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The Cultivation of Biohydrogen-Producing *Tetraselmis subcordiformis* Microalgae as the Third Stage of Dairy Wastewater Aerobic Treatment System

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Abstract: The development of wastewater treatment systems, including competitive methods for nitrogen and phosphorus removal, is focused on intensifying final technological effects with due care taken for economic and environmental concerns. Given the possibility of integrating wastewater treatment processes with biofuel production, the prospective seems to be technologies harnessing microalgal biomass. The present study aimed to verify the feasibility of applying *T. subcordiformis* genus microalgae as the third stage of the dairy wastewater treatment process and to determine microalgae biomass production effectiveness and hydrogen yield in the biophotolysis process. The study proved that microalgae cultivation with dairy wastewater was nearly 35% less effective compared to that with a chemically pure medium. Nitrogen and phosphorus compounds contaminating wastewater were found to represent an available source of nutrients for *T. subcordiformis* population. The volume of hydrogen produced ranged from 116 ± 7 cm^3 to 162 ± 7 cm^3, and the percentage of H_2 content in the biogas ranged from 55.4 ± 2.2% to 57.2 ± 4.1%. A significantly higher hydrogen yield per initial biomass concentration, reaching 69 ± 4.2 $cm^3/g_{o.d.m.}$, was determined in the variant with wastewater accounting for 50% of the culture medium. The respective value noted in the control respirometer was 54 ± 2.1 $cm^3/g_{o.d.m.}$

Keywords: dairy wastewater; treatment technology; *T. subcordiformis*; microalgae biomass; nutrients; cultivation medium; biohydrogen; biofuels

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1. Introduction

The main avenues in the development of wastewater treatment systems include the implementation of solutions which significantly improve the ultimate technological effectiveness, are economically viable, and meet requirements of environmental neutrality [1,2]. Novel solutions should take account of the assumptions of a closed-circuit system, bioeconomy fundamentals, the zero-waste production concept, and determinants of integrated and sustainable biorefinery concepts, as well as go in line with plans for reducing greenhouse gas emissions and developing renewable energetics [3].

Therefore, solutions are searched for in which wastewater is treated as a source of valuable substances and energy [4]. Due to the rising prices of coal, natural gas, crude oil, as well as fertilizers and food, preference is given to integrated treatment systems ensuring nitrogen and phosphorus recovery as well as the production of qualitatively valuable biomass and alternative energy carriers [5]. A crucial aspect in developing technological systems for wastewater biodegradation is the search for energy-saving solutions which

minimize the use of chemical methods posing the risk of re-contamination and are based on environmentally friendly biological methods [6]. It is, therefore, of the utmost importance to develop efficient solutions for the removal of nitrogen and phosphorus compounds implicated in intensifying the eutrophication and degradation of aquatic reservoirs [7,8].

An alternative to the currently deployed methods seems to be solutions based on hydrophytic systems, including dynamically developing microalgae-based technologies [9]. Microalgae-based systems are increasingly considered viable solutions in environmental engineering and protection, including in technologies for wastewater treatment, solid waste neutralization, and biofuel production [10,11]. The potential of microalgae in various applications lies in the high effectiveness of their photosynthesis process, the fast rate of biomass growth, and their capability for consuming and utilizing waste substances [12]. Furthermore, microalgae represent a highly genetically diversified group of organisms, which directly impacts their physiological and biochemical characteristics [13]. This affords the possibility of choosing and adapting specified strains for individual applications, including for use in wastewater treatment systems [14,15]. When choosing microalgae strains for wastewater treatment, their growth rate, resistance to specific contaminants present in wastewater, adaptation capabilities, and contaminant binding effectiveness should be taken into account [16].

Microalgae are capable of accumulating in their biomass multiple bioactive substances featuring a great potential to be harnessed in the industry [17]. They can be used to produce a wide array of cellular metabolites, including high-quality proteins, lipids, carbohydrates, pigments, and vitamins for the food and feedstuff industry, the cosmetic industry, and the alternative energy source sector [18,19]. One of the avenues of research recently spurring great interest among scientists is the possibility of hydrogen production by microalgae. Studies conducted so far have proved microalgae from the species *T. subcordiformis* to be highly prospective in this respect. This strain is capable of photosynthetic hydrogen production via direct biophotolysis, i.e., an enzymatic reaction catalyzed by hydrogenase [20,21]. Two transmembrane peptide complexes, photosystem I (PSI) and photosystem II (PSII), are responsible for the photolysis of water molecules. PSI anaerobically transfers electrons through ferredoxin to the hydrogenase, which initiates the production of hydrogen, while PSII produces O_2. Anaerobic conditions are required to induce hydrogen production and hydrogenase activity. Environments with oxygen levels below 0.1% provide the best conditions for cell systems for hydrogen production [22].

