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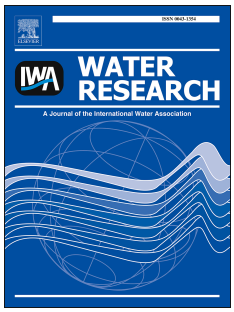

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Autotrophic nitrogen assimilation and carbon capture for microbial protein production by a novel enrichment of hydrogen-oxidizing bacteria

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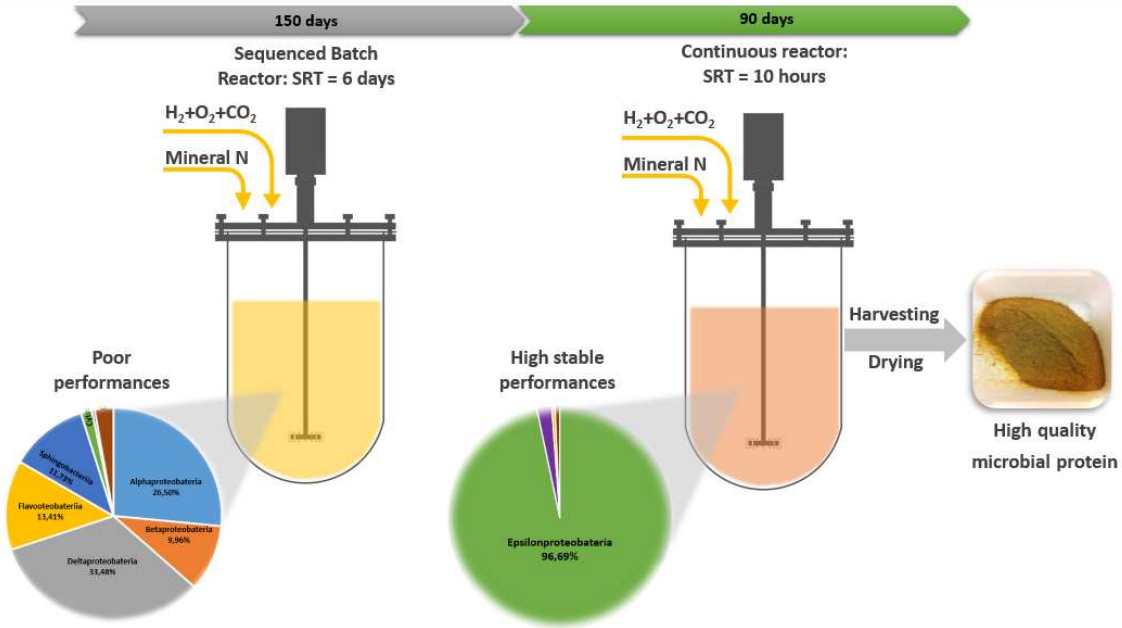
Graphical Abstract



Highlights

- Sequenced batch reactor operations selected a highly diverse culture with low biological performances
- High rate continuous reactor operations attained a culture dominated at 97% by *Sulfuricurvum* spp.
- The *Sulfuricurvum* spp. dominated culture achieved long term stable biological performances
- Ammonium and carbon dioxide were biologically converted into high-quality microbial protein

Abstract

Domestic used water treatment systems are currently predominantly based on conventional resource inefficient treatment processes. While resource recovery is gaining momentum it lacks high value end-products which can be efficiently marketed. Microbial protein production offers a valid and promising alternative by upgrading low value recovered resources into high quality feed and also food. In the present study, we evaluated the potential of hydrogen-oxidizing bacteria to upgrade ammonium and carbon dioxide under autotrophic growth conditions. The enrichment of a generic microbial community and the implementation of different culture conditions (sequenced batch resp. continuous reactor) revealed surprising features. At low selection pressure (i.e. under sequenced batch culture at high solid retention time), a very diverse microbiome with an important presence of predatory *Bdellovibrio* spp*.* was observed. The microbial culture which evolved under high rate selection pressure (i.e. dilution rate $D=0.1h^{-1}$) under continuous reactor conditions was dominated by *Sulfuricurvum* spp*.* and a highly stable and efficient process in terms of N and C uptake, biomass yield and volumetric productivity was attained. Under continuous culture conditions the maximum yield obtained was 0.29 g cell dry weight per gram chemical oxygen demand equivalent of hydrogen, whereas the maximum volumetric loading rate peaked 0.41 g cell dry weight per litre per hour at a protein content of 71%. Finally, the microbial protein produced was of high nutritive quality in terms of essential amino acids content and can be a suitable substitute for conventional feed sources such as fishmeal or soybean meal.

Keywords:

Resource up-cycling; Nitrogen assimilation; Carbon capture; Microbial protein; Hydrogen-oxidizing bacteria; *Sulfuricurvum* spp.

1. Introduction

Primary producers - autotrophic microorganisms - are essential for carbon and nutrients cycling. While fixing inorganic CO_2 into organic biomass they recycle nutrients (N and P) and provide food for higher life forms (Elser et al., 2000). Primary producers such as algae and autotrophic bacteria can serve as alternative protein source in the form of microbial protein (MP) for livestock but also for human consumption (Anupama and Ravindra, 2000; Verstraete, 2015; Walsh et al., 2015). Besides protein, microbes can also accumulate considerable amounts of biocompatible prebiotics such as PHB (Defoirdt et al., 2007), thereby enhancing the nutritional value of the microbial biomass.

After being extensively studied in the past, mainly as means to upgrade fossil fuel (e.g. paraffin, natural gas) to protein supplements (Westlake, 1986), the use of bacteria for microbial protein (MP) production has nowadays re-gained significant interest (Aas et al., 2006; Marit Berge et al., 2005) with natural gas based MP production entering the market economy (Strong et al., 2015). Innovative approaches implementing bacteria to produce MP within the context of resource recovery from used water have also been recently proposed (Lee et al., 2015; Liu et al., 2016; Matassa et al., 2015a). Indeed, the production of MP can allow the up-cycling of nitrogen and carbon dioxide recovered from used water streams, converting them into protein-rich feed and food substances. Different physico-chemical techniques can be implemented in the recovery of N and C substrates. Air stripping or pervaporative processes can recover N from concentrated streams such as anaerobic digestate, whereas pressure swing adsorption (PSA) can concentrate CO_2 from biogas, thus providing the building blocks which are at the base of MP biosynthesis.

