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Article

Production of Endotoxin-Free Microbial Biomass for Food Applications by Gas Fermentation of Gram-Positive H_2-Oxidizing Bacteria

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ABSTRACT: The production of edible microbial biomass in bioreactors is an attractive alternative to agriculture, which is a major consumer of fresh water, an emitter of greenhouse gases, and a cause of eutrophication. This study examines microbial food production by gas fermentation of two endotoxin-free Gram-positive H_2-oxidizing autotrophic bacterial strains, *Nocardioides nitrophenolicus* KGS-27 and *Rhodococcus opacus* DSM 43205. The supply of the gaseous substrates H_2 and O_2 by *in situ* water electrolysis was investigated as an alternative to providing them from an external source. *N. nitrophenolicus* KGS-27 produced ≤9.9 ± 2.0 mg of biomass L^{-1} h^{-1} and was not affected by *in situ* water electrolysis, which potentially produces growth-inhibiting reactive oxygen species. With *R. opacus* DSM 43205, in turn, electrolysis slowed growth considerably and resulted in a volumetric productivity of 2.6 ± 0.8 mg of biomass L^{-1} h^{-1}, which was a quarter of what was obtained with the gases supplied externally. The macromolecular compositions of biomasses produced by the two cultivation modes using the two bacterial strains were rather similar containing approximately 33−37% protein, 28−56% carbohydrates, and 4−5% fatty acids of the cell dry weight. Relatively high protein concentrations and balanced amino acid profiles, comparable to those of casein and soy protein, were achieved with both strains.

KEYWORDS: *Knallgas bacteria, gas fermentation, electrolysis, cellular agriculture, single-cell protein*

INTRODUCTION

It has been estimated that crop production should be doubled between the years 2005 and 2050 to meet the demand for calories and protein by the rapidly growing human population.[1] However, the possibility of increasing the area of arable land is in practice limited. Furthermore, agricultural output is expected to decrease in the future with the reduction of global yields of major crops resulting from climate change.[2] Agriculture is also a major consumer of fresh water, a cause of eutrophication and soil erosion, and a significant emitter of greenhouse gases.[3] In particular, considerable resources are needed for animal protein production, although it accounts for only one-third of global protein production.[4] Therefore, new sustainable ways to produce food are needed.

Because of the high protein content of >30% dry weight, microbial cell biomasses have been considered as food sources and alternatives to animal- and plant-based protein.[5,6] The essential amino acid profiles of yeast, fungi, and microalgae typically fulfill human nutritional needs.[7,8] Although microbial protein provides only a small proportion of current human nutrition, the growing global demand for protein is likely to increase the importance of microbial biomass.[7,9] In addition to protein, microbial biomass can also provide a source of lipids and carbohydrates as well as vitamins and minerals.[10]

The best known examples of commercial microbial food products are the fungal protein product Quorn and some sandwich spreads prepared from spent brewer's yeast. However, these production organisms are grown with starch-derived glucose as the carbon source, and their microbial biomass production is therefore ultimately dependent on agriculture.[5]

Rather than depending on photosynthetic production of sugar feedstocks, CO_2 can also be used directly as the starting material for microbial biomass production. CO_2 can be captured from air or industrial exhaust gases (flue gas) by absorbents followed by desorption[11] or directly assimilated from ambient air by photosynthetic microbes, such as cyanobacteria and other microalgae. The best-known example of algal biomass production for human consumption is the cultivation of cyanobacteria of the genus *Arthrospira*. However, the fundamental problem with systems based on photosynthesis, whether open ponds or bioreactors, is the limited penetration of light into the cultivation medium, requiring large surface areas to efficiently capture the light energy.[12]

CO_2 is also fixed by nonphotosynthetic microbes, such as autotrophic H_2-oxidizing bacteria (HOBs) (so-called "Knallgas" bacteria). HOBs, which assimilate CO_2 concomitantly

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with aerobic H_2 oxidation, have been of considerable interest for the biotechnological production of fuels and different chemicals. The research has mostly focused on the use of the Gram-negative *Cupriavidus necator* (formerly known as *Ralstonia eutropha* and *Alcaligenes eutrophus*) species as the host.[13−16] Bacterial food production by HOBs has been evaluated recently and shown to compare favorably to soy protein production in terms of land and water use as well as prevention of eutrophication.[17,18]

The autotrophic cultivation of HOBs requires a constant supply of the gaseous feedstocks H_2 and O_2, which can be produced sustainably by water electrolysis using wind or solar power.[19] This reaction can be carried out either separately from the cultivation or inside the bioreactor as part of a bioelectrochemical system (BES).[20] The possible benefit of the latter approach is that the evolved H_2 can be delivered directly to the bacteria, at a mass transfer efficiency that is potentially higher than what can be achieved by supply from an external source.[21] However, the downside of *in situ* water electrolysis is that potentially growth inhibiting reactive oxygen species may be produced.[22,23]

Only very few scientific reports have examined HOB cultures as a food source.[8,24] The previously studied HOB species possess a Gram-negative cell-envelope structure, which typically contains lipopolysaccharide endotoxins as major components.[25] Bacterial lipopolysaccharides have been related to diabetes, liver damage, neurological disorders, and chronic gut inflammation.[26] Exposure to lipopolysaccharides has also been recognized as an occupational risk during the production of feed from Gram-negative bacteria.[27] Gram-positive HOBs would seem to be more appropriate production hosts, because they are inherently lipopolysaccharide-free and therefore potentially more suitable for food production. (The Gram-negative and -positive distinction is used here to refer to cell-envelope structure, not to taxonomic classification.) In this study, we have compared a novel Gram-positive isolate, *Nocardioides nitrophenolicus* KGS-27, to the Gram-positive HOB, *Rhodococcus opacus* DSM 43205, for bacterial biomass production by supplying the gaseous substrates externally and by using *in situ* water electrolysis in a BES. We show that high protein concentrations and balanced amino acid profiles can be achieved with both strains. Furthermore, we establish the tolerance of *N. nitrophenolicus* toward *in situ* water electrolysis.

