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Effectiveness of Hydrogen Production by *Bacteroides vulgatus* in Psychrophilic Fermentation of Cattle Slurry

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Abstract: H_2 is a low-impact energy carrier, which the EU hydrogen strategy has positioned as a major component of energy policy. Dark fermentation by psychrophilic bacteria is a promising avenue of H_2 production, though one that requires further study. The aim of this study was to determine the H_2 production performance of a *Bacteroides vulgatus* strain during fermentation of psychrophilic cattle slurry. The test strain was isolated from an inland water body at a depth of 40 ± 5 m. The experimental fermentation process was run at 15 ± 1 °C and yielded 265.5 ± 31.2 cm^3 biogas/g COD removed, including 46.9 ± 2.6 cm^3 H_2/g COD removed. CO_2 was the main constituent of the resultant biogas, at 79.8 ± 1.9%. The gas also contained 17.6 ± 1.4% H_2 and 2.3 ± 0.2% CH_4. Organic matter removal and nutrient take-up from the feedstock were low. Our findings show that practical applicability of this process is hampered by multiple operational hurdles and its relatively poor performance.

Keywords: dark fermentation; biohydrogen; biogas; psychrophilic bacteria; cattle slurry

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1. Introduction

The deteriorating condition of the natural environment and the growing social awareness related to this issue necessitate the search for and implementation of clean energy production technologies [1–3]. Hydrogen meets the criteria of a low-impact energy carrier [4,5], as recognized by energy producers, environmental organizations, policy makers, and politicians alike [6]. On 8 July 2020, the European Commission published its Hydrogen Strategy for a Climate-Neutral Europe [7]. The strategy cites green hydrogen as one of the key energy carriers that can help reach the goals of the European Green Deal [8]. The strategy focuses on stimulating the development of a renewable, green hydrogen sector, with a view of making hydrogen a fully zero-carbon, ubiquitous energy source in the EU by 2050 [7,9]. Promoting hydrogen production and use is intended to form part of the decarbonization and sector-coupling strategy. Through energy storage, hydrogen can also be a way of balancing systems that increasingly rely on renewable energy. This is why the strategy for a climate-neutral EU targets a 13–14% share of hydrogen in the European energy mix, compared to the current 2% [7,10].

H_2 can be used as an electron donor in other processes supporting sustainable and clean energy technologies. An example is the biological methanation process (BMP) based on CO_2 as the sole carbon source. The CO_2-BMP process can be used in many applications such as biogas upgrading, power-to-gas applications, and decentralized energy production, and to convert H_2/CO_2 from process flue gases into value products, e.g., from the ethanol, petroleum, steel, and chemical industries [11]. An important aspect is also optimization of the process by ensuring the correct H_2 partial pressure in the system. This can be achieved by integrating hydrogen dark fermentation with the conversion of CO_2 and H_2 to CH_4 carried out by micro-organisms from the Archea domain. High values of the H_2 partial

pressure in the reactors significantly inhibit the H_2 production efficiency, and thus limit the technological and economic efficiency of the process [12].

Biological technologies are becoming economically viable as methods of hydrogen production [13,14]. Hydrogen production via bacterial dark fermentation is one promising method [15,16]. The types of fermentation most relevant to H_2 production are butyrate/butanol fermentation, common among the *Clostridium* sp., and mixed-acid fermentation, mostly used by the family *Enterobacteriaceae* (*Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Vibrio cholerae*, *Escherichia coli*, and *Shigella dysenteriae*) and *Bacillus* sp. [17]. The fermentation process carried out by psychrophilic bacteria is based on enzymatic processes analogous to mesophilic or thermophilic digestion. H_2 production under anaerobic conditions is common in nature [18]. As mentioned above, a wide range of bacterial strains use the reduction of protons to H_2 in order to remove the products of metabolism from the environment.