Among the various metabolic pathways suitable for MP production, including both eukaryotic and prokaryotic microorganisms (Anupama and Ravindra, 2000), autotrophic hydrogen-oxidizing bacteria (HOB) constitute a special and thus far unexplored metabolic niche with potential for novel applications in resource recovery and upgrade. Even if ubiquitous, autotrophic HOB have only received limited attention, with previous studies focusing on the use of axenic cultures comprising bacteria such as *Alcaligenes eutrophus*, *Ralstonia eutropha, Seliberia carboxydohydrogena* (Ishizaki and Tanaka, 1990; Repaske and Mayer, 1976; Volova and Barashkov, 2010). The metabolic features of autotrophic HOB allow them to grow on hydrogen (electron donor) and oxygen (electron acceptor) while fixing carbon dioxide into cell material and assimilating nitrogen into high quality protein (Parkin and Sargent, 2012; Pohlmann et al., 2006). MP produced by autotrophic HOB is characterized by all the essential amino acids, having an amino acid profile closer to high-quality animal protein rather than to vegetable protein (Volova and Barashkov, 2010). Given this interesting feature, autotrophic HOB were already proposed as possible protein source within biological life support systems for space missions (Bartsev et al., 1996), as well as for human and animal nutrition (Volova and Barashkov, 2010).

An attractive characteristic of MP production with autotrophic HOB is the possibility to exploit the increasing potential of renewable energy generation. A clear example is the use of hydrogen gas produced from water electrolysis, powered by e.g. wind or solar energy, or also from biomass gasification (Ni et al., 2006) Recently, biomethane has also been proposed as possible renewable feedstock for hydrogen production by means of a combined heat, hydrogen and power generation unit (CHHP) (Agll et al., 2013; Hamad et al., 2014). The possibility to implement such technologies on-site and produce hydrogen on demand might enable the direct up-cycling of mineral nitrogen and carbon dioxide recovered from

wastewater treatment plants, as previously mentioned. Moreover, upcoming technological developments and the decrease of hydrogen prices (Ball and Weeda, 2015) justify further research efforts towards the application of autotrophic HOB within resource recovery and up-cycling.

In the present study, we aimed to experimentally determine the feasibility of nitrogen and carbon upgrade into MP by means of a microbial community enriched in HOB using a lab-scale gas. Along the experimental investigation different culture conditions were imposed to the enriched HOB culture (i.e. sequenced batch and continuous). This was done in order to establish how the microbial community was shaped by the process conditions and how this affected the overall biological performance of the system, aiming at maximizing MP production (i.e. biomass yield and volumetric productivities). Nitrogen under the form of ammonium salt and gaseous CO_2 represented the N and C substrates needed for the production of MP protein by means of autotrophic HOB. The study started with the enrichment of a generic aerobic microbial mixed culture with autotrophic HOB under sequencing batch reactor operations. Consequently, the enriched mixed community was cultured in a continuous reactor configuration, resulting in the ongoing evolvement of a highly specific bacterial culture dominated by the genus *Sulfuricurvum.* The efficiency of the process in terms of gas utilization and by-product formation was monitored along the time course of the selective enrichment process. The microbial community analyses of the HOB microbiome under batch and continuous culture systems allowed delineating the evolution of the mixed bacterial community towards a quasi-monoculture dominated by *Sulfuricurvum* spp. Finally, the MP produced was characterized in terms of crude protein content and amino acid profile in order to assess its nutritional value.

2. Material and Methods

2.1 Enrichment of hydrogen-oxidizing bacteria

Aerobic sludge from a local food (potatoes) processing plant (Nazareth, Belgium) was used as an initial mixed culture for the enrichment of autotrophic HOB community. The enrichment was carried out in a 1 L gas fermentor. The fermentor was connected to 3 gas bags supplying a gas mixture composed by $H_2/O_2/CO_2$ with the following composition: 65/20/15 (vol/vol). Prior to use, each gas bag was flushed with Alphagaz 2-grade H_2, O_2 and CO_2 gasses (Air Liquide, Belgium). The gaseous $H_2/O_2/CO_2$ atmosphere was constantly recirculated between the culture vessels and the gas bags by means of a peristaltic pump adapted to gas recirculation (Sci-Q 300, Watson Marlow, Belgium). The reactor was placed in a 28 °C temperature controlled room and shaken at 150 rpm. A volume of 500 mL of mineral media inoculated with 10% of inoculum was used at start. The mineral medium was prepared accordingly to Yu et al. (2013) for HOB isolation and culturing. The growth of HOB was followed by monitoring the increase of cell dry weight (CDW) over the course of the experimental run. When ammonium nitrogen was depleted, 50 mL of bacterial culture was withdrawn and diluted into 450 mL of fresh medium to restart the enrichment. After a stable and reproducible growth was attained in terms of CDW concentrations (2-3 g CDW/L before medium replenishment), the culture was considered enriched and used to start the experimental phase in the final reactor setup.

2.2 Reactor operations and controls

A completely stirred tank reactor (CSTR) (Biostat A plus, Sartorius, Belgium) was used during batch as well as continuous experiments. The 5 L glass vessel, with a working volume of 3 L, was stirred at 700rpm with a 3-blade segment impeller to ensure completely mixed

conditions. Hydrogen gas was produced on site by means of a lab-grade hydrogen generator (Alphagaz™ Flo H_2, Air Liquide, Belgium), while CO_2 from gas bottles was of the same grade of the one used during the initial enrichment of the culture. Compressed air was used to provide the oxygen. Gases were fed separately by means of 3 micro-spargers (Sartorius, Belgium) submerged in the reactor. Gas flows were monitored using gas rotameters (Omega, USA) and kept at H_2: 120 mL/min; CO_2: 25 mL/min; Air: 180 mL/min. The gas collected in the headspace was constantly recirculated by means of a peristaltic pump adapted to gas recirculation (Sci-Q 300, Watson Marlow, Belgium) using a fourth micro-sparger. Utilized gas by the bacteria, was bubbled through an external water lock (imposing an overpressure of 20 mbar) and subsequently vented to the atmosphere by means of a fume hood. Temperature and pH were automatically controlled and kept at 35±1 °C and 6.7, respectively.

2.3 Sequencing batch and continuous reactor culture systems

Sequencing batch reactor (SBR) tests were started by transferring 300 mL of fully grown bacterial culture into 2.7 L of fresh mineral medium, allowing an initial cell dry weight Cell Dry Weight (CDW) concentration of 300 to 500 mg CDW/L. Each sequencing batch test was allowed to evolve for an average of 5 to 6 days before transferring the culture into fresh medium, corresponding to a solid retention time (SRT) of 6±0.5 days. Additional NH_4Cl was added to the standard mineral medium composition in order to achieve initial NH_4^+-N concentration of 1.2 g/L, and simulate higher N concentrations obtainable with recovery techniques such as air stripping or pervaporative systems. The sequencing batch culture was monitored along a period of 5 months.