MATERIALS AND METHODS

Microbial Strains and Cultivation Media. Strain KGS-27 was isolated from a forest soil sample (Espoo, Finland) by continued cultivation at 30 °C in the standard minimal medium DSMZ81[28] under an atmosphere of 11.5% H_2, 60.5% N_2, 4.9% O_2, and 23.1% CO_2.

Identification of the isolate was performed on the basis of its 16S rRNA gene sequence. For this, total DNA was isolated using the NucleoSpin Microbial DNA Mini kit (Macherey-Nagel GmbH & Co. KG, Dueren, Germany). A part of the 16S rRNA gene was amplified using universal primers BSF 8/20 (5′ AGA GTT TGA TCC TGG CTC AG 3′) and BSR 1541/20 (5′ AAG GAG GTG ATC CAG CCG CA 3′) and sequenced at Microsynth SeqLab (Göttingen, Germany). The sequence (1.38 kb) was used as the query in a BLAST search against 16S rRNA gene sequences of type strains in the NCBI nucleotide database.

Cultivations of *N. nitrophenolicus* KGS-27 and the type strain *R. opacus* DSM 43205 were carried out in a modified DSMZ81 mineral medium (without $NaVO_3$) in which the chloride salts were replaced with corresponding sulfate salts to avoid chlorine formation during BES cultivation. The final medium composition per liter was as follows: 2.3 g of KH_2PO_4, 2.9 g of $Na_2HPO_4·2H_2O$, 2.47 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4·7H_2O$, 11.7 mg of $CaSO_4·2H_2O$, 4.27 mg of $MnSO_4·H_2O$, 0.5 g of $NaHCO_3$, 50 mg of ferric ammonium citrate, 0.5 mg of $ZnSO_4·7H_2O$, 0.15 mg of $MnCl_2·4H_2O$, 1.5 mg of H_3BO_3, 1 mg of $CoCl_2·6H_2O$, 50 μg of $CuCl_2·2H_2O$, 0.1 mg of $NiCl_2·6H_2O$, 0.15 mg of $Na_2MoO_4·2H_2O$, 0.5 mg of riboflavin, 2.5 mg of thiamine-HCl, 2.5 mg of nicotinic acid, 2.5 mg of pyridoxine-HCl, 2.5 mg of Ca-pantothenate, 5 μg of biotin, 10 μg of folic acid, and 50 μg of vitamin B_{12}. [Both strains also grow in the absence of the vitamins (data not shown).] The medium for *R. opacus* DSM 43205 was further supplemented with 1.0 g L^{-1} agar to prevent cell aggregation, as the cell aggregates interfered with optical density measurements.

Culture Vessels and Growth Conditions. Shake flask (SF) fermentations were carried out in a 14.5 L airtight metal container with a feed of 60 mL min^{-1} of a gas mixture comprising 13% H_2, 20% CO_2, and 67% air. The cultures were shaken at 130 rpm (shaking diameter of 1.6 cm) in 250 mL Erlenmeyer flasks containing 50 mL of culture at 30 °C. The growth media were inoculated with 5 mL of precultures grown to late exponential phase.

BES cultivations were performed in jacketed 100 mL vessels (URG, Chapel Hill, NC) with custom-made Teflon lids (Figure 1)[29] at a

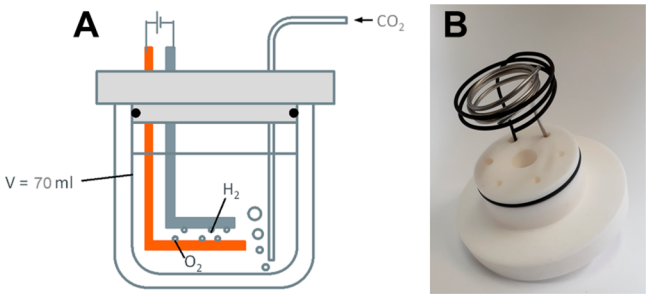

Figure 1. (A) BES reactor. (B) BES electrode coil assembly with a solid steel cathode (inner coil) and an iridium oxide-coated titanium wire anode (outer coil).

constant current of 18 mA and a voltage varying between 2.3 and 2.6 V. The anode was manufactured from titanium wire coated with a thin layer of iridium oxide (Ø 1.5 mm, Magneto Special Anodes, Schiedam, The Netherlands), and a stainless steel capillary (Ø 1.6 mm, 316L-SS, Pfeiffer Vacuum GmbH, Asslar, Germany) was used as the cathode. The surface areas of anode and cathode were 12.3 and 14.1 cm^2, respectively. To achieve electrolysis, the electrodes were bent into helical coils and nested, with the anode coil facing outside. The current was controlled using a Wavenow potentiostat (Pine Research Instrumentation, Grove City, PA) and AfterMath (version 1.3.7060, Pine Research Instrumentation). BES cultivations were initiated by addition of preculture at late exponential phase to 70 mL of cultivation medium to reach an initial OD_{600} of 0.2. The cultivations were performed at 30 °C under stirring at 400 rpm while being sparged at 6 mL/min with a humidified stream of gas containing 20% CO_2 and 80% N_2. Volumetric productivities of the cell mass were calculated from optical densities (OD_{600}) using the correlations 0.3829 ± 0.026 and 0.4522 ± 0.017 (grams of dry weight per liter) OD_{600}^{-1} for *N. nitrophenolicus* KGS-27 and *R. opacus* DSM 43205, respectively.

