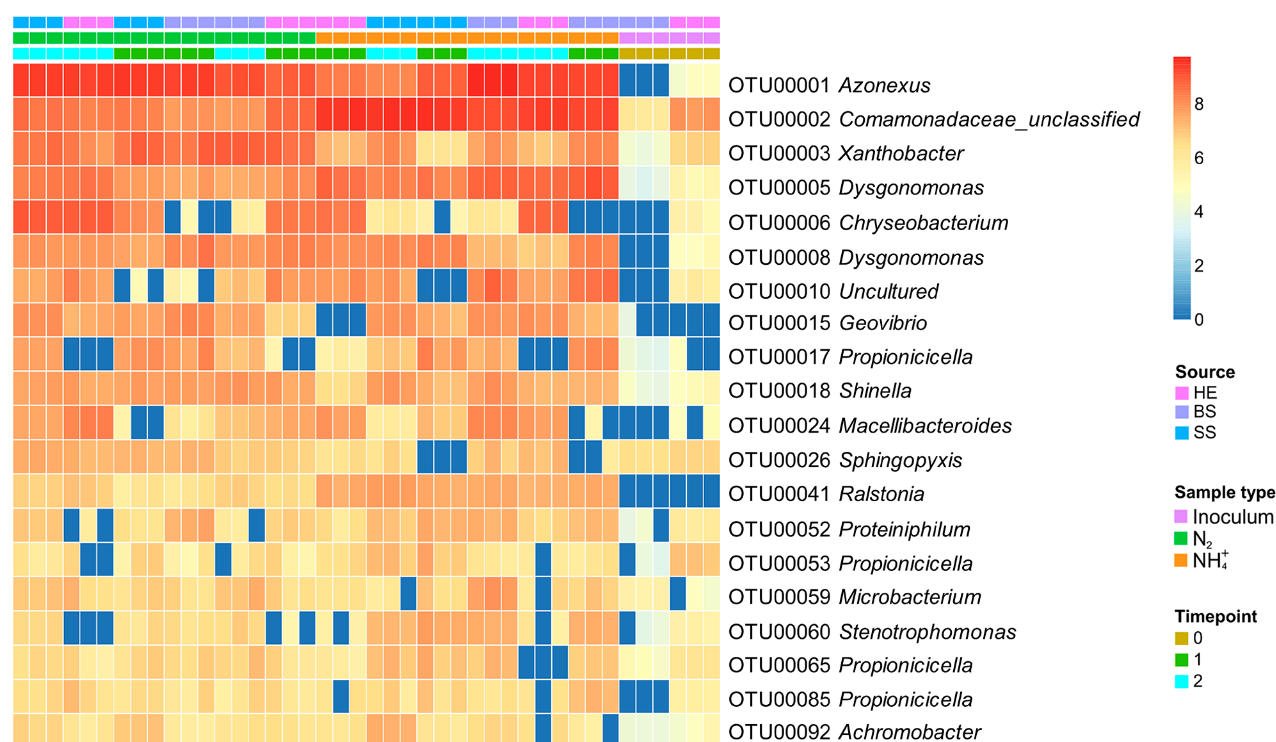


Figure 3. Heat map showing the absolute abundance of top 20 abundant OTUs among all the samples. The color scale ranges from blue to red, corresponding to absolute abundance of 0 to 10 × log10 reads/mL culture. Inoculum sources, sample types, and time points are each represented by a row of color codes on the top of the heatmap. Time points 0, 1, and 2 are day 0, day 44 (day 53 for SS-N_2), and day 80 (day 89 for SS-N_2), respectively. Results of technical triplicates of the inocula and biological triplicates of the enrichments are presented.

https://dx.doi.org/10.1021/acs.est.9b06755
Environ. Sci. Technol. 2020, 54, 3609−3617

Table 2. Comparison of Biomass Yield in This Study with That Obtained with other HOBs

microbial culture	substrate	nitrogen source	biomass yield (g CDW/g COD-H_2)	reference
pure culture	$H_2/O_2/CO_2$	NH_4^+	0.14−0.29	9,44−46
mixed culture	H_2/Air/CO_2	NH_4^+	0.07−0.28	10
mixed culture	$H_2/O_2/CO_2/N_2$	NH_4^+	0.14 ± 0.01 (0.17 ± 0.05)[a,b]	this study
mixed culture	$H_2/O_2/CO_2/N_2$	N_2	0.10 ± 0 (0.09 ± 0)[a,b]	this study

[a]The HOB biomass composition in the study was assumed to be $C_{4.09}H_{7.13}O_{1.89}N_{0.76}$, which is the biomass composition of the most-studied HOB, e.g., *Ralstonia eutropha*.[9] [b]The CDW was calculated via COD or nitrogen concentration (values in brackets) of biomass obtained in SI Table S3.

variable and dominance of one genus over the other was not consistent.