Continuous reactor (CR) operations were set by supplying fresh media with a diaphragm pump (Qdos, Watson Marlow, Belgium), totaling a flow of 7.2 L/day. In the same way, 7.2 L/day of cell culture were constantly withdrawn from the CSTR reactor by means of a similar pump. As for the sequenced batch experiment, additional NH4Cl was added to the standard mineral medium composition in order to achieve initial NH4+-N concentration of 0.5 g/L. The complete absence of biomass recirculation set hydraulic and (SRT) of 10 h. Under these continuous reactor conditions (chemostat), only bacteria with a specific growth rate "μ" equal or higher than the dilution rate D=0.1 h^{-1} could avoid being washed-out from the biological system. The continuous system was operated uninterruptedly for 3 months.

2.4 Analytical methods

NH_4^+-N concentrations were determined by means of cuvette tests (Hach Lange, range 0-47 mg NH_4^+-N/L). Cell Dry Weight (CDW) was measured in duplicate after water was evaporated at 105 °C for 24 h. Prior to analysis, the samples were centrifuged at 12500 g for 10 minutes for three times, each time re-suspending the biomass pellet in demineralized water. Gas samples collected from the reactor headspace were analyzed with a Compact GC (Global Analyser Solutions, Breda, The Netherlands), equipped with a Molsieve 5A pre-column and Porabond column (O_2, H_2 and N_2) and a Rt-Q-bond pre-column and column (CO_2). Concentrations of gases were determined by means of a thermal conductivity detector.

2.5 Analysis and characterization of microbial protein

Kjeldahl nitrogen content of the microbial biomass was analyzed according to Standard methods (APHA et al., 1992). Organic nitrogen was determined as the difference between Kjeldahl nitrogen and ammonium nitrogen. The final protein content of CDW was obtained by multiplying the obtained value by applying a conversion factor of 6.25 as done in previous

studies (Salo-väänänen and Koivistoinen, 1996). The dietary amino acids composition of the microbial biomass was determined by an external accredited commercial laboratory (Eurofins Denmark A/S, Denmark).

2.6 Microbial community analysis

Liquid samples for total DNA extraction were centrifuged for 10 min at 10000 RPM. Subsequently, the supernatant was removed and biomass pellet was stored immediately at −20◦C until further analysis following a protocol adapted from Vilchez Vargas et al. (2013). Cells were lysed with 1 mL lysis buffer (100 mM Tris/HCl pH 8.0, 100 mM EDTA pH 8, 100 mM NaCl, 1% (m/v) polyvinylpyrrolidone and 2% (m/v) sodium docecyl sulphate) and 200 mg glass beads (0.11 mm, Sartorius) in a FastPrepR - 96 instrument (MP Biomedicals, Santa Ana, USA) for two times 40 s (1600 rpm). After removing glass beads by centrifugation (5 min at 10000 RPM), DNA was extracted from supernatant following a phenol–chloroform extraction. DNA was precipitated with 1 volume ice-cold isopropyl alcohol and 0.1 volume 3 M sodium acetate for at least 1h at −20◦C. After removal of isopropyl alcohol by centrifugation (30 min, maximum speed), the DNA pellet was dried and re-suspended in 100 µL 1× TE (10 mM Tris, 1 mM EDTA) buffer. After finishing the extraction protocol, the DNA samples were immediately stored at −20◦C until further processing. Quality of DNA samples was analyzed by 1% (w/v) agarose (Life technologies, Madrid, Spain) gel electrophoresis. The PCR amplicons were purified with the innuPREP PCR pure kit (Analytik Jena, Jena, Germany), and sequenced with the primers used for PCR. 16s rRNA Illumina and Sanger sequencing analyses were conducted for each sample in triplicate by external commercial laboratories (Analytik Jena, Jena, Germany).

2.8 Calculations

206 The gas conversion efficiency was calculated as:

207 $$Gas\ conversion\ efficiency\ (\%) = \frac{Gas\ inlet\ (mol/min) - Gas\ outlet\ (mol/min)}{Gas\ inlet\ (mol/min)} \times 100 \quad (1)$$

208

209 With hydrogen gas as the electron donor for the HOB, the biomass yield on H_2 gas is
210 expressed in terms of Chemical Oxygen Demand (COD) hydrogen gas equivalent. The yield is
211 calculated as:

212 $$Y_{H_2}\left(\frac{g\ CDW}{g\ H_2 - COD}\right) = \frac{CDW\ (g/L)}{H_2\ gas\ uptake\ (mol) \times 16\ (g\ COD/mol)} \times Liquid\ volume\ (L) \quad (2)$$

213

214 The biomass yield on carbon dioxide is calculated as:

215 $$Y_{CO_2}\left(\frac{g\ CDW - C}{g\ CO_2 - C}\right) = \frac{CDW\ (g/L) \times 0.5\ (g\ C/g\ CDW)}{CO_2\ gas\ uptake\ (mol) \times 12\ (g\ C/mol)} \times Liquid\ volume\ (L) \quad (3)$$

216

217 The mineral nitrogen upgrade efficiency is calculated as:

218 $$N\ upgrade\ effieicncy\ (\%) = \frac{NH4 - N\ in\ (g/L) - X - N\ out\ (g/L)}{NH4 - N\ in} \times 100 \quad (4)$$

219

220 Where *NH_4-N in* indicates the amount of NH_4-N fed to the reactor, respectively to the SBR
221 and the CR systems and *X-N out* indicates the amount of dissolved nitrogen under the form
222 of NH_4^+, NO_2^- or NO_3^- at in at the end of each SBR test and in the effluent of the CR system.

223

3. **Results**

3.1 Sequencing batch and continuous reactor performances

The enriched HOB culture was first cultivated under sequencing batch reactor (SBR) conditions, with a SRT of 6±0.5 days. The same experimental setup was then adapted to grow the HOB culture under continuous reactor (CR) configuration, imposing a SRT of 10 h. The main parameters analyzed both under SBR and CR configurations were: volumetric productivities (g CDW/L·h), biomass yields on hydrogen (g CDW/g COD-H_2) and carbon dioxide (g CDW-C/g CO_2-C) and hydrogen gas conversion efficiencies (%), as shown in Table 1.