Catalase Test. The cultures were grown on Trypticase Soy Agar (BD) at 30 °C until colonies were formed. The cell mass was spread on a microscopic slide, and a drop of 3% hydrogen peroxide was suspended with the cell mass. The suspension was monitored for immediate effervescence, indicating O_2 evolution and catalase activity.[30]

Electron Microscopy of Autotrophically Grown HOB Cells. For electron microscopy, bacterial cells were grown autotrophically at 30 °C for 4 days in shake flasks and fixed by adding 1 volume of a fixative solution including 5% glutaraldehyde (EM-grade) and 2% formaldehyde in 0.2 M sodium cacodylate buffer (pH 7.4). After incubation for 1 h at room temperature, fixation was continued for an

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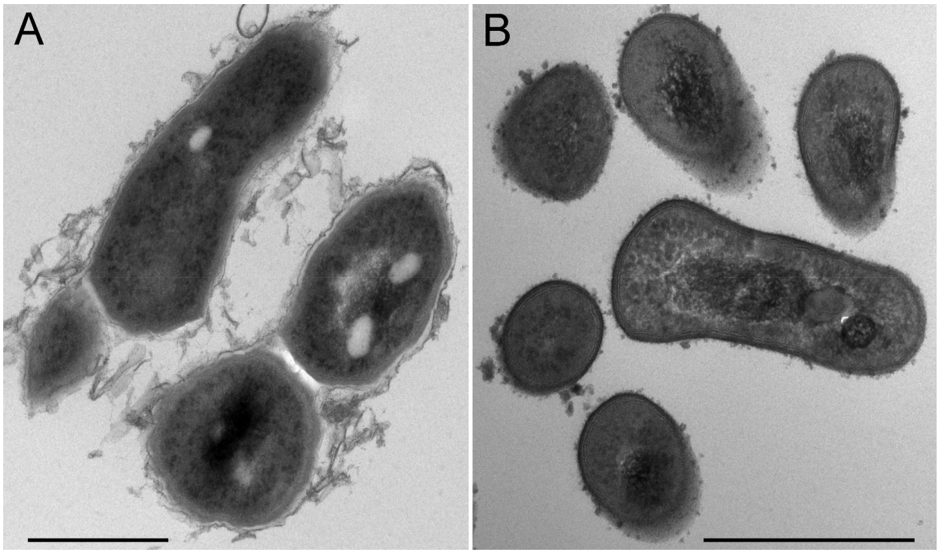

Figure 2. TEM electron micrographs of cells of (A) *R. opacus* DSM 43205 and (B) *N. nitrophenolicus* KGS-27. A scale bar (500 nm) is shown at the bottom (black line).

additional 1 h in a fixative solution including 2% glutaraldehyde, 1% formaldehyde, and 0.1% ruthenium red in 0.1 M sodium cacodylate buffer (pH 7.4). Samples were further postfixed with 2% osmium tetroxide supplemented with 0.1% ruthenium red for 3 h at room temperature. After the samples had been washed with buffer and distilled water, the samples were dehydrated through a series of transfers to ethanol and acetone and gradually infiltrated into epoxy resin (low-viscosity resin, TAAB, T044). After polymerization of resin overnight at 60 °C, a pyramid was made using a razor blade, and 60 nm thin sections were cut and collected on Pioloform-coated single-slot copper grids. Sections were poststained with uranyl acetate and lead citrate and examined using a model JEM-1400 transmission electron microscope (Jeol Ltd., Tokyo, Japan) operated at 120 kV. Images were collected with an Orius SC 1000B CCD camera (Gatan Inc., Pleasanton, CA).

Compositional Analysis of the Microbial Biomass Product. For compositional analysis, cells were harvested at stationary phase by centrifugation (*R. opacus* DSM 43205 in BES and shake flasks at 530 and 383 h, respectively, and *N. nitrophenolicus* KGS-27 at 336 and 504 h in BES and shake flasks, respectively), washed twice with deionized water, and freeze-dried for further analysis.

The elemental composition of the dried cell biomass was analyzed as described previously.[31] Briefly, C, H, N, and S were determined from the lyophilized samples with the combustion method using a FLASH 2000 series analyzer. The oxygen content was determined by pyrolysis in the same analyzer. The results were quantified using Certified Elemental Microanalysis standards using K factor (CHNS/CHNS-O Standards Kit, cystine, sulfanilamide, methionine, and BBOT, 33840010, Thermo Fischer Scientific, Waltham, MA).

The amino acid content of the cell biomass was analyzed as described previously.[32] Samples were oxidized with fresh performic acid and hydrolyzed using 6 N HCl. For the determination of tryptophan (Trp), a separate alkaline hydrolysis was performed using 4 M NaOH. Hydrolyzed samples were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reagent. Ultra-performance liquid chromatography (UPLC) analysis was performed on an Acquity UPLC system (Waters, Milford, MA) with an Acquity UPLCTM BEH C18 column (2.1 mm × 100 mm, 1.7 μm). Norvaline (10 μL) was used as an internal standard for the amino acids quantified from the acid hydrolysate. In acid hydrolysis, asparagine (Asn) is converted to aspartic acid (Asp) and glutamine (Gln) to glutamic acid (Glu). Cysteine (Cys) and methionine (Met) were determined as cysteic acid and methionine sulfone after the oxidation procedure. The total protein content of the cell biomass was derived from the sum of amino acids. Amino acid scores for essential amino acids were calculated by dividing the content of each amino acid by its content in a reference protein representing the requirements of adults as reported by the World Health Organization (WHO).[33]

The fatty acid (carbon chain lengths of ≤22) content of the cell biomass was determined according to a previously described protocol.[34] Aliquots (5 mg) of dried cell biomass samples were spiked with internal standards (21.6 μg of glyceryl triheptadecanoate and 5.5 μg of heptadecanoic acid). Fatty acids were transesterified with 0.5 M sodium methoxide (at 45 °C for 5 min), and the samples were acidified with 15% $NaHSO_4$. Fatty acid methyl esters (FAMEs) and free fatty acids (FFAs) were extracted with petroleum ether before analysis by GC-MS (Agilent 7890A gas chromatograph combined with a 5975C MSD instrument) equipped with an Agilent FFAP silica capillary column (25 m × 0.2 mm × 0.33 μm).

Sugars were quantified from hydrolyzed cell biomass samples for carbohydrate analysis. Approximately 20 mg of dried cell biomass was incubated at 30 °C for 1 h in the presence of 200 μL of 70% (w/w) H_2SO_4, followed by addition of 5.6 mL of deionized H_2O and autoclavation at 120 °C for 50 min. The samples were filtered through a 0.45 μm membrane, and monosaccharides were quantified by high-performance anion exchange chromatography (HPAEC) with pulse amperometric detection (Dionex ICS 3000A instrument equipped with a Dionex CarboPac PA20 column). The carbohydrate content in the samples was determined from the monosaccharide amounts using an anhydro correction of 0.88 for pentoses and 0.9 for hexoses.