4. DISCUSSION

4.1. Biomass Production of the N_2-Enrichments. Microbial enrichments that could grow on N_2, H_2, O_2, and CO_2 in N-free liquid medium were obtained, and H_2 oxidation activity could be maintained in a stable manner over around 50 days. Utilization of N_2 as the nitrogen source for biomass growth was further confirmed by $^{15}N_2$ assimilation into cells and increasing nitrogen content in biomass during growth. This capacity of simultaneous N_2 fixation and H_2 oxidization of the N_2-enrichments was similar to that of genus *Xanthobacter*,[26] which is the N_2-fixing HOB genus that has been studied most. The close correlation between change of H_2/O_2 consumption and biomass growth indicated that CO_2 and N_2 fixation into biomass consumed energy from H_2 oxidation.[26,41]

The conversion from gases to biomass was quite efficient as little synthesized material was released as soluble compounds in medium. The characteristic of fixing nitrogen from the gas mainly into biomass was also observed in the pure strain of *Xanthobacter autotrophicus* growing in a similar medium and atmosphere.[26] However, biological N_2 fixation is an energy-expensive process which requires at least 16 mol ATP (equivalent to 2.3 mol H_2[41]) to reduce 1 mol N_2 to NH_4^+.[42] Thus, instead of direct NH_4^+ assimilation, part of the energy from H_2 oxidation needs to be diverted to N_2 fixation, which could explain the lower biomass yields in the N_2-enrichments. In this research, the biomass yield of HE-N_2 based on H_2 consumption was 30% to 50% lower than that of HE-NH_4^+ (Table 2). This kind of reduced growth caused by the extra energy demand for N_2 fixation was also observed with bioethanol-producing strain of *Zymomonas mobilis* which showed 50% less heterotrophic growth with N_2 compared to that with NH_4^+.[43]

Biomass yield of HE-N_2 growing on N_2 was on the same order of magnitude of observed yields in pure or mixed cultures of HOB supplied with NH_4^+ as the nitrogen source (Table 2), indicating its potential application in MP production. However, under the current nonoptimized growth conditions, the biomass yield of HE-N_2 was situated in the lower range of the reported values of the NH_4^+-based HOBs.

4.2. Characteristics of Bacterial Communities of the Enrichments. Different nitrogen sources resulted in differentiation of the bacterial community composition between the N_2-enrichments and the NH_4^+-enrichments. Despite the difference between the two groups of enrichments, they had dominant taxa in common having limited diversity. Therefore, regardless of the selective pressure of the nitrogen sources, other parameters of the growth condition, such as the supply of H_2 as the energy source and microaerobic environment, might also play an important role in the taxa selection. The presence of the same OTUs in both N_2-enrichments and NH_4^+-enrichments implies that the versatile metabolism of those bacteria might allow them to shift between N_2-fixing and non N_2-fixing HOB metabolism. The synthesis of uptake hydrogenase, which can oxidize H_2, in N_2-fixing bacteria may allow these bacteria to grow as HOB with N_2 or ammonium as the nitrogen source.[42,47]

The consistently higher absolute abundance of the OTU that represented the unclassified *Comamonadaceae* in the NH_4^+-enrichments compared to the N_2-enrichments could contribute to the differentiation of the two groups. By comparison of the partial 16S rRNA gene sequence of the unclassified *Comamonadaceae* with the database of the Basic Local Alignment Search Tool (BLAST), it could be a bacterium related to the genus *Hydrogenophaga*, mainly known as HOB growing with NH_4^+,[20] with an identity of 99.53%. Some strains of *Hydrogenophaga* were proven to be N_2-fixing HOB[24] and the presence of genus *Hydrogenophaga* in the N_2-enrichments also indicates its potential as a N_2-fixer. However, the growth of the unclassified *Comamonadaceae* might still be limited by the deficiency of reactive mineral nitrogen, leading to its lower abundances in the N_2-enrichments in this study.