During the bacterial heterotrophic growth, the organic substrates are degraded to provide compounds and metabolic energy for growth. This process generates electrons that must be removed to maintain the electrical neutrality of the environment [19]. In anaerobic digestion, the electron acceptors are protons, which are reduced to H_2. Alternative electron acceptors in anaerobic conditions can be nitrates reduced to N_2, sulfates transformed to H_2S, or organic compounds in microbial production of butanol by reduction of butyric acid [20]. The ability to reduce nonoxygen electron acceptors requires the presence of a specific enzyme system based on hydrogenases. The biohydrogen production through biological anaerobic processes involves the reduction of protons by hydrogenase, using electrons donated by ferredoxin. The electrons are released by the degradation of glucose to pyruvate, which is then oxidized to Acetyl-CoA and CO_2 [21].

The dark fermentation produces a maximum of 4 moles H_2/mole of glucose, with an energy production of 206 kJ/mole of glucose. This is enough to support the growth of the anaerobic bacterial population. The rest of the H_2 is converted into acetate or ethanol, lactate, or alanine, as by-products of the process [22]. The efficiency of H_2 production during anaerobic digestion is influenced by many factors, including the presence and quality of available organic matter, the presence of minerals, temperature, light, pH, salinity, redox potential, H_2 partial pressure, and synergistic or antagonistic effect of the microbial population [23]. The environmental conditions and technological parameters of the process influence the activity of the microbial population, and thus the concentration and diversity of end products, which include CO_2, H_2, H_2O, NO_3, CH_4, etc. [24].

Hydrogen fermentation under thermophilic or mesophilic conditions (35 °C–70 °C), though widely used, is hampered by microbial vulnerability to variable environmental parameters and costs of bioreactor heating [25]. Technologies that utilize a chosen genus of psychrotrophic micro-organisms may prove to be a viable alternative [26]. Such solutions could reduce the expenditure on heating reactors and enable wider use of fermentation technology in regions with less-than-ideal climatic conditions [27]. Hydrogen-producing bacteria, which are strict anaerobes, are broad-spread in the natural biocenosis [28,29]. They are able to survive and metabolize at negative temperatures [30]. This resilience of psychrophiles is borne out of mutations in genes coding for ribosome proteins and enzymatic proteins [31].

Micro-organisms are affected by temperature levels, either directly—when the temperature modifies their growth rates, enzyme activity, cellular characteristics, and nutrient requirements—or indirectly, as the temperature changes the solubility of intracellular molecules, ion transport, diffusion, and the osmotic properties of cell membranes [32]. Enzymes of cold-adapted micro-organisms have been shown to exhibit higher rates of catabolic activity than those of the mesophilic or thermophilic micro-organisms [33]. It has also been observed that cold-adapted micro-organisms tend to produce more enzymes at suboptimal temperatures to compensate for the potentially slower reaction rates [34]. Psychrophilic and psychrotrophic micro-organisms exhibit high enzyme activity and catalytic capacity, as well as considerable adaptability across the temperature range of 0–20 °C [35].

The enzymes secreted by psychrophilic and psychrotrophic bacteria are distinguished from those produced by mesophiles by lower optimum temperature, activation energy for substrate hydrolysis, and greater thermal stability [36].

The aim of this study was to determine how a *Bacteroides vulgatus* strain performs in terms of hydrogen production and primary pollutant removal during fermentation of psychrophilic cattle slurry.

2. Materials and Methods

2.1. Materials

The *Bacteroides vulgatus* strain used in the experiment was isolated from the benthic water of Lake Hańcza (the deepest inland water body in Poland) at a depth of 40 ± 5 m. Initial concentration of *B. vulgatus* biomass in the bioreactors was 180.0 mg DM/dm^3 (DM—dry mass). Cattle slurry was used as the organic feedstock for the experiment. The slurry was sourced from the Teaching and Research Station of the University of Warmia and Mazury in Olsztyn (Bałdy village, Poland). The entirety of the subject slurry was collected from a 50 m^3 nonoutflow tank that served as a retention chamber for discharging the manure from the cowsheds and water used to rinse the station's milking system. The profile of the slurry (original and dis-solved) used for the experiments is presented in Table 1. The slurry solution was pasteurized (30 min, 90 °C) before being fed into the anaerobic reactors in order to remove competing micro-organisms.