Table 1. Parameters of HOB cultivation obtained under SBR tests (averaged over three different sequencing batch tests) and CR operations (over 90 days of continuous operations). Maximum values were calculated for each batch for the data points which maximized volumetric productivity and biomass yield, whereas average values were calculated over the whole period.

Parameter		**Sequence Batch reactor**	**Continuous reactor**
Volumetric productivity (g CDW/L·h)	Average	0.078 ± 0.012	0.375 ± 0.015
	Maximum	0.187 ± 0.045	0.406
Y_{H2} (g CDW/g COD-H_2)	Average	0.073 ± 0.007	0.280 ± 0.010
	Maximum	0.157 ± 0.037	0.290
Y_{CO2} (g CDW-C/g CO_2-C)	Average	0.153 ± 0.023	0.427 ± 0.013
	Maximum	0.246 ± 0.058	0.456
H_2 gas conversion efficiency	Average	65% ± 4%	81% ± 2%
	Maximum	71% ± 3%	87%
N upgrade efficiency	Average	100%	87% ± 4%
	Maximum	100%	97%
Protein content (%CDW)	Average	66% ± 5%	71% ± 5%
	Maximum	73%	76%

The average values for each individual SBR test reported in Table 1 were calculated, by considering the initial and final point of each test over the duration of the experimental run

(i.e. t=0 to t=120-144 h). Three subsequent SBR experimental run (t=41, 82, 120 days) were averaged together to summarize the values obtained along the SBR cultivation period. Maximum values indicate the maximum single data point measured during each individual SBR test For the CR operations, samples were taken for analysis three times per week over a period of 90 days (n=35). The average values reported in table 1 show the average of the total amount of samples taken.

Under SBR conditions, an average volumetric productivities of 0.078 ± 0.012 g CDW/L·h was achieved. The latter value increased about 5-fold under CR configurations, reaching an average of 0.375 ± 0.015 g CDW/L·h. Biomass yields in terms of g CDW/g COD-H_2 increased from 0.073 ± 0.007 to 0.280 ± 0.010 g CDW/g COD-H_2, when changing from a SBR to a continuous operation mode. In the same way, CO_2–based yield increased from the minimum of 0.153 ± 0.023 g CDW-C/g CO_2-C observed during SBR cultivation to the maximum of 0.427 ± 0.013 g CDW-C/g CO_2-C. Hydrogen gas was also converted more efficiently when the reactor operated continuously, with an increase of 16% compared to SBR operations, reaching 81 ± 2%. Maximum values observed under CR were almost double than observed under SBR conditions. A different trend was observed for the nitrogen upgrade efficiency. SBR conditions allowed the complete conversion of the ammonium nitrogen supplied into MP, which reached an average of 65 ± 5% of the microbial biomass CDW. Under CR operation, instead, about 13% of the total mineral ammonium nitrogen supplied was still present in dissolved form in the CR effluent, whereas the average protein content of the produced biomass was 71 ± 5% (%CDW).

3.2 Microbial community analysis

In order to assess the composition of the microbial community, DNA samples from the SBR (after 120 days of operations) and from the CR configurations (after 20 days of operations) were analyzed by means of 16S rRNA Illumina sequencing.

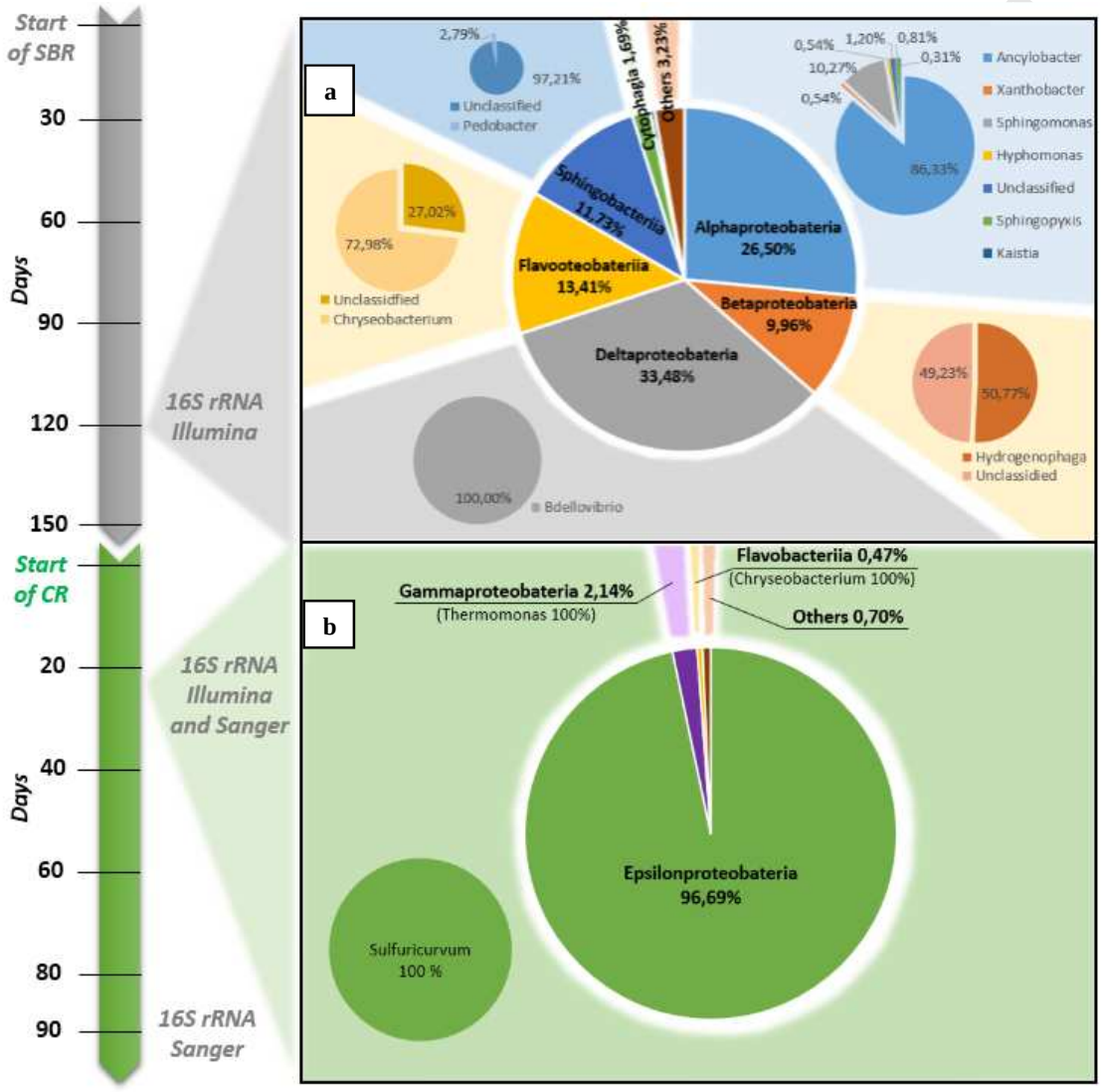


Figure 1. Phylogenetic composition of the HOB microbiome during SBR **(a)** and CR **(b)** operations, assessed by 16S rRNA Illumina sequencing. The timeline indicates the duration of each phase: SBR and CR, and when DNA samples were processed for 16S rRNA Illumina and Sanger sequencing. The central graph resumes the percentage of each class within the microbial community. Each class is then characterize in terms of genera composition in the external graphs **(a, b)** or within brackets **(b)**.