RESULTS AND DISCUSSION

Isolation and Identification of a Novel HOB Strain from an Environmental Sample. The Finnish environmental isolate KGS-27 was assigned to the species *N. nitrophenolicus* based on 99.35% 16S rRNA sequence identity (European Nucleotide Archive accession number PRJEB42617) with the type strain *N. nitrophenolicus* NSP41 (NCBI GenBank accession number NR_024847). To the best of our knowledge, *N. nitrophenolicus* NSP41 has not been previously shown to belong to HOB. As presented in the phylogenetic tree in Figure S1, HOBs are organized in well-separated clades, and the actinobacteria *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 are very distinct from the most widely studied HOB, the β-proteobacterium *C. necator*. *R. opacus* DSM 43205 was chosen for our studies, because it is a well-established Gram-positive HOB.[35,36] Furthermore, *R.*

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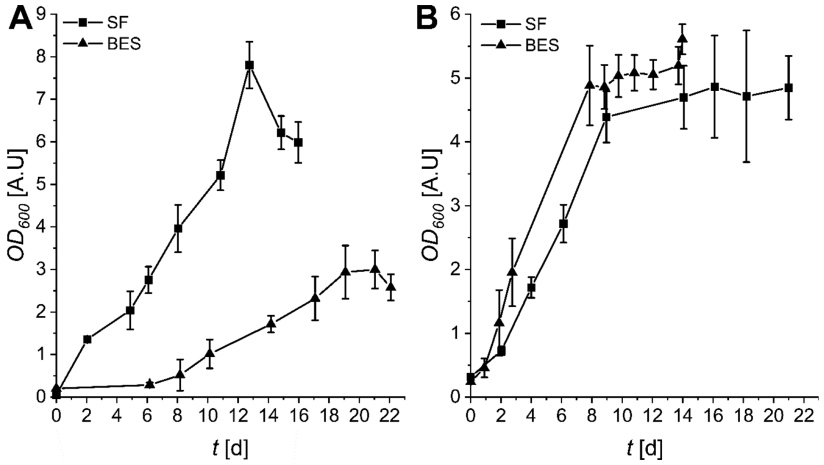

Figure 3. Autotrophic growth of (A) *R. opacus* DSM 43205 and (B) *N. nitrophenolicus* KGS-27 through hydrogen oxidation in a bioelectrochemical system (BES) or a shake flask (SF). The error bars refer to standard errors calculated from three parallel cultivations.

opacus strains have been of recent interest for the production of bacterial mass for food from agricultural wastes.[37]

Electron microscopy images of autotrophically grown *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 cells are presented in Figure 2. The presence of the structures visible around the *R. opacus* DSM 43205 cells suggests the synthesis of extracellular polymeric substances (EPS). Production of EPS, consisting mainly of polysaccharides, has been previously reported for other *R. opacus* strains, as well.[38] The *N. nitrophenolicus* KGS-27 cells exhibited coccoid and rod form morphology, typical also for the corresponding type strain NSP41T.[39]

Shake Flask and BES Cultivation of *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27. *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 were cultivated in the mineral medium sparged with CO_2 and N_2 using the BES reactor equipped with an *in situ* electrolyzer for H_2 and O_2 generation as well as in shake flasks under an atmosphere of H_2, CO_2, and air. Because *R. opacus* DSM 43205 cells tended to flocculate, presumably at least partly because of EPS formation, the growth medium was supplemented with agar to increase its viscosity and thereby reduce the level of cell aggregation. It was verified in shake flask cultivations that *R. opacus* DSM 43205 was not able to use agar as a carbon source (data not shown). Despite the addition of agar, *R. opacus* DSM 43205 cells still adhered to some extent to the walls of the reactor and the flasks.

In the BES cultivation of *R. opacus* DSM 43205, a lag phase of approximately 7 days could be observed, but in other cultivations, growth started quickly after inoculation, as presented in Figure 3. The results indicate that growth of *R. opacus* DSM 43205 was significantly slower in BES than in shake flasks. In contrast, with *N. nitrophenolicus* KGS-27, there was no clear difference between the growth rates in the two cultivation modes.

Final cell densities and volumetric productivities of cell biomass determined at the onset of the stationary phase are presented in Table 1. The slower growth rate and long lag phase of *R. opacus* DSM 43205 in BES were also reflected by the significantly lower volumetric productivity, which was less than one-third of what was achieved in the other cultivations (*R. opacus* DSM 43205 in a shake flask and *N. nitrophenolicus* KGS-27 in shake flasks and in BES). The final cell densities (as dry cell weight per volume) were, within the error limits

Table 1. Growth Parameters of *N. nitrophenolicus* KGS-27 and *R. opacus* DSM 43205 Cultivated in Shake Flasks (SF) and in the Bioelectrochemical System (BES)

strain	cultivation mode	final cell density[a] (g L^{-1})	volumetric productivity (mg L^{-1} h^{-1})
N. nitrophenolicus	SF	2.10 ± 0.08	7.8 ± 1.3 (at 215 h)
N. nitrophenolicus	BES	2.17 ± 0.29	9.9 ± 2.0 (at 189 h)
R. opacus	SF	2.82 ± 0.11	11.5 ± 1.3 (at 306 h)
R. opacus	BES	1.99 ± 0.38	2.6 ± 0.8 (at 458 h)

[a]Freeze-dried cell mass per volume.

(Table 1), the same in the *N. nitrophenolicus* KGS-27 cultivations in BES and in shake flasks and in *R. opacus* DSM 43205 cultivation in BES. However, an approximately 40% higher cell density was achieved in the *R. opacus* DSM 43205 shake flask cultivation than in the other cultivations.

The growth rates of *N. nitrophenolicus* KGS-27 in BES reactors and shake flasks were very similar. It therefore seems likely that hydrogen can be supplied to the cells by the current *in situ* electrolysis system in amounts that are comparable to those provided by the continuous stream of gas used in the shake flask cultivations.

So far, only a few studies have attempted to determine the volumetric productivity of cell mass in autotrophic HOB cultivations. However, on the basis of the available data and approximate correlation factor between cell biomass and optical density given for a *C. necator* strain engineered for isopropanol production, a rough estimate for the volumetric product of 18 mg L^{-1} h^{-1} in a BES cultivation can be calculated.[22] This value is in the same order of magnitude as that obtained in the study presented here.