Table 1. Profile of raw and dis-solved cattle slurry used during the experiments.

Feedstock	Parameter	Unit	Mean Value	Standard Variation
Raw slurry	COD	[mgO_2/dm^3]	50,742.8	4092.6
	BOD_5	[mgO_2/dm^3]	39,739.6	0.5
	TN	[mg N/dm^3]	10,600	199.7
	TP	[mg P/dm^3]	694.2	72.7
	pH	-	7.12	0.08
	Dry matter	[g/dm^3]	18.92	2.55
	Volatile substances	[g/dm^3]	15.72	1.78
	Minerals	[g/dm^3]	3.20	0.99
Dis-solved slurry 100.0 g/dm^3	COD	[mgO_2/dm^3]	5093	230
	BOD_5	[mgO_2/dm^3]	3499	190
	TN	[mg N/dm^3]	403	32
	TP	[mg P/dm^3]	77.2	12.6
	pH	-	7.09	0.19

2.2. Experimental Set-Up

New Brunswick BioFlo 310 batch bioreactors were used in the experiment, with internal temperature maintained at 15 ± 1 °C. The reactor content was mixed with a vertical paddle agitator at a rate of 100 rpm. The active volume was 500 cm^3, retention time—30 days. The nitrogen-purged (5 min, 150 dm^3/h), diluted cattle slurry was fed into the reactor, then inoculated with *B. vulgatus*.

2.3. Microbiological Identification Procedure

The samples were fixed with 4% paraformaldehyde, then left at 4 °C for 24 h. After fixation, the biological material was selected on polycarbonate filters (Millipore GTTP, pore size = 0.2 mm, diameter = 47 mm). Selected groups of micro-organisms were identified by fluorescence in situ hybridization (FISH). The probe EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′) was used. The preparations were analyzed under immersion conditions in an epifluorescence microscope, using two types of filters for DAPI and Cy3 [27].

Psychrophilic bacteria were isolated on Brucella agar supplemented with defibrinated blood, hemin, and vitamin K. The micro-organisms were obtained after centrifugation at 10 °C/4000 rpm/15 min. The treatments were performed without oxygen in the BACTRON

chamber. The biomass was incubated at 8 °C. Identification of selected strains was carried out using bioMerieux API 20A tests intended for anaerobic bacteria. The bacteria were typed by sequencing their 16S rDNA using the BigDye Thermiantor v3.1 kit on an ABI 3730x genetic analyzer (Applied Biosystems, Coster City, CA, USA).

2.4. Analytical Methods

The feedstock and reactor effluent were analyzed for COD (Chemical Oxygen Demand), TN, TP, using a DR 5000 spectrophotometer and an HT 200 s mineralizer (Hach Company, USA). BOD_5 (Biological Oxygen Demand) was monitored using an Oxi-top control system (Wissenschaftlich-Technische Werkstätten (WTW), Weilheim in Oberbayern, Germany). Sample pH was measured with a 1000 L pH meter (VWR, Radnor, PA, USA). Biogas output was measured using a mass flow meter (Aalborg Instruments and Controls, Inc., Orangeburg, NY, USA). Qualitative composition was determined chromatographically with a GC 7890 A chromatograph (Agilent Technologies, Santa Clara, CA, USA). Biogas measurements were converted to normal conditions.

2.5. Statistical Analysis

The experiment was conducted in triplicate. Statistical analysis was performed using Statistica 13.1 PL. Distribution of variables was verified using Shapiro–Wilk's W test. The Tukey honestly significant difference (HSD) test and ANOVA were applied to determine significant differences between the variables. Results were considered significant at $p = 0.05$.