The enriched microbial community cultivated under SBR conditions was characterized by a rather high diversity. Amongst the 12 genera identified, *Ancylobacter* (Morita, 1999), *Xanthobacter* (Wilde and Schlegel, 1982) and *Hydrogenophaga* (Willems et al., 1989) have been already documented as able to carry out autotrophic oxyhydrogen metabolism. The latter constitute less than one-third of the quantitative genera distribution of the microbial community. No direct evidence of aerobic hydrogen oxidation is available in literature for the other genera present. Notably, the microbial community was dominated (one-third of the whole quantitative genera distribution) by *Bdellovibrio*, a genus of the class of *Deltaproteobacteria* encompassing predatory bacteria able to invade and lyse various other Gram-negative bacteria (Rendulic et al., 2004). The remaining genera detected (about 30% relative abundance) were mainly composed by the classes of *Falvobacteriia* and *Sphingobacteriia*, known as aerobic chemoorganotrophic bacteria (Vandamme et al., 1994; Yabuuchi et al., 1983).

Following the SBR cultivation period, the effect of the first 20 days of CR operations on the microbial community was investigated by means of a second 16S rRNA Illumina sequencing analysis. As shown in Fig. 1.b, the simple implementation of high rate ($D=0.1\ h^{-1}$) continuous reactor operations led to a remarkable selection within the microbial community, with almost 97% of the total community composed by a single genus: *Sulfuricurvum*. Almost 80% of the remaining 3% was composed by only other two genera: *Gammaproteobacteria* (*Thermomonas*) and *Flavobacteriia* (*Chryseobacterium*).

The DNA sample used for the 16S rRNA Illumina sequencing analysis of the CR was subsequently analyzed by means of 16S rRNA sequencing, together with a second sample taken after 90 days of continuative CR operations. The latter was done in order to confirm

the stability of the microbial community composition and to gain more in depth information on the dominating *Sulfuricurvum* genus. For both samples the analysis indicated similarities at the level of 98 and 99% to *Sulfuricurvum kujiense* strains *YK-2*, *YK-3* and *YK-4*, as well as to other uncultured *Epsilonproteobacteria* when compared using NCBI BLAST under default settings (Han et al., 2012).

3.3 Protein and amino acid profile

The bacterial biomass grown under CR configurations was harvested at day 90 (i.e. at the end of the CR cultivation period) and analyzed for crude protein content as well as for essential amino acids composition.

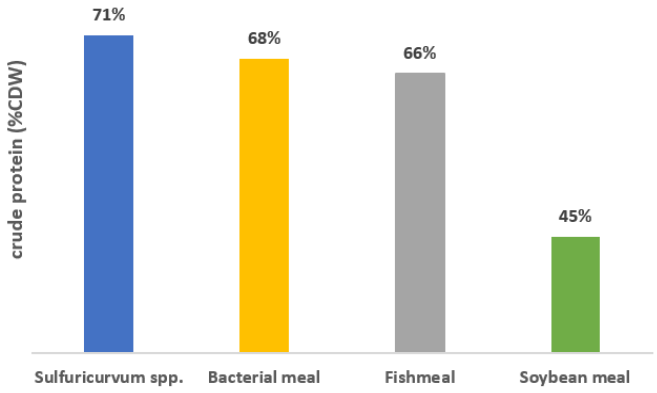


Figure 2. Crude protein content on CDW basis of the microbial biomass produced under CR configuration by the *Sulfuricurvum* spp. dominated culture (this study) compared with other microbial protein (bacterial meal), animal protein (fishmeal) and vegetable protein (soybean meal) (Øverland et al., 2010).

Figure 2 compares the results obtained in this study with reference protein feed additives such as fishmeal, soybean meal and bacterial meal. The latter is a MP product obtained from methane oxidizing bacteria (*Methylococcus capsulatus* grown in association with other heterotrophic bacteria) already produced at pilot scale and tested in several feed trials involving monogastric animals as well as aquaculture species, for which the EU already

approved the use in animal nutrition (Øverland et al., 2010). Fishmeal and soybean meal were chosen as a reference for animal and vegetable protein, respectively. Bacterial meal allows to benchmark the MP produced in this study with another know similar product (i.e. already tested and legally approved MP).

As demonstrated in Fig. 2, the crude protein content of 71% of the *Sulfuricurvum* spp. microbial culture is slightly higher than bacterial meal (68%) and fishmeal (66%) and substantially higher than the average crude protein content of soybean meal (45%).

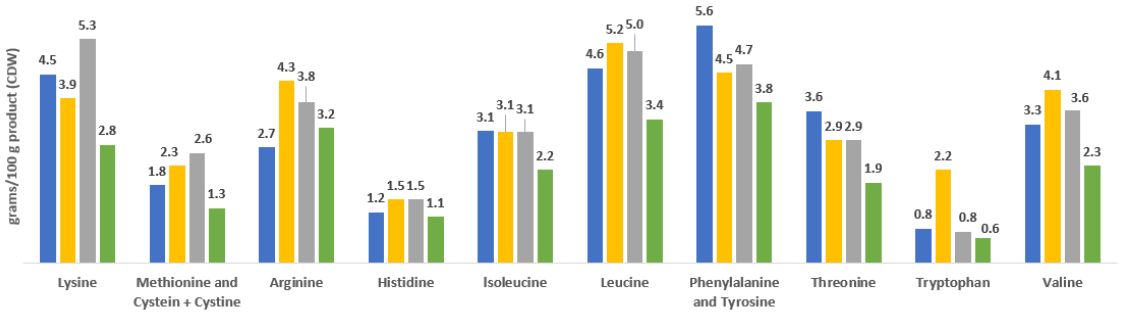


Figure 3. Essential amino acids profile of the microbial biomass produced under CR configuration by the *Sulfuricurvum* spp. dominated culture (blue) (this study) compared with bacterial meal (yellow), fishmeal (grey) and soybean meal (green) as reported from Øverland et al. (2010).