The HOB cultivation experiments of this study were performed in shake flasks and in a prototypical BES apparatus. These setups do not probably reveal the full potential of HOB for bacterial biomass production. Significantly higher volumetric productivities of biomass, ranging from 0.28 g L^{-1} h^{-1} for *Ideonella* sp. 0-1[40] to 2.3 g L^{-1} h^{-1} for *C. necator*,[41] have been reported for some Gram-negative strains in batch cultures sparged with mixtures of H_2, O_2, and CO_2. Furthermore, final biomass concentrations as high as 91 g L^{-1}, far exceeding those obtained in this study, have been achieved for *C. necator*.[41] However, these cultivations were carried out in bioreactors

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Table 2. Elemental Composition of Bacterial Biomasses Produced by Autotrophical Growth of *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 during Cultivation in a Bioelectrochemical System (BES) and Shake Flasks (SF)

	elemental composition (% of cell dry weight)			
	R. opacus		*N. nitrophenolicus*	
element	BES	SF	BES	SF
carbon	47.2 ± 0.7	46.7 ± 0.3	43.8 ± 0.1	42.3 ± 0.5
hydrogen	6.9 ± 0.1	7.0 ± 0.1	6.5 ± 0.1	6.2 ± 0.1
nitrogen	7.6 ± 0.8	8.0 ± 0.2	9.3 ± 0.2	9.0 ± 0.5
sulfur	0.13 ± 0.01	0.13 ± 0.02	0.79 ± 0.03	0.77 ± 0.13
oxygen	32.0 ± 1.5	31.0 ± 2.4	28.0 ± 0.7	30.2 ± 0.3
total	94	93	88	88

Table 3. Macromolecular Composition of Bacterial Biomass Produced by Autotrophic Growth of *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 during Cultivation in the Bioelectrochemical System (BES) and Shake Flasks (SF)

		biomass composition (% of cell dry weight)			
strain	cultivation system	fatty acids	proteins	carbohydrates	total
R. opacus	BES	3.8 ± 0.6	32.7 ± 1.7	27.8 ± 0.2	64
R. opacus	SF	3.6 ± 0.0	32.5 ± 0.8	56.2 ± 1.4	92
N. nitrophenolicus	BES	4.8 ± 0.1	36.9 ± 1.1	29.9 ± 3.2	72
N. nitrophenolicus	SF	4.0 ± 0.1	36.8 ± 1.8	38.3 ± 5.5	79

equipped with special stirring systems providing high volumetric mass transfer coefficients for the gases, unattainable with the cultivation methods used in this study. In addition to making the distribution of the gases to the medium more efficient, optimizing their ratios in the feed would most likely increase the growth rate. Improved growth can also been obtained by cultivation under an increased gas pressure.[42]

The gaseous HOB growth substrates H_2 and O_2 can be either generated *in situ* by water electrolysis or sparged into the cultivation from an external reservoir or electrolyzer. Comparing these two approaches was a key target of this work. Although *in situ* electrolysis has benefits, such as a simpler construct and potentially more efficient mass transfer of the gases to the cells, it carries the risk that reactive oxygen species, such as hydrogen peroxide and more short-lived hydroxyl and superoxide radicals, toxic to the cells are produced at the cathode.[22,23,43]

It has been shown for *C. necator* grown in BES with *in situ* electrolysis that full cell viability can be achieved by the removal of the cathodically produced hydrogen peroxide by added catalase.[22] Catalase activity could be detected in both *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 cells by the qualitative catalase test used, although only the growth of *R. opacus* DSM 43205 was slowed in BES. However, it is possible that under the conditions of the BES cultivation, the catalase levels in *N. nitrophenolicus* KGS-27 are higher than in *R. opacus* DSM 43205 and the toxic hydrogen peroxide is therefore more efficiently degraded.

Elemental and Macromolecular Composition of Cell Biomass. The macromolecular composition of microbial cells varies with growth phase. In the stationary phase, protein and RNA contents typically decrease whereas the DNA content remains approximately constant.[44−46] The maximal protein content can therefore most likely be achieved by harvesting the cells in the deceleration phase, which follows exponential growth. In this study, such timing was not attempted, but cells were harvested in the stationary phase to simplify the comparison of the cultivation modes and strains. It should also be noted that protein is not the only valuable macronutrient produced by the cells. Carbohydrates and lipids are also an important part of the bacterial mass product.

Foods are a diverse and complex group of organic materials. Determination of the elemental composition provides the basis for comparison of the chemistries of different foods and, in conjunction with the macromolecular analysis, allows for the study of effects of different treatments on the food materials. As shown in Table 2, there were no significant differences between the elemental compositions of *R. opacus* DSM 43205 or *N. nitrophenolicus* KGS-27 cell biomasses from cultivations in shake flasks and in BES. The observed cell biomass carbon content of 42−47% corresponds well with those reported for other bacteria (45−50%).[47] The contents of the elements determined were also similar between the two strains, with the exception that the *N. nitrophenolicus* KGS-27 biomass contained 5 times more sulfur than the biomass of *R. opacus* DSM 43205. This difference cannot be explained by differences in the contents of the sulfur-containing amino acids cysteine and methionine, because their abundance was virtually the same in both bacteria (Table 4). Nevertheless, sulfur comprised only a minor fraction of the total elements. The unaccounted residue of the elemental analysis of approximately 10% would correspond mostly to ash components (minerals and phosphorus). The ash content of bacteria has been reported to represent approximately 3−7% of bacterial dry weight.[48]

The protein, fatty acid, and carbohydrate contents of the bacterial cell biomasses are presented in Table 3. In terms of protein contents, the cells produced by the two cultivation modes appeared to be very similar. Furthermore, comparable levels of fatty acids were obtained in BES and shake flask cultivations, although the fatty acid content of *N. nitrophenolicus* KGS-27 cells grown in a BES was ∼20% higher than in cells obtained by shake flask cultivations.

However, a considerably increased carbohydrate content was apparent for *R. opacus* DSM 43205 grown in shake flasks (56.2%) when comparing it with the composition of biomass that was obtained from BES cultivations (27.8% carbohydrates). Possibly, the growth conditions promoted increased production of carbohydrate-containing EPS in the shake flasks.