The source, form, and availability of N and P have been deemed the key parameters affecting the growth and profiling of the chemical composition and enzymatic activity of microalgae [23]. Kim et al. (2016) investigated the effect of nine various nitrogen sources, i.e., NH_4HCO_3, NH_4Cl, NH_4NO_3, KNO_3, $NaNO_3$, urea, glycine, and yeast extract (organic nitrogen), on the effectiveness of *T. subcordiformis* biomass production. They demonstrated that organic N derived from the yeast extract and glycine enabled achieving the highest microalgae concentration in photobioreactors, reaching 2230 mg/dm^3 and 1620 mg/dm^3, respectively. In contrast, the use of $NaNO_3$ yielded a biomass concentration of barely 1450 mg/dm^3. The cited study proved nitrogen source and availability to be the key determinants of the ultimate technological effect [24]. Another research scrutinized the feasibility of harnessing municipal sewage before and after nitrification for *T. subcordiformis* production. Thus, it evaluated the impact of the mineral form of nitrogen (ammonia nitrogen and nitrates) on the course and the ultimate efficiency of microalgae cultures. Biomass productivity in both variants was similar under continuous culture conditions and approximated 343 $\pm$ 53 mg/dm^3·d, whereas biomass concentration reached 1900 $mg_{o.d.m.}$/dm^3 [25]. Nitrogen is used for protein synthesis, and the lack of it stops the synthesis of polypeptides [26]. Ammonia, due to the speed of uptake and the low energy needed for transport across the cell membrane, is the most common option chosen by the cell among all forms of nitrogen. In addition, the external concentration of ammonia is a signal for the activation of this substance's metabolism [27]. Phosphorus is involved in the formation of nucleotides and ATP. Some species of microalgae can over-take up phosphorus and store it as polyphos-

phates, and other microorganisms store nitrogen sources in the form of nitrates in their vacuoles. The uptake of these elements depends on their concentration in the environment. When it is low, the microalgae accumulate more carbohydrate substances and fewer N and P compounds, so the increase in biomass decreases. In the case of the availability of nutrients rich in N and P, microalgae store less carbon-rich substances and more with a high content of N and P, which results in an increase in biomass [26]. The direction consistent with the circular economy and biorecycling is the use of N and P compounds from wastewater.

The study goal was to determine the possibility of harnessing microalgae from the genus *Tetraselmis subcordiformis* as the third stage of dairy wastewater treatment in a technology based on a sequence of biological processes in the aerobic activated sludge—hydrophytes—microalgae system. Experimental works aimed to evaluate the effectiveness of nitrogen and phosphorus compound removal, verify the impact of applying pre-treated dairy wastewater on the effectiveness of *T. subcordiformis* biomass production, and determine the effect of the experimental variants tested on the yield of hydrogen production by microalgae. The dynamics of microalgal population development and hydrogen production yield were referred to as the results achieved with a culture medium prepared based on pure chemical reagents.

2. Materials and Methods

2.1. Experimental Design

The investigations were conducted on three experimental variants differing in the volume of dairy wastewater pre-treated in the activated sludge-hydrophytic system and fed to photobioreactors (PBRs) operating to proliferate *T. subcordiformis* microalgae. In variant 1 (control), the culture medium was made of deionized water enriched with pure chemical reagents. In variant 2, dairy wastewater and deionized water were fed to the photobioreactors in a 50%:50% ratio (*v*:*v*). In variant 3, wastewater accounted for 100% of the culture medium. In variants 2 and 3, the culture medium was supplemented with selected microelements. The experimental series was made up of four stages. In stage 1, wastewater was treated in a hybrid bioreactor; stage 2 involved the analysis of the impact of the experimental variants tested on the production effectiveness of *T. subcordiformis* microalgae biomass in PBR and on the efficiency of nitrogen and phosphorus compound removal from wastewater. Stage 3 involved *T. subcordiformis* biomass separation to remove the culture medium rich in sulfur compounds, which determined the biohydrogen production process. Stage 4 aimed to verify the effect of culture variants on the volume of hydrogen produced in the biophotolysis process. Figure 1 presents the organizational scheme of the research works.