A similar trend can be observed in Fig. 3 for the amino acid profile. The profile for the *Sulfuricurvum* spp. microbial culture was comparable to that of bacterial meal and fishmeal and systematically better (at the exception of Arginine) than the one of soybean meal.

4. Discussion

4.1 Sequenced Batch Reactor

Following the enrichment, the SBR operations confirmed that the microbial culture effectively oxidized hydrogen coupled with assimilation of carbon dioxide and mineral nitrogen (i.e. ammonium nitrogen) into cell biomass. Consistent biomass growth was observed, allowing to operate the SBR at a SRT of about 6 days. Also, the NH_4-N fed at the beginning of each SBR test was completely (100%) converted into organic nitrogen for microbial biomass build up. Nevertheless, the average performances observed in terms of volumetric productivities and biomass yield on hydrogen were far from being optimal. More specifically, the mixed culture grown under SBR conditions gave average biomass yields and productivities lower than values previously reported using specific bacterial strains (see Table 2).

Table 2. Comparison of results obtained in this study for SBR and CR grown cultures with data from literature on single HOB strains.

Microbial culture / Strains	Substrate	Culture method	Biomass productivity (g CDW/L·h)	Biomass yield (g CDW/g COD-H_2)	Reference
Alcaligenes eutrophus	$H_2/O_2/CO_2$	Batch	2.28	-	(Tanaka et al., 1995)
***Alcaligenes eutrophus* ATCC 17697^T**	$H_2/O_2/CO_2$	Batch	0.71	0.28	(Ishizaki and Tanaka, 1990)
***Ideonella sp.* O-1**	$H_2/O_2/CO_2$	Batch	0.27	0.20	(Tanaka et al., 2011)
Pseudomonas hydrogenovora	$H_2/O_2/CO_2$	Batch	0.50	0.16	(Goto et al., 1977)
Mixed culture (SBR)	H_2/Air/CO_2	Batch	0.08	0.07	This study (average values)
Alcaligenes eutrophus ATCC17697	$H_2/O_2/CO_2$	Continuous	0.40	0.29	(Morinaga et al., 1978)
Alcaligenes hydrogenophilus	$H_2/O_2/CO_2$	Continuous	0.33	0.23	(Miura et al., 1982)
Cupriavidus eutrophus B-10646	$H_2/O_2/CO_2$	Continuous	-	0.14	(Volova et al., 2013)
***Sulfuricurvum* spp. (CR)**	H_2/Air/CO_2	Continuous	0.38	0.28	This study (average values)

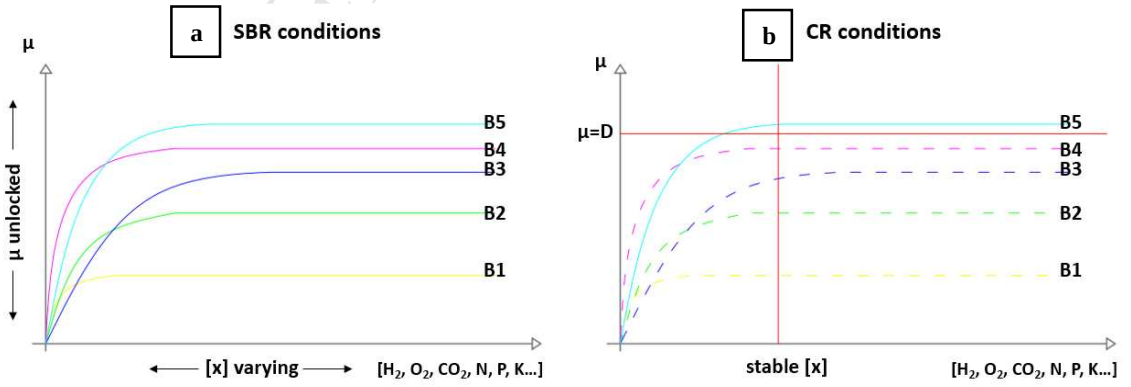
The average volumetric productivity of 0.08 g CDW/L·h observed under SBR conditions, was 28.5, 9.1, 3.5, 6.4 and 3.3 times lower than the values reported for autotrophic growth of *Alcaligenes eutrophus, Alcaligenes eutrophus ATCC 17697T, Ideonella sp. O-1* and *Pseudomonas hydrogenovora*, respectively. Equally, the biomass yield on hydrogen gas was 3.8, 2.7 and 2.2 times lower than vales reported for *Alcaligenes eutrophus ATCC 17697^T, Ideonella sp. O-1* and *Pseudomonas hydrogenovora* grown under batch conditions.

The analysis of the community composition revealed a surprising fractionation of the HOB enriched community into three distinct compartments: autotrophic HOB, heterotrophic bacteria and predatory bacteria, each sharing about 1/3 of the relative abundance of the overall community. The association between primary producers (autotrophic bacteria) and secondary consumers (heterotrophic bacteria) has already been documented in full scale MP production as well as reported and investigated in recent scientific studies (Aas et al., 2006; Ho et al., 2014). In the context of MP production, a clear example is represented by a methylotrophic bacterium (*Methylococcus capsulatus*) cultured in association with other heterotrophic bacteria. Such microbial fermentation is used in pilot-scale bioconversion of natural gas into MP (bacterial meal), eventually used as high-quality feed in aquaculture (Aas et al., 2006; Marit Berge et al., 2005). The coexistence of different microbial species offers benefits such as the removal of inhibiting byproducts or cell lysates, as well as the regulation of oxygen level (Ho et al., 2014; Strong et al., 2015).

Quite unexpected was the 33% relative abundance of *Bdellovibrio* spp., by far the most abundant genus dominating the mixed culture after 120 days of continuous SBR operations. The fact that such genus comprises predatory bacteria thriving on invasion and consumption of other Gram-negative bacteria (Rendulic et al., 2004) offers a reasonable yet remarkable

366 explanation for the low performances of the HOB enriched community observed under SBR
367 conditions. It is likely that the predatory activity of *Bdellovibrio* spp. imposed a major stress
368 on the primary producers HOB, which were actively oxidizing hydrogen and fixing carbon
369 dioxide into new microbial biomass then partly lysed by predatory activity. The lysed
370 biomass might have also served as growth substrate for heterotrophic bacteria (Van
371 Loosdrecht and Henze, 1999), in fact occupying the remaining 1/3 of the microbial
372 community.