On the contrary, *Azonexus* could grow faster than the unclassified *Comamonadaceae* in the N_2-enrichments. Members of the genus *Azonexus* have the *nif*H gene encoding dinitrogenase[48] which is the enzyme known to catalyze N_2 fixation, indicating that *Azonexus* could have the advantage of surviving with limited reactive mineral nitrogen due to its potential N_2-fixing capacity. However, the high abundances of *Azonexus* in most NH_4^+-enrichments implied that *Azonexus* could also perform actively as HOB with NH_4^+ as nitrogen source. This versatile metabolism was also observed in some N_2-fixing bacteria such as *Rhizobium japonicum* and *Derxia gummosa*, which could grow autotrophically on exogenous H_2 as an energy source with either N_2 or ammonium as nitrogen source.[25] The subclustering in cluster 1 and cluster 2 could be attributed to *Azonexus* due to the fact that subcluster 1b had higher abundance of *Azonexus* than subcluster 1a while subcluster 2b had higher abundance of *Azonexus* than subcluster 2a. The dynamic changes in the two groups of enrichments during the enrichment process could result from the interaction of various genera in the communities.

The restricted diversity of genera in the N_2-enrichments indicates potentially versatile metabolism and collaboration of bacteria under the nutrient-limiting condition. In a study of pure cultures of *Xanthobacter*, aerobic H_2 oxidation was shown to provide energy for CO_2 fixation and N_2 fixation while biological N_2 fixation provided fixed nitrogen for bacterial growth.[26] For the hydrogenotrophic growth of mixed communities under the reactive mineral nitrogen deficient condition, the possible mechanism could be versatile, e.g., the individual activity of N_2-fixing HOB or the cooperation of non-N_2-fixing HOB and heterotrophic N_2-fixers similar to the

symbiosis between biological N_2 fixation and legume roots.[49] In the latter case, N_2-fixers could benefit from the organics released from non-N_2-fixing HOB while non-N_2-fixing HOB could utilize the released nitrogen from N_2-fixers. However, whether a specific taxon performed as N_2-fixing HOB, heterotrophic N_2-fixers, or non-N_2-fixing HOB or switched from one metabolism to another in the mixed communities still needs further examination, e.g., by isolation of pure cultures from the enrichments and characterization of the isolates.

4.3. Potential of N_2-Fixing H_2-Oxidizing Bacteria in MP Production. The protein content of the HOB biomass produced without addition of reactive mineral nitrogen was about 10% lower than that of HOB biomass relying on reactive mineral nitrogen while the essential amino acid profile was comparable between them.[10] In addition, the protein quality of N_2-fixing HOB in terms of both protein content and essential amino acid profile was similar to MP from methane-oxidizing bacteria[50] as well as animal protein from fishmeal (Norse-LT)[51] and higher than vegetable protein from soybean seeds[52] (SI Table S4). Therefore, HOB growing on N_2 constitute a promising alternative source of high-quality protein.

However, the process of using simultaneous N_2 fixation and H_2 oxidation for MP production is not yet optimized, and additional H_2 is needed to drive biological N_2 fixation. The energy requirement for MP production by N_2-fixing HOB, i.e., HE-N_2, is estimated to be 50% higher than that by HE-NH_4^+ growing on nitrogen from either Haber-Bosch nitrogen fertilizer or ammonia recovered via electrolysis and stripping (SI S5). Although N_2-fixing HOB were not more energy-efficient than HOB growing on reactive mineral nitrogen, it could be more sustainable because it can rely mainly on energy from renewable H_2 and avoid addition of industrial nitrogen fertilizers which cause detrimental effects to the environments and require land space for the construction of manufacturing plants. Furthermore, wide availability of N_2 from air combined with potential in situ H_2 production powered by renewable energy makes in situ MP production possible.