3. Results and Discussion

Many studies have proven that anaerobic digestion is an effective H_2 production technology [37]. This is based on the exhaustive understanding of the metabolic pathways of the process, the optimal technological parameters, and the characteristics of the substrates used, as well as the availability of suitable equipment and buildings (including large-scale facilities) [38]. There are numerous examples where organic feedstock is a substrate for biological hydrogen production, including sewage sludge, manure and slurry, sewage, and municipal and food waste [39,40]. A large majority of these processes incorporate mesophilic or thermophilic fermentation [41]. Wu et al. (2010) investigated fermentation of swine manure supplemented with glucose and determined hydrogen production at 2.25 $dm^3/dm^3 \cdot d$ and hydrogen content of the biogas at 36.9% [42]. In turn, in the study by Kim et al. (2008), food waste was fermented using *Clostridium beijerinckii* KCTC 1785 at 40 °C [43], which allowed for the achieving of a hydrogen production of 128 cm^3/g $COD_{removed}$ and a hydrogen yield close to 110 $cm^3/dm^3 \cdot h$. Finally, Song et al. (2012) processed cow dung using dark fermentation and obtained a hydrogen yield of 290.8 cm^3/dm^3 culture. The feedstock input into the system was 10 g/dm^3 and the initial pH was around 7.0. The dominant hydrogen producers were *Clostridium* sp. and *Enterobacter* sp. [44].

Despite the above information, however, there are not enough data to determine whether psychrophilic and psychrotrophic micro-organisms can be used to produce hydrogen from waste organic substrates while maintaining high yields and cost-effectiveness. The present study achieved a total biogas production of 58.8 ± 4.0 cm^3 after 30-day fermentation (Figure 1a), which means that, relative to the initial *B. vulgatus* biomass in the bioreactor, the production was 653.3 ± 44.1 cm^3/gDM (Figure 1b). The H_2 in the biogas amounted to 17.6 ± 1.4% (Figure 2a) or, in nominal terms—10.3 ± 1.5 cm^3 H_2 (Figure 1a) and 114.7 ± 16.7 cm^3 H_2/gDM (Figure 1b). The CO_2 fraction was 79.8 ± 1.9% (Figure 2a), which translates to 46.9 ± 2.2 cm^3 CO_2 (Figure 1a) or, on a dry-matter basis—521.4 ± 21.3 cm^3 CO_2/gDM (Figure 1b). CH_4 fraction was 2.3 ± 0.2% (Figure 2a), which translates to a yield of 1.4 ± 0.1 CH_4 (Figure 1a) and 15.0 ± 1.1 cm^3 CH_4/gDM (Figure 1b). The output of other gases was negligible: 84,000 ± 9000 ppm O_2, 2070 ± 190 ppm H_2S, and 20,900 ± 1970 ppm NH_3 (Figure 2b).