373 The high metabolic diversity and the low performances characterizing the microbial
374 community under SBR condition can be also explained by speculating over the degrees of
375 freedom of the biological systems in terms of growth rate and substrates concentrations, i.e.
376 from a Monod-like point of view. Under SBR conditions, low constrains were imposed to the
377 specific growth rate of the different bacteria present, which were therefore able to coexist in
378 the same biological context. Also, the depletion of nutrients as well as the varying
379 concentration of gasses as affected by the changing microbial activity over the batch culture
380 (i.e. lag, log and decay phase), resulted in continuously changing growth conditions,
381 potentially favoring different bacteria over time (see Fig. 4.a).



382

383 **Figure 4.** Hypothetic resume of growth rates depending on substrates concentrations in SBR **(a)** and CR **(b)**
384 conditions. **(a)** SBR culture conditions do not impose a strong growth rate to the system and substrate levels
385 vary over the growth period, hence bacteria with different specific growth rates and affinities can freely co-

evolve. **(b)** CR conditions are characterized by a strong dilution rate, which imposes a specific growth rate to the overall biological system. In the CR configuration substrates concentrations are well defined and become constant over time. Therefore only bacteria able to cope with the imposed dilution rate and having high affinity with the set of substrates concentrations can evolve in the system.

In view of the sub-optimal performances, the SBR culture system did not seem to offer the best solution between process efficiency and stability. Moreover, the diverse microbial community would be difficult to control in terms of constancy of composition, and the presence of different bacterial strains of uncertain nutritional composition would affect the quality of the HOB microbiome as such for MP production for feed and food purposes.

4.2 Continuous Reactor

The continuous operation at a dilution rate of 0.1 h^{-1} allowed to select for the evolvement of a more performing microbial culture in terms of biomass yields and volumetric productivities. Indeed the CR culture system selected for bacteria able to implement maximum substrate conversion at the specific growth rate imposed by the dilution D at which the bioreactor is operated (Goldberg, 1985). Thus microorganism having high specific growth rate can outcompete others not able to cope with the dynamics of the system. Such configuration can be summarized in the following two conditions:

1) $\mu \geq D=0.1\ h^{-1}$: Continuous growth

2) $\mu < D=0.1\ h^{-1}$: Wash-out

Fig. 4.b offers a virtual example of how the CR reactor impacted on the initial diverse community. The dilution rate of 0.1 h^{-1} required a corresponding specific growth rate of the same value. Moreover, the constant supply of nutrients and substrates to a biological system growing in steady conditions allowed to set a quite specific environment able to naturally select for the more adaptive and fast growing bacteria. In other terms, only bacteria

410 possessing a specific growth rate higher than the dilution rate imposed, as well good
411 affinities with the substrates provided were not washed-out.

412 As revealed by the microbial community analyses, within three weeks of operation the high
413 dilution rate resulted in the selection of a highly specific microbial culture, dominated for
414 more than 96% by *Sulfuricurvum* spp. The latter genus is known as encompassing a specific
415 type of bacteria predominantly active towards sulfur oxidation in crude oil deposits (Han et
416 al., 2012). *Sulfuricurvum kujiense* YK-1^T was first isolated from oil sands and characterized as
417 a facultative anaerobic sulfur oxidizing bacteria (sulfide, elemental sulfur and thiosulfate)
418 also able to use hydrogen as electron donor. Electron acceptors were described to be nitrate
419 and oxygen under anaerobic and aerobic conditions, respectively. Aerobic growth though
420 was limited to microaerophilic ranges (with maximum 1% in the headspace)(Kodama and
421 Watanabe, 2004). Three other strains of *Sulfuricurvum kujiense* were already reported, but
422 only strain YK-1T was cultured and characterized in its whole genome (Han et al., 2012).
423 Recently, the complete genome of *Candidatus Sulfuricurvum* sp. RIFRC-1 was assembled de
424 novo from an aquifer-derived metagenome, confirming the importance and the link
425 between sulfur and hydrogen metabolism in terrestrial subsurface environments. The latter
426 finding also points out how more strains of the *Sulfuricurvum* spp. genus might still be
427 discovered and characterized.

428 The *Sulfuricurvum* spp. dominating the culture studied in the present work is closely related
429 to *Sulfuricurvum kujiense*, yet the exact identity of the strain is still unclear. The mineral
430 medium used to culture the HOB both under sequenced batch and continuous reactor
431 conditions did not contain any reduced sulfur compound such as sulfide, elemental sulfur or
432 thiosulfate. Only oxidized sulfur under the form of sulfate ($MgSO_4 \cdot 7H_2O$ (0.5 g/L)) was

433 supplied to the reactor with the influent mineral medium. The development of the high
434 enrichment of *Sulfuricurvum* spp. without any available reduced sulfur substrate points out
435 that the most plausible metabolism for biomass growth within the biological system was
436 hydrogen as electron donor and oxygen as electron acceptor. Therefore, when analyzing the
437 conditions under which hydrogen oxidation was carried out, the fact that partial pressures of
438 O_2 in the headspace of the CR were constantly higher than 1%, reaching 5-6% for long
439 periods, constitutes a first important difference with the strain YK-1^T as characterized by
440 Kodama and Watanabe (2004). The latter observation is supported by the fact that dissolved
441 oxygen levels between 0.5 and 1.2 mg O_2/L could be measured in the effluent of the
442 continuous reactor. Although the abovementioned physiological characterization reported
443 the use of H_2 as electron donor in combination with microaerophilic O_2 concentrations, it did
444 not identify the possibility of exploiting such bacterium for high rate autotrophic hydrogen
445 oxidation, as experimentally demonstrated in this study has not been described before.