If H_2 is produced through solar powered water electrolysis and used by N_2-fixing HOB for MP production, then the proposed process would be comparable to the photosynthetic growth of soybean that can obtain most of its nitrogen source from bacterial N_2 fixation and soil.[53] The estimated energy conversion efficiency from solar energy to biomass of N_2-fixing HOB (1.3−1.5%) was over twice that of soybean seeds (0.55%) (SI S6). The superiority of the energy conversion efficiency of N_2-fixing HOB was enhanced in terms of protein production due to the 70% higher protein content than soybeans. In addition, more efficient solar-to-H_2 conversion[54,55] may improve the overall energy efficiency of the proposed process and integration of H_2 supply from other processes, e.g., wind powered water electrolysis and hybrid thermochemical cycles, is also possible.[56−58] As one of the most important protein sources in the world,[59−61] soybean crops represent about 9% of the world's arable land[62] and supplementary nitrogen fertilizer is necessary to improve the soybean yield.[63] Therefore, MP produced via the proposed process, which can serve as an alternative to feed or food supplied by soybean, can have promising applications. In nutrient-limiting conditions, e.g., life support systems for long-term travel or habitation in space, N_2-fixing HOB can be used to reuse H_2, waste produced in these systems, and fix N_2 escaping into the atmosphere without entailing high pressure and temperature to balance the nitrogen cycle and supply food for the crew.[64]

The proposed process in this research provides a promising alternative shortcut for protein-rich food/feed production. The enriched microbial communities obtained in this study also provide microbial resources to study the mechanism of bacterial interactions and construct more efficient cultures for MP production. Further research will be necessary to study the engineering of this process and optimize the performance in terms of biomass yield and protein quality.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b06755.

Sources of inocula; overview of the operational parameters during the enrichment process; the scaling-up setup for microbial protein production; the scheme of the scaling-up setup; analysis of the quality of microbial protein; calculations of biomass yield; gas consumption rates of the enrichments originated during enrichment phase; change of H_2 oxidation rates and cell density during the enrichment phase; change of TN, COD, and cell density of the cultures during a batch incubation; average biomass yield of the enrichments HE-N_2 and HE-NH_4^+; crude protein and essential amino acid content of HE-N_2 biomass, HE-NH_4^+ biomass, soybean meal, bacterial protein meal, and fish meal (Norse-LT); evolution of the bacterial community structures of enrichments; differentially abundant OTUs between N_2-enrichments and NH_4^+-enrichments; energy requirement of MP production by HE-N_2 vs HE-NH_4^+; and solar energy conversion efficiency in the production of soybean seed and N_2-fixing HOB biomass (PDF)

OTU table of Illumina sequencing (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

Nico Boon − *Center for Microbial Ecology and Technology (CMET), Ghent University, 9000 Gent, Belgium;* orcid.org/0000-0002-7734-3103; Phone: +32 (0)9 264 59 76; Email: Nico.Boon@UGent.be; Fax: +32 (0)9 264 62 48

Authors

Xiaona Hu − *Center for Microbial Ecology and Technology (CMET), Ghent University, 9000 Gent, Belgium;* orcid.org/0000-0002-4323-6932

Frederiek-Maarten Kerckhof − *Center for Microbial Ecology and Technology (CMET), Ghent University, 9000 Gent, Belgium*

Justien Ghesquière − *Bio- and Chemical Systems Technology, Reactor Engineering, and Safety (CREaS) Division, Department of Chemical Engineering, KU Leuven, 3001 Leuven, Belgium*

Kristel Bernaerts − *Bio- and Chemical Systems Technology, Reactor Engineering, and Safety (CREaS) Division, Department of Chemical Engineering, KU Leuven, 3001 Leuven, Belgium*

Pascal Boeckx − *Isotope Bioscience Laboratory (ISOFYS), Ghent University, 9000 Gent, Belgium*

Peter Clauwaert − *Center for Microbial Ecology and Technology (CMET), Ghent University, 9000 Gent, Belgium*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.9b06755

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by the project of Geconcerteerde Onderzoeksactie (GOA) of Ghent University (BOF15/GOA/006). X.H. is supported by the China Scholarship Council (201606260046) and Special Research Fund (BOF) of Ghent University (01SC4518). J.G. holds a Ph.D. grant for Strategic Basic Research from the Research Foundation—Flanders (FWO-1SA5719N). The authors thank Samuel Bodé, Marijn Bauters, and Katja Van Nieuland for nitrogen isotope analysis, Tim Lacoere for DNA extraction, and Kenneth Simoens for amino acid analysis. We acknowledge Amanda Luther for critically reading the manuscript.