2.2. Materials

2.2.1. Microalgae Biomass

The study used *T. subcordiformis* microalgae (UTEX 171) sourced from the UTEX Culture Collection of Algae. As part of the preparation stage, *T. subcordiformis* cultures were performed to produce sufficient biomass for the experiment. The microalgae were grown in sterilized Falcon tubes with an active volume of 50 cm^3. The tubes were pasteurized using a Tuttnauer 2840 EL-D autoclave at 121 °C for 15 min.

2.2.2. Dairy Wastewater

The wastewater used in the experiment was derived from a milk processing plant. It was pre-treated in the installation and exploited in the fractional–technical scale operating based on a hybrid system of activated sludge and a hydrophyte—narrow-leaf cattail (*Typha angustifolia)*. The characteristics of the raw wastewater and wastewater pre-treated in the activated sludge–hydrophyte system is presented in Table 1. The organic load rate of the activated sludge tank was at A = 0.10 $mgBOD_5/mg_{d.m.}\cdot d$.

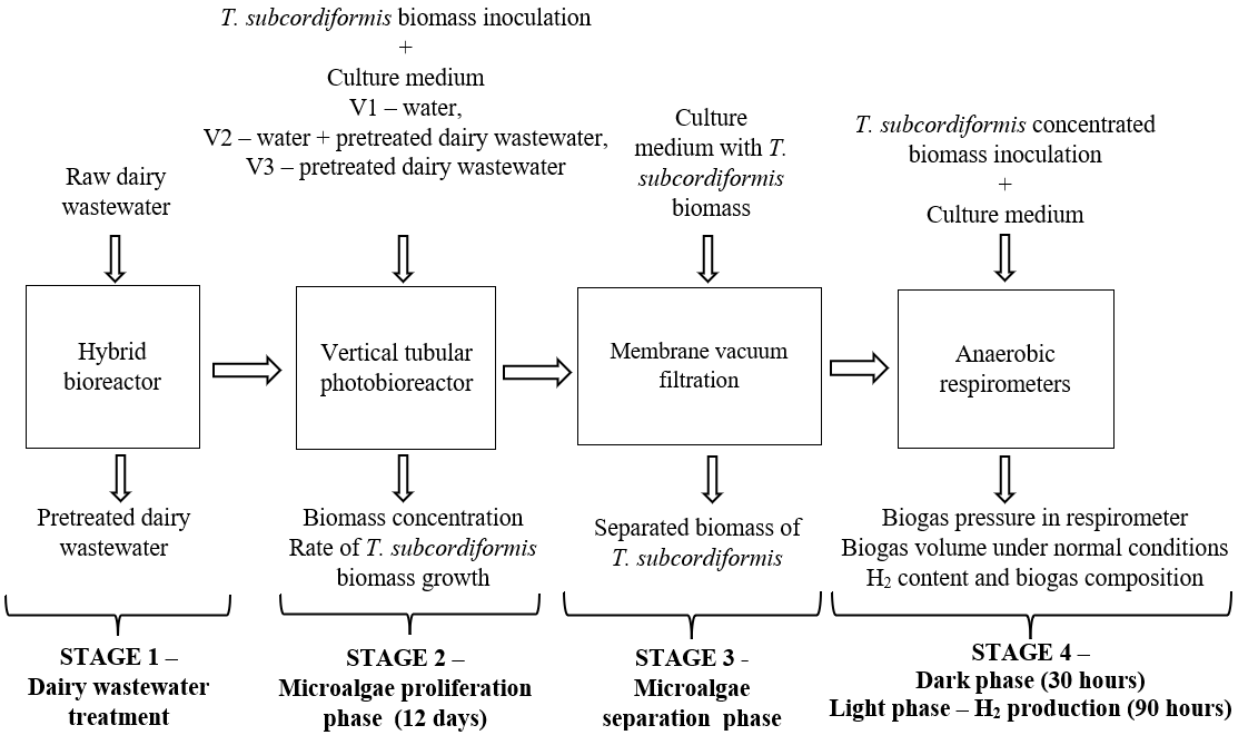

Figure 1. Scheme of experimental works.