446 As reported in Table 2, the cultured *Sulfuricurvum* spp. displayed biomass yields and
447 volumetric productivities comparable to the ones reported for *Alcaligenes eutrophus*
448 *ATCC17697* from (Morinaga et al., 1978), outscoring the values available in other studies for
449 continuous cultures of *Alcaligenes hydrogenophilus* and *Cupriavidus eutrophus* B-10646
450 (Miura et al., 1982; Volova et al., 2013). *Alcaligenes eutropuhs*, currently known as
451 *Cupriavidus necator* can be also regarded as reference for a possible overall stoichiometry of
452 carbon dioxide and ammonium nitrogen assimilation into bacterial biomass within the
453 biological system driven by hydrogen oxidation (Ishizaki and Tanaka, 1990):

454 $$21.36\ H_2 + 6.21\ O_2 + 4.09\ CO_2 + 0.76\ NH_3 \rightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76} + 18.70\ H_2O \quad (5)$$
455

456 The molar ratios which can be calculated from the stoichiometry are in fact close to the gas
457 ratios used in the present experiment. More specifically, from the stoichiometry a ratio of 3.4
458 can be calculated between H_2 and O_2, which is comparable to the ratio of 3.2 at which the
459 enrichment of *Sulfuricurvum* spp. was obtained. Similarly, the ratio of 4.8 between H_2 and
460 CO_2 used in the present investigation was similar to the 5.2 obtained from the stoichiometry
461 of *Cupriavidus necator*.

462 The fact that the culture dominated by *Sulfuricurvum* spp. matched efficiencies in terms of
463 biomass yield and volumetric productivities of other well-known HOB strains while being fed
464 with gas mixtures suitable for HOB growth, represents an interesting and novel finding and
465 holds the potential to expand the biotech applications of autotrophic hydrogen oxidation to
466 unexplored bacteria. Further research is warranted to investigate its potential in more detail.

467 Interestingly, the microbial composition was stable over the course of the experiments (90
468 days) and dominated by the same genus (see Figure 1). This finding is important as this
469 implies that the fermentation process can be easily managed without cumbersome sterility
470 precautions (e.g. media autoclaving, gas filtering). The latter feature can be of interest in
471 allowing the direct upgrade of used resources such as carbon dioxide and ammonia gas
472 recovered e.g. from biogas and anaerobic digestate, respectively (Matassa et al., 2015b),
473 without requiring strict subsequent axenic processing conditions and related operational
474 costs. Further research is required to understand how such operational setting is resistant to
475 external invasion and destabilization. Indeed, the latter can have biotechnological
476 applications which go beyond the aim of this study.

477 In relation to the other bacteria coexisting with the *Sulfuricurvum* spp., the spectrum was
478 composed by heterotrophic bacteria pertaining to the classes of *Gammaproteobacteria*
479 (*Thermomonas*) and *Flavobacteriia* (*Chryseobacterium*). It is therefore likely that under high

480 rate CR reactor configuration, these bacteria were benefitting from organic metabolites
481 from the HOB, in this case *Sulfuricurvum* spp. Yet, this equilibrium achieved under CR
482 conditions was totally different from the almost equal relative abundance between HOB and
483 heterotrophs under SBR conditions. The magnitude of the residual heterotrophic niche (in
484 terms of relative abundance) might be indeed depending on the growth conditions, and
485 more specifically on the dilution rate imposed to the system. Further research efforts might
486 aim at establishing whether or not such niche would be completely diminished at higher
487 dilution rates, not allowing the secondary heterotrophic consumers to take advantage of the
488 primary autotrophic carbon fixation activity.

489 *4.3 Nitrogen assimilation efficiency, protein and amino acids profile*

490 In terms of nitrogen assimilation and conversion efficiency, the system operated in batch-
491 mode was able to convert 100% of NH_4-N nitrogen into MP at 66% or more protein content
492 on CDW basis. In case of the CR configuration, the N-usage efficiency was lower, in the order
493 of 87% on CDW basis. The aim of the high rate CR operation was to attain maximum biomass
494 growth and MP accumulation, avoiding nutrient limitation. As result, nitrogen was added in
495 a slight excess with some nitrogen was still present (unused) in the effluent of the reactor. It
496 is likely that higher efficiencies could be obtained imposing more carefully N limiting
497 conditions and varying the initial nitrogen loading rate.

498 The biomass produced under constant CR operation revealed a high protein content of more
499 than 70%. The latter is in agreement with other studies on HOB for MP production (Volova
500 and Barashkov, 2010), and confirms that *Sulfuricurvum* spp. might be suitable as a MP
501 producing bacterium. The overall protein content is higher than the 68% reported for
502 bacterial meal as well as than the one of fishmeal, regarded as high-quality additive in

503 nutrition and also than the one of soybean meal, the reference vegetable protein for
504 livestock. In the same way, the amino acids profile of the produced MP revealed a close
505 compatibility to the one of bacterial meal as well as fishmeal, outscoring the one of soybean
506 meal. Bacterial meal, as already produced from natural gas could also be used to directly
507 upgrade the biogas produced from anaerobic digestion of sewage into MP. As discussed in a
508 recent review (Matassa et al., 2015b), more than being self-excluding the hydrogen and the
509 methane platforms can be seen as complementary, depending on the availability of each
510 resource on-site. Like for bacterial meal, which already received positive feedback from feed
511 trials, preliminary in vitro tests on the nutritional digestibility of our MP were also positive
512 (data not shown). Clearly, the findings obtained in the study need to be complemented by
513 detailed animal studies in which aspects of long-term gastro-intestinal uptake and putative
514 nutritional side effects are scrutinized. However, the current findings clearly show the
515 potential of using the produced MP as high-quality feed/food additive, offering a valid
516 alternative to the high land, water, nutrients and carbon footprint of conventional vegetable
517 protein production (Walsh et al., 2015). If this would be done by upgrading nitrogen
518 recovered from used water the benefits in terms of avoided N losses and emissions could be
519 even higher (Matassa et al., 2015a).

520 **5. Conclusions**

521 In this study, we aimed at assessing the potentialities of autotrophic hydrogen oxidation to
522 recover and upgrade of resources under different operating conditions. The evolution of
523 HOB from a generic mixed microbial community under different operating conditions
524 allowed to reveal interesting and novel aspects, with potential for application in industrial
525 contexts. The key findings are:

- Under SBR conditions the enriched mixed culture revealed the coexistence of a diversity of microbial actuators
- Under high rate CR culture conditions the microbiome narrowed down to *Sulfuricurvum* spp. dominated culture which was both stable and highly productive
- Mineral nitrogen and carbon dioxide were directly upgraded into microbial biomass, rich in protein, by using hydrogen and oxygen with high efficiency under CR culture conditions;
- The nutritional properties of the produced MP are comparable to the high-quality fishmeal and surpass those of vegetable soybean meal.

Microbial biosynthesis of useful commodities from carbon dioxide is amongst the most challenging yet promising routes of the future bioeconomy. The exploration of renewable energy generation combined with technology advances in hydrogen production might enable on-site recovery and upgrading of valuable resources by means of HOB, produced under appropriate microbial resource management (MRM) conditions (Verstraete, 2015; Verstraete et al., 2007).

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