■ REFERENCES

(1) FAO. *The Future of Food and Agriculture—Trends and Challenges*; Food and Agriculture Organization of the United Nations: Rome, 2017.

(2) United Nations Population Division. *World Population Prospects 2019: Highlights*; Department of Economic and Social Affairs, The United Nations: New York, 2019.

(3) Bouwman, A. F.; Beusen, A. H. W.; Billen, G. Human alteration of the global nitrogen and phosphorus soil balances for the period 1970−2050. *Global Biogeochem. Cycles* **2009**, *23* DOI: 10.1029/2009GB003576.

(4) Bodirsky, B. L.; Popp, A.; Lotze-Campen, H.; Dietrich, J. P.; Rolinski, S.; Weindl, I.; Schmitz, C.; Muller, C.; Bonsch, M.; Humpenoder, F.; Biewald, A.; Stevanovic, M. Reactive nitrogen requirements to feed the world in 2050 and potential to mitigate nitrogen pollution. *Nat. Commun.* **2014**, *5* DOI: 10.1038/ncomms4858.

(5) Matassa, S.; Batstone, D. J.; Hulsen, T.; Schnoor, J.; Verstraete, W. Can. Direct Conversion of Used Nitrogen to New Feed and Protein Help Feed the World? *Environ. Sci. Technol.* **2015**, *49* (9), 5247−5254.

(6) Pikaar, I.; Matassa, S.; Bodirsky, B. L.; Weindl, I.; Humpenoder, F.; Rabaey, K.; Boon, N.; Bruschi, M.; Yuan, Z. G.; van Zanten, H.; Herrero, M.; Verstraete, W.; Popp, A. Decoupling Livestock from Land Use through Industrial Feed Production Pathways. *Environ. Sci. Technol.* **2018**, *52* (13), 7351−7359.

(7) El Abbadi, S. H.; Criddle, C. S. Engineering the Dark Food Chain. *Environ. Sci. Technol.* **2019**, *53* (5), 2273−2287.

(8) Aragno, M.; Schlegel, H. G. The Hydrogen-Oxidizing Bacteria. In *The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria*; Starr, M. P., Stolp, H., Trüper, H. G., Balows, A., Schlegel, H. G., Eds.; Springer: Berlin, Heidelberg, 1981; pp 865−893.

(9) Ishizaki, A.; Tanaka, K. Batch Culture of Alcaligenes-Eutrophus Atcc 17697t Using Recycled Gas Closed-Circuit Culture System. *J. Ferment. Bioeng.* **1990**, *69* (3), 170−174.

(10) Matassa, S.; Verstraete, W.; Pikaar, I.; Boon, N. Autotrophic nitrogen assimilation and carbon capture for microbial protein production by a novel enrichment of hydrogen-oxidizing bacteria. *Water Res.* **2016**, *101*, 137−146.

(11) Volova, T. G.; Barashkov, V. A. Characteristics of Proteins Synthesized by Hydrogen-Oxidizing Microorganisms. *Appl. Biochem. Microbiol.* **2010**, *46* (6), 574−579.

(12) Industrial Efficiency Technology Database. http://www.iipinetwork.org.

(13) Bicer, Y.; Dincer, I.; Vezina, G.; Raso, F. Impact Assessment and Environmental Evaluation of Various Ammonia Production Processes. *Environ. Manage.* **2017**, *59* (5), 842−855.

(14) Hasler, K.; Broring, S.; Omta, S. W. F.; Olfs, H. W. Life cycle assessment (LCA) of different fertilizer product types. *Eur. J. Agron.* **2015**, *69*, 41−51.

(15) Stabnikova, O.; Wang, J. Y.; Ding, H. B.; Tay, J. H. Biotransformation of vegetable and fruit processing wastes into yeast biomass enriched with selenium. *Bioresour. Technol.* **2005**, *96* (6), 747−751.

(16) Gelinas, P.; Barrette, J. Protein enrichment of potato processing waste through yeast fermentation. *Bioresour. Technol.* **2007**, *98* (5), 1138−1143.

(17) Ippersiel, D.; Mondor, M.; Lamarche, F.; Tremblay, F.; Dubreuil, J.; Masse, L. Nitrogen potential recovery and concentration of ammonia from swine manure using electrodialysis coupled with air stripping. *J. Environ. Manage.* **2012**, *95*, S165−S169.