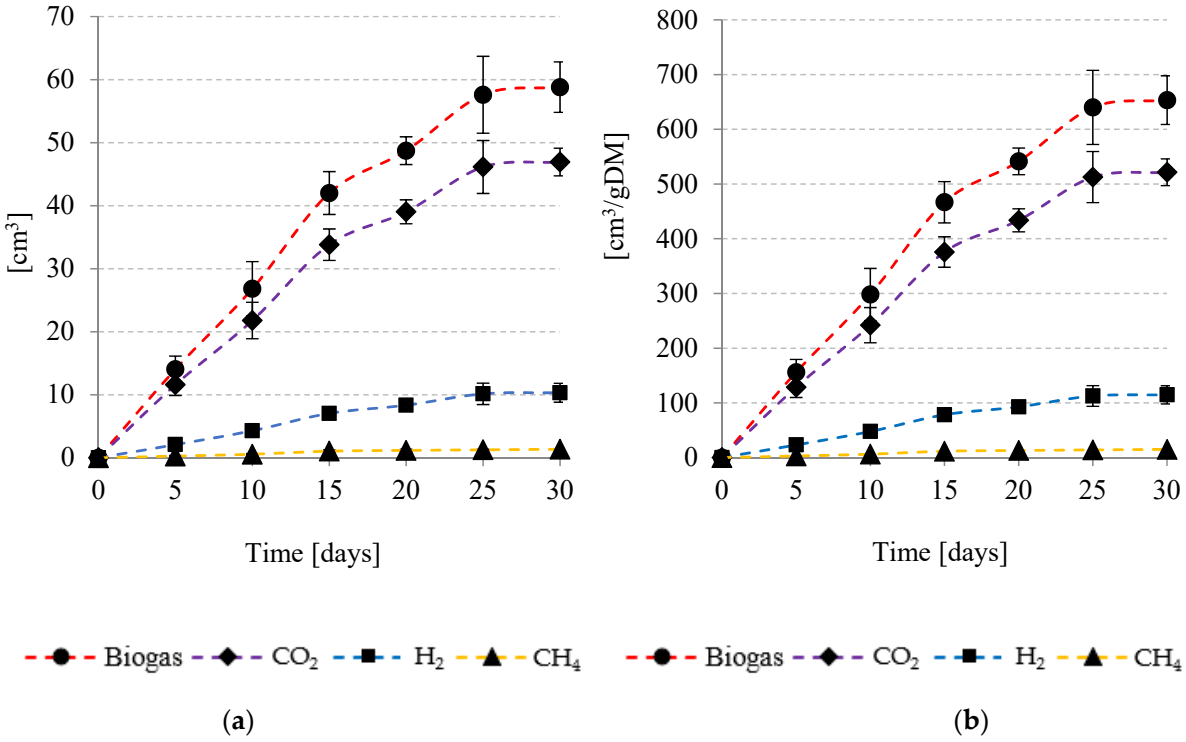

Figure 1. Build-up of biogas and its main constituents (**a**) total (**b**) per unit.

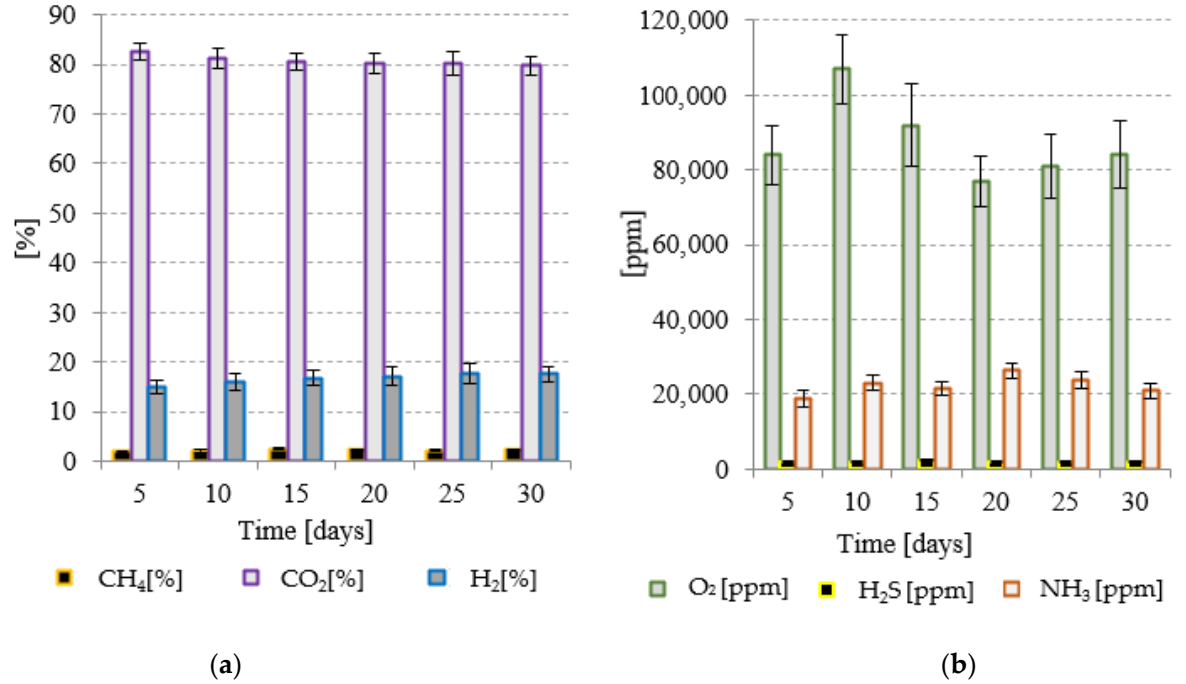

Figure 2. Biogas fractions (**a**) CH_4, CO_2, and H_2, (**b**) O_2, H_2S, and NH_3.