Table 1. Characteristics of dairy wastewater used in the experiment.

Indicator	Unit	Raw Wastewater	Pre-Treated Wastewater
BOD_5	mgO_2/dm^3	3010 ± 258	15.1 ± 3.2
COD	mgO_2/dm^3	4280 ± 299	55.7 ± 9.8
$N_{tot.}$	mg N/dm^3	192 ± 9.6	23.6 ± 4.3
$N_{org.}$	mg $N_{org.}/dm^3$	160 ± 12.1	2.2 ± 0.3
$N\text{-}NO_3$	mg $N\text{-}NO_3/dm^3$	1.9 ± 0.3	20.7 ± 3.7
$N\text{-}NO_2$	mg $N\text{-}NO_2/dm^3$	1.2 ± 0.5	1.7 ± 0.4
$N\text{-}NH_4$	mg $N\text{-}NH_4/dm^3$	29 ± 6.2	1.9 ± 0.5
$P_{tot.}$	mg P/dm^3	48.2 ± 3.3	15.8 ± 2.4
$P_{org.}$	mg $P_{org.}/dm^3$	33.7 ± 4.1	3.6 ± 1.1
$P\text{-}PO_4$	mg $P\text{-}PO_4/dm^3$	15.6 ± 2.5	12.3 ± 2.9
pH	-	7.06 ± 0.14	7.06 ± 0.22
Total suspended matter	mg/dm^3	12.9 ± 1.6	4.2 ± 2.6

2.2.3. Culture Medium

The composition of the synthetic medium used in variant 1 was established according to Guan et al. [28] as follows: 0.36 mg/dm^3 $MnCl_2$, 1.30 mg/dm^3 $FeCl_3$, 33.60 mg/dm^3 H_3BO_3, 20.00 mg/dm^3 NaH_2PO_4, 45.00 mg/dm^3 EDTA, 0.21 mg/dm^3 $ZnCl_2$, 100.00 mg/dm^3 $NaNO_3$, 0.20 mg/dm^3 $CuSO_4$, 0.20 mg/dm^3 $CoCl_2$, 0.09 mg/dm^3 $(NH_4)_4Mo_7O_{24}$, 1.00 $\mu g/dm^3$ VB1, and 0.10 $\mu g/dm^3$ VB12. Its pH was 8.00–8.20, and the salinity was 30–33 ppt. In variants 2 and 3, the culture medium was supplemented with: 0.20 mg/dm^3 $CuSO_4$, 0.20 mg/dm^3 $CoCl_2$, 0.21 mg/dm^3 $ZnCl_2$, 0.09 mg/dm^3 $(NH_4)_4Mo_7O_{24}$, 1.00 $\mu g/dm^3$ VB1, and 0.10 $\mu g/dm^3$ VB12, and its salinity was ensured at 30–33 ppt. Table 2 presents the characteristics of the culture media used in particular experimental variants.

Table 2. Characteristics of culture media used in experimental variants.

Indicator	Unit	Variant 1	Variant 2	Variant 3
BOD_5	mgO_2/dm^3	3.4 ± 0.3	7.9 ± 1.7	15.1 ± 3.2
COD	mgO_2/dm^3	11.7 ± 1.4	28.6 ± 3.1	55.7 ± 9.8
$N_{tot.}$	mg N/dm^3	22.8 ± 0.9	12.1 ± 1.4	23.6 ± 4.3
$N_{org.}$	mg $N_{org.}/dm^3$	0.0 ± 0.0	1.2 ± 0.4	2.2 ± 0.3
$N\text{-}NO_3$	mg $N\text{-}NO_3/dm^3$	20.4 ± 0.8	10.9 ± 1.6	20.7 ± 3.7
$N\text{-}NO_2$	mg $N\text{-}NO_2/dm^3$	1.4 ± 0.4	0.9 ± 0.1	1.7 ± 0.4
$N\text{-}NH_4$	mg $N\text{-}NH_4/dm^3$	0.0 ± 0.0	1.1 ± 0.3	1.9 ± 0.5
$P_{tot.}$	mg P/dm^3	8.2 ± 0.5	8.1 ± 1.3	15.8 ± 2.4
$P_{org.}$	mg $P_{org.}/dm^3$	0.0 ± 0.0	1.7 ± 0.9	3.6 ± 1.1
$P\text{-}PO_4$	mg $P\text{-}PO_4/dm^3$	8.2 ± 0.5	6.4 ± 2.0	12.3 ± 2.9
pH	-	7.1 ± 0.1	7.09 ± 0.1	7.06 ± 1.62
Total suspended matter	mg/dm^3	0.8 ± 0.2	2.3 ± 1.0	4.2 ± 2.6