(18) Desloover, J.; Woldeyohannis, A. A.; Verstraete, W.; Boon, N.; Rabaey, K. Electrochemical Resource Recovery from Digestate to Prevent Ammonia Toxicity during Anaerobic Digestion. *Environ. Sci. Technol.* **2012**, *46* (21), 12209−12216.

(19) Maurer, M.; Schwegler, P.; Larsen, T. A. Nutrients in urine: energetic aspects of removal and recovery. *Water Sci. Technol.* **2003**, *48* (1), 37−46.

(20) Willems, A.; Busse, J.; Goor, M.; Pot, B.; Falsen, E.; Jantzen, E.; Hoste, B.; Gillis, M.; Kersters, K.; Auling, G.; Deley, J. Hydrogenophaga, a New Genus of Hydrogen-Oxidizing Bacteria That Includes Hydrogenophaga-Flava Comb-Nov (Formerly Pseudomonas-Flava), Hydrogenophaga-Palleronii (Formerly Pseudomonas-Palleronii), Hydrogenophaga-Pseudoflava (Formerly Pseudomonas-Pseudoflava and Pseudomonas-Carboxydoflava), and Hydrogenophaga-Taeniospiralis (Formerly Pseudomonas-Taeniospiralis)N. *Int. J. Syst. Bacteriol.* **1989**, *39* (3), 319−333.

(21) Pohlmann, A.; Fricke, W. F.; Reinecke, F.; Kusian, B.; Liesegang, H.; Cramm, R.; Eitinger, T.; Ewering, C.; Potter, M.; Schwartz, E.; Strittmatter, A.; Voss, I.; Gottschalk, G.; Steinbuchel, A.; Friedrich, B.; Bowien, B. Genome sequence of the Bioplastic-producing "Knallgas" bacterium Ralstonia eutropha H16. *Nat. Biotechnol.* **2006**, *24* (10), 1257−62.

(22) Willems, A., The Family Comamonadaceae. In *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*; Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., Thompson, F., Eds.; Springer: Berlin, Heidelberg, 2014; pp 777−851.

(23) Wiegel, J., The Genus Xanthobacter. In *The Prokaryotes: Vol. 5: Proteobacteria: Alpha and Beta Subclasses*; Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E., Eds.; Springer: New York, NY, 2006; pp 290−314.

(24) Jenni, B.; Isch, C.; Aragno, M. Nitrogen-Fixation by New Strains of Pseudomonas-Pseudoflava and Related Bacteria. *Microbiology* **1989**, *135*, 461−467.

(25) Malik, K. A.; Schlegel, H. G. Chemolithoautotrophic Growth of Bacteria Able to Grow under N-2-Fixing Conditions. *FEMS Microbiol. Lett.* **1981**, *11* (1), 63−67.

(26) Liu, C.; Sakimoto, K. K.; Colon, B. C.; Silver, P. A.; Nocera, D. G. Ambient nitrogen reduction cycle using a hybrid inorganic-biological system. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114* (25), 6450−6455.

(27) Mus, F.; Crook, M. B.; Garcia, K.; Costas, A. G.; Geddes, B. A.; Kouri, E. D.; Paramasivan, P.; Ryu, M. H.; Oldroyd, G. E. D.; Poole, P. S.; Udvardi, M. K.; Voigt, C. A.; Ane, J. M.; Peters, J. W. Symbiotic Nitrogen Fixation and the Challenges to Its Extension to Non-legumes. *Appl. Environ. Microbiol.* **2016**, *82* (13), 3698−3710.

(28) Ehsani, E.; Dumolin, C.; Arends, J. B. A.; Kerckhof, F. M.; Hu, X.; Vandamme, P.; Boon, N. Enriched hydrogen-oxidizing microbiomes show a high diversity of co-existing hydrogen-oxidizing bacteria. *Appl. Microbiol. Biotechnol.* **2019**, *103* (19), 8241−8253.

(29) Prevoteau, A.; Clauwaert, P.; Kerckhof, F. M.; Rabaey, K. Oxygen-reducing microbial cathodes monitoring toxic shocks in tap water. *Biosens. Bioelectron.* **2019**, *132*, 115−121.