Alvarez-Guzmán et al. (2020) were one of the few to examine whether psychrophilic bacteria can be used for fermentative hydrogen production from organic waste. Using the psychrophilic bacterium GA0F, the researchers obtained 73.5 ± 10 cm^3 H_2/g from whey powder, 43.6 ± 2 cm^3 H_2/g from wheat straw hydrolysate, and 52.4 ± 4 cm^3 H_2/g from cane molasses [45]. Zieliński et al. (2017) have tested the utility of psychrophilic bacteria from the phyla *Proteobacteria* (*Rahnella aquatilis*, *Raoutella terrigena*) and *Firmicutes* (*Carnobacterium maltaromaticum*, *Clostridium algidixylanolyticum*) with regard to photofermentative

hydrogen production from cheese whey. The microbes were isolated from underground water and demersal lake water. The H_2 in the biogas ranged from 32.61% to 43.21%, with nominal H_2 production between 20.1 and 58.1 cm^3 H_2/g COD. The highest hydrogen yields—at 16.64 cm^3 H_2/g bacterial biomass—were achieved when *Rahnella aquatilis* was used [46]. Dębowski et al. (2014) tested photofermentative hydrogen production from cheese whey using psychrophilic bacteria from the class *Gammaproteobacteria—Rahnella aquatilis* (nine strains) and the *Firmicutes* species: *Carnobacterium maltaromaticum*, *Trichococcus collinsii*, and *Clostridium algidixylanolyticum*. The study found that biogas production varied greatly—between 126.48 and 477.72 cm^3/g bacterial biomass—and was highly strain-specific. *R. aquatilis* isolated from demersal lake water performed the best in terms of hydrogen production, with H_2 fractions in the biogas of 65.15–69.12% and H_2 yields of 1587.47–3087.57 cm^3/g. Conversely, *Firmicutes* proved to be the poorest hydrogen producers, with only 15.46% to 20.70% H_2 in the gas metabolites [40].

No significant reduction in organic matter in the cattle slurry solution was noted in the course of fermentation. The initial COD of 5093 ± 230 mgO_2/dm^3 dropped to 4874 ± 190 mgO_2/dm^3 (Figure 3). The biodegradation efficiency was no more than 4.3 ± 0.2%. BOD_5, and fell from 3499 ± 120 to 3128 ± 130 mgO_2/dm^3 (Figure 3), which translates to a removal efficiency of approx. 10.6 ± 0.4%. The observed degradation of organic matter (as expressed by BOD_5) was statistically significant ($p \leq 0.05$). Nitrogen levels showed a reduction of 15.1 ± 1.1%—from the initial level of 403 ± 32 mg N/dm^3 to 342 ± 24 mg N/dm^3 (Figure 3). Phosphorus removal was 8.9 ± 0.1%, with *p* levels starting at 77.2 ± 1.2 mg P/dm^3 and dropping to 70.3 ± 0.8 mg P/dm^3 (Figure 3).