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Table 4. Amino Acid Composition of Bacterial Protein Produced during Autotrophic Growth of *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 in the Bioelectrochemical System (BES) and Shake Flasks (SF)[a]

	amino acid content (% of total)						
	R. opacus		*N. nitrophenolicus*				
amino acid	BES	SF	BES	SF	*C. necator* Z1[8]	soy protein isolate	casein
histidine*	2.49 ± 0.13	2.43 ± 0.08	2.59 ± 0.08	2.36 ± 0.11	2.0	2.6	2.6
isoleucine*	4.45 ± 0.24	4.51 ± 0.09	3.99 ± 0.15	3.77 ± 0.16	4.5	4.9	4.8
leucine*	8.51 ± 0.44	8.70 ± 0.18	9.06 ± 0.34	8.52 ± 0.32	8.6	8.2	8.8
lysine*	4.85 ± 0.29	4.58 ± 0.14	4.37 ± 0.09	4.93 ± 0.21	7.0	6.3	7.4
methionine*	2.33 ± 0.12	2.21 ± 0.11	2.35 ± 0.07	2.13 ± 0.09	2.6	1.3	2.6
phenylalanine*	4.91 ± 0.27	5.02 ± 0.13	4.14 ± 0.14	3.97 ± 0.19	4.4	5.2	5.0
threonine*	5.18 ± 0.28	4.94 ± 0.13	5.34 ± 0.17	5.21 ± 0.15	5.3	3.7	3.8
tryptophan*	0.46 ± 0.02	0.44 ± 0.03	0.27 ± 0.13	0.63 ± 0.07	1.4	1.3	1.2
valine*	6.75 ± 0.45	6.78 ± 0.15	7.06 ± 0.25	6.67 ± 0.22	6.4	5.1	5.7
alanine	9.36 ± 0.61	9.28 ± 0.22	10.19 ± 0.38	10.10 ± 0.20	9.1	4.3	2.6
arginine	6.87 ± 0.37	6.53 ± 0.21	8.14 ± 0.27	7.35 ± 0.29	7.3	8.4	3.6
asparagine and aspartic acid	9.39 ± 0.56	8.74 ± 0.26	10.14 ± 0.32	10.37 ± 0.34	10[b]	12	6.5
cysteine	1.14 ± 0.06	1.14 ± 0.05	0.85 ± 0.02	0.92 ± 0.06	0.28	1.3	0.4
glutamine and glutamic acid	18.74 ± 1.19	20.55 ± 0.52	14.70 ± 0.68	15.95 ± 1.78	13[c]	19	21
glycine	4.58 ± 0.25	4.47 ± 0.14	6.05 ± 0.20	5.98 ± 0.06	6.1	4.2	1.8
proline	3.97 ± 0.24	3.74 ± 0.11	4.71 ± 0.14	4.62 ± 0.24	4.6	5.2	11.7
serine	4.33 ± 0.31	4.56 ± 0.07	4.36 ± 0.08	4.09 ± 0.24	4.0	5.1	5.4
tyrosine	1.67 ± 0.51	1.37 ± 0.04	1.70 ± 0.06	2.44 ± 0.72	3.6	3.9	5.3

[a]The compositions of soy protein isolate[53] and casein[54] and the Gram-negative HOB, *C. necator* (*A. eutrophus*) Z1, are presented for comparison. Asparagine and aspartic acid as well as glutamine and glutamic acid are presented as combined, because it was not possible to discern them with the analytical methods used. Essential amino acids are marked with asterisks. [b]Only aspartic acid. [c]Only glutamic acid.

In addition, the carbohydrate content of *N. nitrophenolicus* was 28% higher in cells grown in shake flasks than in those from BES.

Overall, the protein contents were lower and the carbohydrate contents clearly higher than those commonly reported in studies that aimed to produce bacterial biomass for food. It is possible that higher protein contents could have been achieved by harvesting the cells in the exponential growth phase. Biomasses from autotrophically grown Gram-negative HOB (*C. necator* and *Pseudomonas carboxydohydrogena*) have been reported to contain 46−71% protein, 5.0−6.7% carbohydrates, and 6.0−9.1% lipids.[8] The best-known example of edible microbes, the *Arthrospira* cyanobacteria, comprises on average 62% (per dry weight) protein, 22% carbohydrates, and 2.2% lipids.[49] According to the summary by Miller and Litsky, bacterial cells typically contain 50−65% (per cell dry weight) protein,[48] but lower contents (<40%) have been reported elsewhere.[5] Heterotrophically grown *R. opacus* strains have also been studied previously for protein production. Protein contents of 42−57% were achieved by cultivation with various agricultural wastes as the substrates.[37] Comparison of the reported lipid values to the results of this work is not straightforward, because instead of lipids, total amounts of fatty acids with chain lengths of up to C_{22} were analyzed here. This excludes, for instance, the long chain α-alkyl-β-hydroxy fatty acids (mycolic acids) with average chain lengths of 28−52 that are associated with the cell envelope of the *Rhodococcus* genus.[50] Fatty acid contents of 3.6−4.8% determined in this work are nevertheless at levels similar to the lipid contents reported previously for potentially edible bacterial biomasses (ranging from 1%[48] to 9%[51]).

It should be noted that the results presented in different studies might not be strictly comparable, because different analytical methods have been used. In the work presented here, protein was determined by analysis of the total amino acid content, whereas in many studies, an estimate for the protein content is obtained by multiplying the elemental nitrogen content by a conversion factor, which gives less accurate values.[52] For instance, differences in the ratios of nucleic acids to protein would affect the results. For comparison, when the typical conversion factor of 6.25 is used, the apparent protein content in the biomass samples of *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 increases to 50% and 60% (in cell dry weight), respectively, which are closer to the previously reported values. The macromolecular composition of the cell may also be dependent on the growth phase,[45,46] which complicates the comparison between different studies.

The sum of the macromolecules presented in Table 3 leaves 8−36% unaccounted for. Most of this residue presumably consists of minerals and nucleic acids (RNA and DNA), the latter of which have been reported to represent approximately 8−12% of bacterial dry weight.[48,49] Furthermore, fatty acids with carbon chain lengths of ≥23 were not analyzed.