(30) Mineral medium for chemolithotrophic growth. https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium81.pdf.
(31) *Standard Methods for the Examination of Water and Wastewater*, 20th ed.; American Public Health Association, American Water Works Association, Water Environment Federation: Washington DC, 1999.
(32) Van Nevel, S.; Koetzsch, S.; Weilenmann, H. U.; Boon, N.; Hammes, F. Routine bacterial analysis with automated flow cytometry. *J. Microbiol. Methods* **2013**, *94* (2), 73−76.
(33) Vilchez-Vargas, R.; Geffers, R.; Suarez-Diez, M.; Conte, I.; Waliczek, A.; Kaser, V. S.; Kralova, M.; Junca, H.; Pieper, D. H. Analysis of the microbial gene landscape and transcriptome for aromatic pollutants and alkane degradation using a novel internally calibrated microarray system. *Environ. Microbiol.* **2013**, *15* (4), 1016−1039.
(34) Kozich, J. J.; Westcott, S. L.; Baxter, N. T.; Highlander, S. K.; Schloss, P. D. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Appl. Environ. Microbiol.* **2013**, *79* (17), 5112−5120.
(35) Schloss, P. D.; Gevers, D.; Westcott, S. L. Reducing the Effects of PCR Amplification and Sequencing Artifacts on 16S rRNA-Based Studies. *PLoS One* **2011**, *6* (12), e27310.
(36) Props, R.; Kerckhof, F. M.; Rubbens, P.; De Vrieze, J.; Sanabria, E. H.; Waegeman, W.; Monsieurs, P.; Hammes, F.; Boon, N. Absolute quantification of microbial taxon abundances. *ISME J.* **2017**, *11* (2), 584−587.
(37) Gaujoux, R.; Seoighe, C. A flexible R package for nonnegative matrix factorization. *BMC Bioinf.* **2010**, *11*, 1 DOI: 10.1186/1471-2105-11-367.
(38) vegan: Community Ecology Package. https://CRAN.R-project.org/package=vegan.
(39) Love, M. I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15* (12) DOI: 10.1186/s13059-014-0550-8.
(40) McMurdie, P. J.; Holmes, S. Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. *PLoS Comput. Biol.* **2014**, *10* (4), e1003531.
(41) Yu, J.; Dow, A.; Pingali, S. The energy efficiency of carbon dioxide fixation by a hydrogen-oxidizing bacterium. *Int. J. Hydrogen Energy* **2013**, *38* (21), 8683−8690.
(42) Hoffman, B. M.; Lukoyanov, D.; Yang, Z. Y.; Dean, D. R.; Seefeldt, L. C. Mechanism of Nitrogen Fixation by Nitrogenase: The Next Stage. *Chem. Rev.* **2014**, *114* (8), 4041−4062.
(43) Kremer, T. A.; LaSarre, B.; Posto, A. L.; McKinlay, J. B. N-2 gas is an effective fertilizer for bioethanol production by Zymomonas mobilis. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (7), 2222−2226.
(44) Tanaka, K.; Miyawaki, K.; Yamaguchi, A.; Khosravi-Darani, K.; Matsusaki, H. Cell growth and P(3HB) accumulation from CO2 of a carbon monoxide-tolerant hydrogen-oxidizing bacterium, Ideonella sp O-1. *Appl. Microbiol. Biotechnol.* **2011**, *92* (6), 1161−1169.
(45) Morinaga, Y.; Yamanaka, S.; Ishizaki, A.; Hirose, Y. Growth Characteristics and Cell Composition of Alcaligenes eutrophus in Chemostat Cultures. *Agric. Biol. Chem.* **1978**, *42* (2), 439.
(46) Volova, T. G.; Kiselev, E. G.; Shishatskaya, E. I.; Zhila, N. O.; Boyandin, A. N.; Syrvacheva, D. A.; Vinogradova, O. N.; Kalacheva, G. S.; Vasiliev, A. D.; Peterson, I. V. Cell growth and accumulation of polyhydroxyalkanoates from CO2 and H2 of a hydrogen-oxidizing bacterium, Cupriavidus eutrophus B-10646. *Bioresour. Technol.* **2013**, *146*, 215−222.