2.2.4. Biohydrogen Production Medium

Deionized water supplemented with the culture medium, in which sulfur was replaced with chloride compounds, served as the culture medium for hydrogen production in all of the experimental series of stage 2. The composition of the culture medium was established based on the literature data [28] and was as follows: 0.667 mg/dm^3 KCl, 27.23 mg/dm^3 NaCl, 1.123 mg/dm^3 $CaCl_2$, 5.079 mg/dm^3 $MgCl_2$, 0.002 mg/dm^3 $CuCl_2$, 0.024 mg/dm^3 $SrCl_2$, 0.003 mg/dm^3 NaF, 0.098 mg/dm^3 KBr, 0.098 mg/dm^3 H_3BO_3, and 0.196 mg/dm^3 $NaHCO_3$. Its pH was 7.90–8.00.

2.3. Experimental Stations

2.3.1. Bioreactor for Wastewater Treatment

The hybrid bioreactor used for dairy wastewater pre-treatment had the shape of a pipe; it was 2.0 m high and had a 0.2 m internal diameter (Figure 2). It was made of transparent Plexiglas and was composed of two tanks that served various functions: a bottom tank for activated sludge and an upper tank for hydrophyte growth. The tanks were separated by a supporting grate for placing and rooting the *Typha angustifolia* shoots. The activated sludge tank was 1.0 m high. A magnetic stirrer was mounted at its bottom, which was propelled by a drive located in the axis of the reactor underneath its bottom. Fifty mm above the magnetic stirrer, an aeration diffuser was installed to which compressed air was supplied. The air pump with a capacity of 150 L/h was mounted inside a sealed casing. Both parts of the reactor, the sludge tank and the hydrophyte growth tank, were tightly connected to each other. The activated sludge tank was filled with liquid to the level of the supporting grate on which plant rhizomes were placed. A ventilation valve with a diameter of 1 inch was installed in the dome of the plant growth tank. When opened, it allowed for free gas exchange between the tank and the outer environment. The technical parameters of the reactor used for wastewater treatment were as follows: total height $H_{tot.}$ = 220 cm, active height $H_{act.}$ = 200 cm, height of activated sludge tank $H_{slu.}$ = 100 cm, height of hydrophyte growth tank $H_{hydr.}$ = 100 cm, internal diameter $D_{in.}$ = 20 cm, active volume of sludge tank $V_{slu.}$ = 31.4 dm^3, and active volume of hydrophyte plant growth tank $V_{hydr.}$ = 47.1 dm^3.

Throughout exploitation, the activated sludge tank operated as an SBR-type sequential reactor. The reactor's work cycle was 1 day. The 20-h aeration was followed by 2-h sedimentation and effluent discharge. Afterwards, the reactor was filled with raw wastewater, and then its contents were stirred, both operations lasting 2 h, and the cycle was repeated (Figure 3). In the aeration phase, the aeration ensured a continuous supply of atmospheric air to the activated sludge tank.