Amino Acid and Fatty Acid Composition. The amino acids were analyzed to determine the overall protein contents (Table 3) and the amino acid profiles of the bacterial biomasses (Table 4). The amino acid profiles are important determinants of the food protein quality, because protein synthesis in the human body depends on the availability of adequate amounts of each amino acid building block. Of particular importance is the presence of essential amino acids, which humans cannot synthesize *de novo*. The profiles of soy protein isolate[53] and casein[54] are presented for comparison in Table 4. These protein sources are commonly used references for so-called complete protein, which means that they have sufficient proportions of each essential amino acid. In addition, the amino acid profile of the Gram-negative *C. necator* (*A. eutrophus*) Z1 strain[8] is shown for comparison in Table 4.

With both strains studied, the differences in the relative amounts of amino acids were mostly small (<10%) between

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Table 5. Essential Amino Acid Scores of *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 Proteins from the Bioelectrochemical System (BES) and Shake Flasks (SF)[a]

	essential amino acid scores						
	R. opacus		*N. nitrophenolicus*				
amino acid	BES	SF	BES	SF	*C. necator* Z1[8]	soy protein isolate	casein
histidine	1.66 ± 0.09	1.62 ± 0.05	1.73 ± 0.05	1.57 ± 0.07	1.3	1.7	1.7
isoleucine	1.48 ± 0.08	1.5 ± 0.03	1.33 ± 0.05	1.26 ± 0.05	1.5	1.6	1.6
leucine	1.44 ± 0.07	1.47 ± 0.03	1.54 ± 0.06	1.44 ± 0.05	1.5	1.4	1.5
lysine	1.08 ± 0.06	1.02 ± 0.03	0.97 ± 0.02	1.1 ± 0.05	1.6	1.4	1.6
methionine and cysteine	1.58 ± 0.08	1.52 ± 0.07	1.45 ± 0.04	1.39 ± 0.07	1.3	1.2	1.4
phenylalanine and tyrosine	1.73 ± 0.21	1.68 ± 0.04	1.54 ± 0.05	1.69 ± 0.24	2.1	2.4	2.7
threonine	2.25 ± 0.12	2.15 ± 0.06	2.32 ± 0.07	2.27 ± 0.07	2.3	1.6	1.7
tryptophan	0.77 ± 0.03	0.73 ± 0.05	0.45 ± 0.22	1.05 ± 0.12	2.3	2.2	2
valine	1.73 ± 0.12	1.74 ± 0.04	1.81 ± 0.06	1.71 ± 0.06	1.6	1.3	1.5

[a]Calculated by dividing the content of each amino acid by its content in a reference protein meeting the nutritional requirements of adults, as reported by the WHO.[33] Values of ≥1.0 suggest that the amino acid is present at a sufficient level.

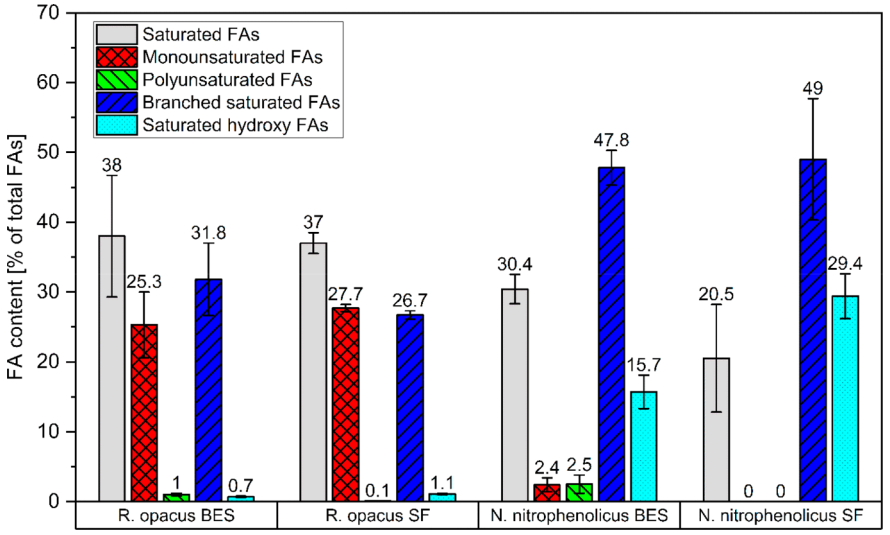

Figure 4. Fatty acid composition of bacterial biomass produced by autotrophic growth of *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 during cultivation in either a bioelectrochemical system (BES) or a shake flask (SF). Error bars represent the standard deviation (n = 3).

the two cultivation modes. The tryptophan content in BES-grown cells was >2 times the content in cells cultivated in shake flasks with *N. nitrophenolicus* KGS-27. However, it should be noted that the experimental error in tryptohan analysis of the BES-grown *N. nitrophenolicus* KGS-27 cells was rather high in this case. With *R. opacus* DSM 43205, the difference between the tryptophan contents was negligible. The cultivation mode does not appear to affect the amino acid profiles in any consistent or significant way.

As shown in Table 4, tryptophan was the scarcest of the amino acids with both strains and cultivation modes. Asparagine and aspartic acid as well as glutamine and glutamic acid are presented as combined because it was not possible to discern them with the analytical methods used. *R. opacus* DSM 43205 biomass contained amounts of glutamine and glutamic acid similar to those of soy beans and casein, while *N. nitrophenolicus* KGS-27 biomass contained considerably smaller amounts. Alanine was the most abundant amino acid in both biomass samples. The relatively high alanine content is also the most striking difference between the amino acid profiles of the HOBs of our study and the profiles of soy protein isolate and casein. This difference may, at least partly, be due to the presence of alanine in the bacterial cell wall peptidoglycan.[55] High alanine contents have also been reported previously for some other microorganisms such as yeast and microalgae.[8] In addition, the alanine content for the Gram-negative HOB, *A. eutrophus* Z1, was equally high (Table 4). The amino acid profile of *A. eutrophus* Z1 cells[8] was in general comparable to those of *N. nitrophenolicus* KGS-27 and *R. opacus* DSM 43205. However, the cysteine and tryptophan contents of the strains in our study were >3 times and less than half of that of *A. eutrophus* Z1, respectively.