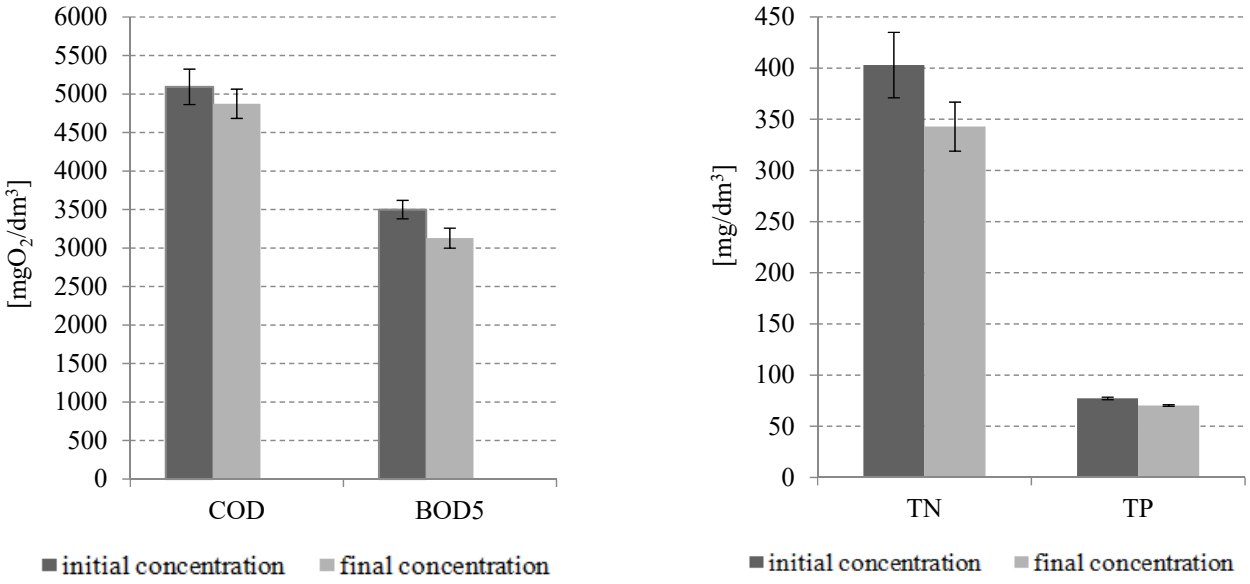

Figure 3. Trends in (**a**) organic levels, (**b**) nutrient levels.

According to Fu et al. (2021), organic matter in waste is largely underutilized during dark fermentation due to the rigid structure of microbial walls [47]. A study by Yang et al. (2019) has demonstrated that anaerobic fermentation systems perform at less than 40% VSS reduction [48]. The released organic substances are only partially taken up to produce hydrogen. Though the anaerobes absorb a small number of soluble substrates to fuel their growth and survival, a large proportion of soluble organics is left in the fermentation liquid [47].

The pH in the fermented cattle slurry was stable throughout the process, oscillating around 7.09 ± 0.19. It has been shown that optimal pH for efficient hydrogen production ranges from 5.0 to 6.0 [49]. Lower pH values cause microbes to switch their metabolism

toward other biochemical processes, and pH under 4.0 can inhibit microbial growth [50]. Conversely, increased pH can induce methanogenic bacteria to grow, consuming hydrogen to produce methane [51]. In our study, pH > 7 did not affect the species composition of the hydrogen-producing bacteria inoculum, thanks to the use of a pure, isolated strain and presterilized organic substrate.

4. Conclusions

The experiment served to assess the applicability of the *Bacteroides vulgatus* strain for fermentative hydrogen production from psychrophilic cattle slurry. Our findings show that the practical value of this process is hampered primarily by its poor technological performance. The experiment yielded 265.5 ± 31.2 cm^3 biogas/g COD removed, including only 46.9 ± 2.6 cm^3 H_2/g COD removed. CO_2 was the main constituent of the biogas, accounting for 79.8 ± 1.9%. The biogas also contained 17.6 ± 1.4% H_2 and 2.3 ± 0.2% CH_4.

The H_2 output was also very poor when expressed relative to the initial organic load. The obtained values were not competitive compared to those reported for mesophilic and thermophilic fermentation in the literature. This stems from the low concentrations of the isolated *Bacteroides vulgatus* biomass in the bioreactors (180 mg DM/dm^3). It appears that more bacterial biomass in the system would be required to improve organic matter biodegradation and the biogas/hydrogen output.

It seems that there are multiple and significant barriers to successful application of fermentation processes based on isolated, single-species cultures of psychrophilic bacteria. These include problems with industrial-level isolation/cultivation of strains, scaled-up production of bacterial biomass, and ensuring the purity of the bacterial community when supplying and operating large facilities. Overcoming these issues, while possible, would be associated with high costs, high process complexity, and operational complications.

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