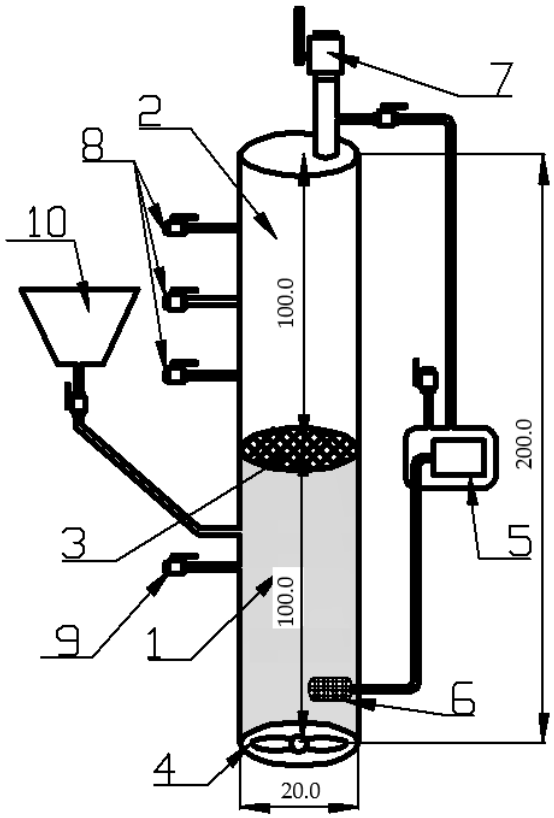

Figure 2. Single experimental station scheme 1—activated sludge tank, 2—hydrophyte growth tank, 3—plant supporting grate, 4—stirrer, 5—aerating pump in tight casing, 6—aerator, 7—ventilation valve, 8—gas sampling ports, 9—effluent discharge, 10—raw wastewater tank.

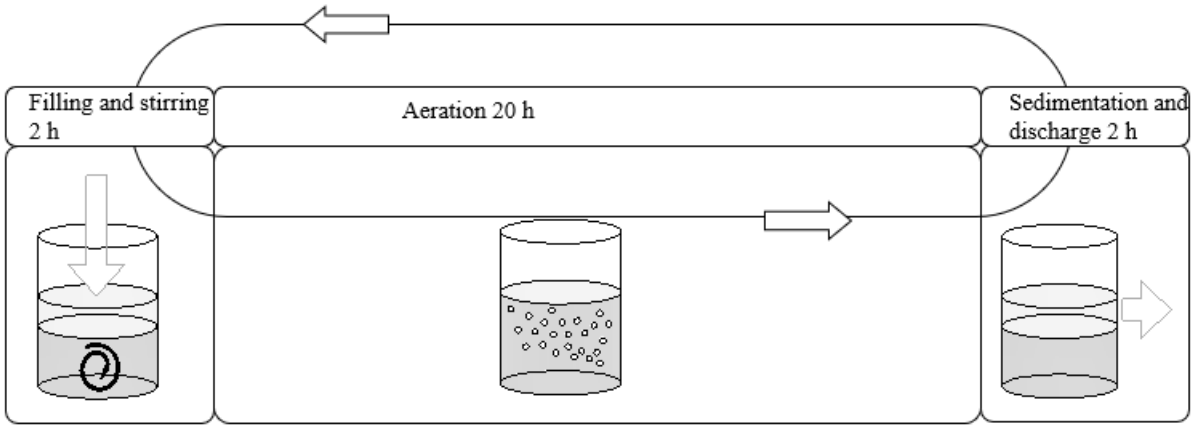

Figure 3. Scheme of SBR work cycle.

2.3.2. Microalgae Biomass Cultivation in PBR

T. subcordiformis was cultured in column PBRs (Figure 4) that had an active volume of 1.0 dm^3, at a temperature of ca. 25 °C, under varying environmental conditions, with light:dark cycles of 14:10 h. In the light phase, the PBRs were illuminated with white light with an intensity of 5 klux. The light was sourced from a fluorescent tube (Philips Lighting MASTER TL-D Super 80). The color temperature corresponded to daylight, reaching 6500 K, and the power reached 58 W. Air was supplied to the reactor by means of Mistral 200 peristaltic pumps with a performance of 200 dm^3/h. The goal of this technological treatment was to mix PBR contents and provide carbon dioxide. The initial microalgal biomass concentration, measured as dry organic matter inside the reactors,

was kept at 200 $mg_{o.d.m.}/dm^3$. *T. subcordiformis* was cultured for 12 days for all of the experimental variants.