In Table 5, the essential amino acid contents of *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 from BES and shake flask cultivations are compared to requirements of adults as reported by the WHO[33] by calculating the amino acid score values (see Materials and Methods). Values of ≥1.0 suggest that the amino acid is present at a sufficient level. Cysteine and tyrosine can be regarded as conditionally essential, because they can be synthesized from methionine and phenylalanine, respectively.[56] Therefore, pools of methionine and cysteine, representing the sulfur-containing amino acids, and phenylalanine and tyrosine, representing the aromatic amino acids, are typically used in evaluating the nutritional value of the protein in question.

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The results suggest that proteins contained in biomass samples of both strains fulfill and exceed the nutritional needs for most amino acids. Only the tryptophan content of *R. opacus* DSM43205 protein produced in BES and in shake flasks and that of *N. nitrophenolicus* KGS-27 produced in BES clearly lag behind what is required. Lysine was contained at sufficient but not excessive levels with an amino acid score of 1.0−1.1. All other amino acid scores range between approximately 1.3 and 2.3. The sulfur-containing amino acids methionine and cysteine are claimed to limit the nutritional value of proteins of legumes[57] but appear to be produced by *R. opacus* DSM43205 and *N. nitrophenolicus* KGS-27 at levels above the recommended amino acid intake threshold. The amino acid profile of the Gram-negative HOB strain *A. eutrophus* Z1 appears to be more balanced than those of *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27, because both lysine and tryptophan are present in excess in *A. eutrophus* Z1.

Because the fatty acid contents of the bacterial biomasses produced were rather low [between 3.6% and 4.8% (Table 3)], their fatty acid distributions are of minor nutritional importance. Nevertheless, as shown in Figure 4, there were significant differences between the fatty acid profiles of the two strains. With *R. opacus* DSM 43205, the unsaturated (mono and poly) fatty acids prevailed, whereas with *N. nitrophenolicus* KGS-27, saturated fatty acids (including saturated branched and hydroxy fatty acids) were predominant. The most noticeable difference between the strains is that saturated hydroxy fatty acids were virtually absent from *R. opacus* DSM 43205 biomass whereas they represented approximately 15% and 30% of total fatty acids of *N. nitrophenolicus* KGS-27 cells cultivated in BES and shake flasks, respectively.

A conspicuous difference between the fatty acid profiles of *N. nitrophenolicus* KGS-27 and *R. opacus* DSM 43205 and those of animal and plant lipids is the high content of branched chain fatty acids in the HOB biomasses. Branched chain fatty acids represented approximately one-half and one-third of the *N. nitrophenolicus* KGS-27 and *R. opacus* DSM 43205 fatty acids, respectively. Branched chain fatty acids are recognized as characteristic components of bacterial membrane lipids. A main source of branched chain fatty acids in human diet is milk fat in which they, presumably originating from ruminal bacteria, represent 1.7−3.4% of the total fatty acids. Although branched chain fatty acids have been suggested to have beneficial effects on human gut microbiota and skin, their influence on human health is still largely unexamined.[58]

Potential of Gram-Positive HOB and the BES Setup for Food Production. Production of bacterial protein for food and feed [so-called single cell protein (SCP)] has been of increasing interest during the past two decades.[59] This has led to the foundation of start-up companies, such as Solar Foods (Finland), Air Protein (United States), and Deep Branch Biotechnology (U.K.), exploring commercial production. The suitability of many of the strains examined for the production for human consumption in the literature is, however, questionable. Gram-negative bacteria,[5,8,60] which typically produce endotoxic lipopolysaccharides, mixed cultures,[61] and even pathogenic bacteria,[62] have been suggested for SCP production. In terms of bacterial food production, Gram-positive strains such as *R. opacus* DSM 43205 and *N. nitrophenolicus* KGS-27 are better candidates, because they are devoid of endotoxins. Other major concerns common for both Gram-negative and -positive species include the origin of the microorganism, the possible presence of exotoxins, and the cytotoxic and genotoxic properties of the cell masses, which should be examined before considering food use. A further common concern of edible microbial biomass is its high nucleic acid content (7−12%),[5] which is due to the high rate of proliferation of the cells. Nucleic acids are composed of pyrimidine and purine nucleotides. The metabolic breakdown product of dietary purines is uric acid, accumulation of which may eventually lead to gout or generation of kidney stones. However, nucleic acid contents can be efficiently reduced, e.g., by heat treatment.[5]

As discussed above, the BES system offers obvious benefits, such as a simpler design and potentially more efficient mass transfer, over the use of a separate water electrolysis unit. The use of BES apparently had no significant or negative effects on the nutritional quality of the bacterial biomasses in comparison to the biomasses produced by supplying the bacteria with the gases externally. The potential downside of BES is the production of toxic reactive oxygen species. Interestingly, there seem to be differences in how different HOB strains tolerate these compounds. The results presented in this study suggest that some bacteria, such as the novel *N. nitrophenolicus* strain, are not necessarily affected by these electrolysis byproducts, indicating the potential of the BES approach. It should be noted that production of hydrogen by electrolysis, whether *in situ* or in a separate unit, for powering the metabolism of HOB is relatively energy consuming. It has been estimated that, if all hydrogen needed by the bacteria would be produced by polymer electrolyte membrane electrolysis, 537−902 GW of energy would be needed for 175−307 Mt of bacterial mass with a protein content of 70%. This indicates that renewable energy, such as wind or solar power, is needed to make such processes sustainable.[63]

Despite the relatively close phylogenetic relationship, there were differences in the fatty acid profiles and, more importantly, in the amino acid profiles between the two Gram-positive HOBs studied. The essential amino acid contents of the proteins from the two strains, with the exception of the low tryptophan content, appeared, nevertheless, to be exceeding that of the recommended intake values, indicating sufficient nutritional value. However, the bioavailability of the proteins for digestion, the quality of the carbohydrates (digestible vs nondigestible), and the micronutrient composition are issues to be elucidated in future studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.0c00129.

Phylogenomic clustering of HOB strains (PDF)

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Notes

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