(47) Tamagnini, P.; Axelsson, R.; Lindberg, P.; Oxelfelt, F.; Wunschiers, R.; Lindblad, P. Hydrogenases and hydrogen metabolism of cyanobacteria. *Microbiol Mol. Biol. R* **2002**, *66* (1), 1−20.
(48) Chou, J. H.; Jiang, S. R.; Cho, J. C.; Song, J.; Lin, M. C.; Chen, W. M. Azonexus hydrophilus sp nov., a nifH gene-harbouring bacterium isolated from freshwater. *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 946−951.
(49) Dhole, A.; Shelat, H.; Vyas, R.; Jhala, Y.; Bhange, M. Endophytic occupation of legume root nodules by nifH-positive non-rhizobial bacteria, and their efficacy in the groundnut (Arachis hypogaea). *Ann. Microbiol.* **2016**, *66* (4), 1397−1407.
(50) Overland, M.; Kjos, N. P.; Olsen, E.; Skrede, A. Changes in fatty acid composition and improved sensory quality of backfat and meat of pigs fed bacterial protein meal. *Meat Sci.* **2005**, *71* (4), 719−729.
(51) Norse-LT fishmeal. http://www.norsildmelinnovation.no/product_mp01_norseLT94.html.
(52) Baker, K. M.; Liu, Y.; Stein, H. H. Nutritional value of soybean meal produced from high protein, low oligosaccharide, or conventional varieties of soybeans and fed to weanling pigs. *Anim. Feed Sci. Technol.* **2014**, *188*, 64−73.
(53) La Menza, N. C.; Monzon, J. P.; Specht, J. E.; Grassini, P. Is soybean yield limited by nitrogen supply? *Field Crop Res.* **2017**, *213*, 204−212.
(54) Buttler, A.; Spliethoff, H. Current status of water electrolysis for energy storage, grid balancing and sector coupling via power-to-gas and power-to-liquids: A review. *Renewable Sustainable Energy Rev.* **2018**, *82*, 2440−2454.
(55) Green, M. A.; Dunlop, E. D.; Levi, D. H.; Hohl-Ebinger, J.; Yoshita, M.; Ho-Baillie, A. W. Y. Solar cell efficiency tables (version 54). *Prog. Photovoltaics* **2019**, *27* (7), 565−575.
(56) Rahmouni, S.; Negrou, B.; Settou, N.; Dominguez, J.; Gouareh, A. Prospects of hydrogen production potential from renewable resources in Algeria. *Int. J. Hydrogen Energy* **2017**, *42* (2), 1383−1395.
(57) Average Tested Heat Rates by Prime Mover and Energy Source, 2008−2018. https://www.eia.gov/electricity/annual/html/epa_08_02.html.
(58) Dincer, I.; Acar, C. Review and evaluation of hydrogen production methods for better sustainability. *Int. J. Hydrogen Energy* **2015**, *40* (34), 11094−11111.
(59) Rizzo, G.; Baroni, L. Soy, Soy Foods and Their Role in Vegetarian Diets. *Nutrients* **2018**, *10* (1), 43.
(60) Mottet, A.; de Haan, C.; Falcucci, A.; Tempio, G.; Opio, C.; Gerber, P. Livestock: On our plates or eating at our table? A new analysis of the feed/food debate. *Glob Food Secur-Agr* **2017**, *14*, 1−8.
(61) Fry, J. P.; Love, D. C.; MacDonald, G. K.; West, P. C.; Engstrom, P. M.; Nachman, K. E.; Lawrence, R. S. Environmental health impacts of feeding crops to farmed fish. *Environ. Int.* **2016**, *91*, 201−214.
(62) FAOSTAT. http://www.fao.org/faostat/en/#home.
(63) Salvagiotti, F.; Cassman, K. G.; Specht, J. E.; Walters, D. T.; Weiss, A.; Dobermann, A. Nitrogen uptake, fixation and response to fertilizer N in soybeans: A review. *Field Crop Res.* **2008**, *108* (1), 1−13.
(64) Clauwaert, P.; Muys, M.; Alloul, A.; De Paepe, J.; Luther, A.; Sun, X. Y.; Ilgrande, C.; Christiaens, M. E. R.; Hu, X. N.; Zhang, D. D.; Lindeboom, R. E. F.; Sas, B.; Rabaey, K.; Boon, N.; Ronsse, F.; Geelen, D.; Vlaeminck, S. E. Nitrogen cycling in Bioregenerative Life Support Systems: Challenges for waste refinery and food production processes. *Progress in Aerospace Sciences* **2017**, *91*, 87−98.