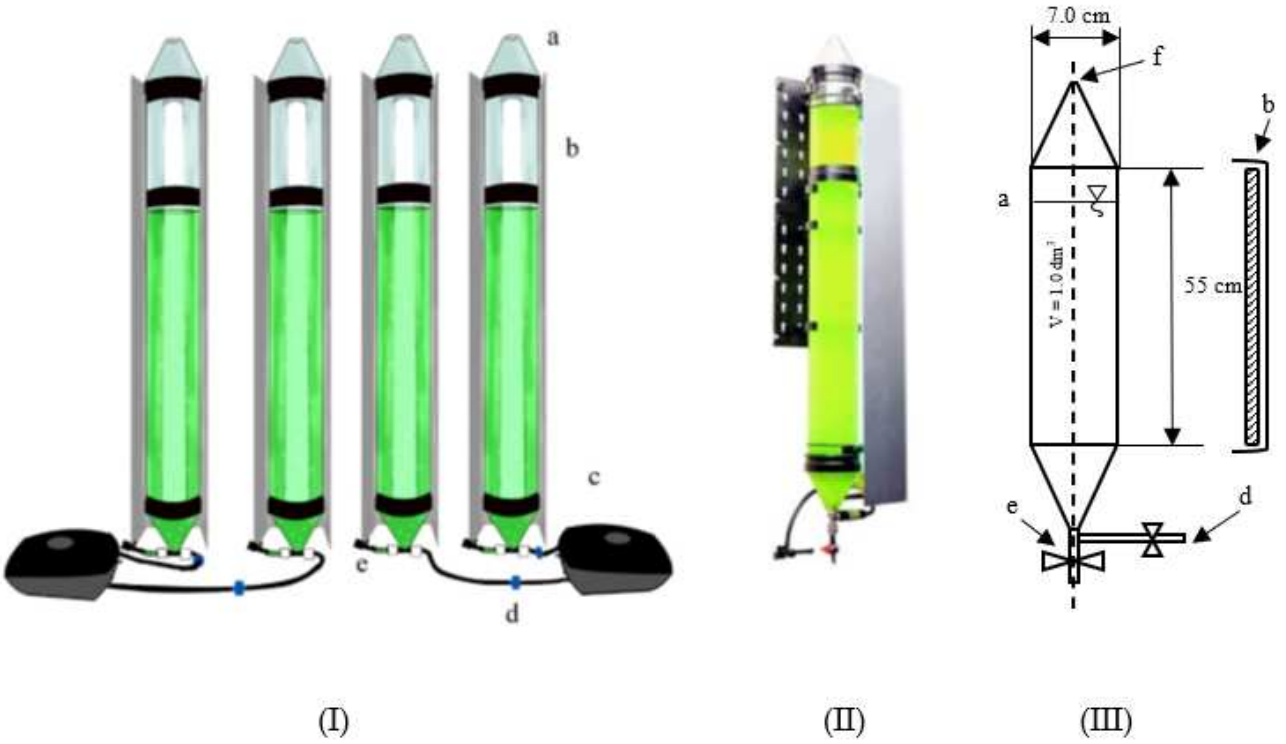

Figure 4. Scheme of the *T. subcordiformis* biomas production stand ((**I**)—visualization of the test stand, (**II**)—photography, (**III**)—basic dimensions): a—vertical photobioreactor, b—light source and reflector, c—air pump, d—air supply, e—discharge valve, f—gas outflow.

2.3.3. Biomass Separation Station

The *T. subcordiformis* biomass was separated via vacuum membrane filtration (MDS 1 set, Whatman). The filtration set was equipped with a filter cartridge of mixed cellulose esters with a diameter of 50 mm and a porosity of 8.0 µm. The microalgae biomass was separated from the culture medium in the filtration partition by applying a negative pressure of 0.5 atm using a Mobil 20 vacuum pump.

2.3.4. Hydrogen Production in PBR

The separated microalgae biomass was fed to respirometric reactors with an active volume of 1.0 dm^3 equipped with a system for monitoring changes in the partial pressure of the gases inside the reactor. The pressure values were registered every 10 h. The concentration of *T. subcordiformis* biomass in the subsequent experimental variants depended on the biomass production effectiveness in stage 1, which was 3.0 $g_{o.d.m.}/dm^3$ in variant 1 and 2.0 $g_{o.d.m.}/dm^3$ in variants 2 and 3. The contents of the respirometers were mixed with the yield of 100 rpm using VMS—C4 Advanced magnetic stirrers. Measurements were carried out at a temperature of 25 ± 1 °C for 120 h, including the first 30 h in the dark regime and the next 90 h in the light regime, with light sourced from a fluorescent tube (Philips Lighting MASTER TL-D Super 80). The color temperature corresponding to daylight reached 6500 K, and the power was 58 W.

2.4. Analytical Methods

The biomass volume and concentrations of the nitrogen and phosphorus compounds were determined every 2 days. Changes in the partial pressure were registered every 10 h. For the determination of biogas potential, the ideal gas law was used, and the pressure changes inside the bottles were converted to the biogas volumes produced under normal

conditions. Taxonomic analysis of microalgae biomass was conducted by means of an MF 346 biological microscope with an Optech 3 MP camera. The concentrations of dry matter, organic dry matter, and mineral dry matter were determined with the gravimetric method. The concentrations of contaminants in the culture medium were assayed using Hach Lange cuvette tests and a UV/VIS DR 5000 spectrophotometer. Culture medium salinity was measured using Marine Control Digital by Aqua Medic. Light intensity was measured using a HANNA HI 97,500 luxometer, whereas hydrogen content was measured by means of a GC Agillent 7890 A gas chromatograph (Santa Clara, CA, USA), incorporating the two Hayesep Q columns (80/100 mesh), two molecular sieve columns (60/80 mesh), and Porapak Q column (80/100) operating at a temperature of 70 °C was used to analyze the biogas composition. The GC was also equipped with a thermal conductivity detector. A sample injection and detector ports had a temperature of 50 °C and 250 °C, respectively. The carrier gasses were helium and argon, with a flow rate of 15 mL/min.

2.5. Statistical Analysis

Analyses were conducted in four replications. The statistical analysis of the experimental results and calculation of determination coefficients (R^2) were carried out in STATISTICA 13.1 PL (ANOVA) package. The distribution of the analyzed variables was determined by means of the W Shapiro–Wilk test, homogeneity of variance in groups was checked with the Levene test, whereas the significance of differences between variables was verified with the HSD Tukey test. The probability level was set at $p = 0.05$.

3. Results and Discussion

3.1. Wastewater Treatment in the Bioreactor

The efficiency of contaminant removal from the wastewater in the bioreactor was high, reaching 87 ± 1.1% and 95 ± 0.6% in the case of COD and BOD_5, respectively. The mean COD concentration in the effluent was 55.7 ± 9.8 mgO_2/dm^3, and that of BOD_5 was 15.1 ± 3.2 mgO_2/dm^3 (Table 1). The initial $N_{tot.}$ concentration of 192 ± 9.6 mgN/dm^3 was reduced to 23.6 ± 4.3 mgN/dm^3 in a treatment cycle. In turn, the concentration of $P_{tot.}$ was reduced by 67.2 ± 4.1% to the value of 15.8 ± 2.4 mgP/dm^3 (Table 1). The effluent contained mainly mineral forms of nitrogen and phosphorus, i.e., nitrates and orthophosphates (Table 1). A three-fold reduction was noted in the concentration of total suspended solids in the effluent, i.e., to the value of 4.2 ± 2.6 mg/dm^3. The pH value remained stable throughout the treatment cycle and averaged 7.6 in both raw and treated wastewater (Table 1). The determined efficiency of wastewater treatment was comparable to those noted in other systems based on activated sludge or constructed wetlands. The study by Karolinczak et al. [29] demonstrated the 97% removal of organic matter as well as 88% and 89.5% removal efficiency of nitrogen and phosphorus, respectively, in SBR-type reactors for the full-scale treatment of dairy wastewater. The concentrations of these contaminants at the outflow from the reactor were 66 mgO_2/dm^3, 11 mgN/dm^3, and 2 mgP/dm^3, respectively [29]. The use of the biofilm allowed achieving high removal efficiencies of COD (84.5%), $N_{tot.}$ (82.4%), and $P_{tot.}$ (30.2%) in the biofilm with a depth of 0.65 m and similar removal efficiencies of COD (87.5%), $N_{tot.}$ (76.5%), and $P_{tot.}$ (40.6%) in the bed with a depth of 1.0 m [30]. A study by Dębowski et al. [31] tested the feasibility of dairy wastewater treatment in an integrated technology entailing activated sludge and a hydrophyte system of common reed (*Phragmites australis*) or common cattail (*Typha latifolia*). Experiments were conducted in an innovative reactor exploited on the fractional–technical scale. The integrated treatment system allowed for the improvement of the removal efficiency of the biogenes expressed by $N_{tot.}$ and $P_{tot.}$ concentrations. In contrast, this system had no significant effect on the reduction of concentrations of organic compounds in hydrophyte filters [31]. Often, the treatment efficiency fails to ensure the appropriate quality of the effluent, especially considering concentrations of nitrogen and phosphorus [32]. In such cases, the treatment system needs to be expanded, or chemical precipitation of phosphorus needs to be implemented [33]. These operations may, however, lead to secondary wastewa-

ter contamination with the chemicals used for coagulation [34]. Photobioreactors (PBRs) have proven to offer a viable alternative in this respect, as they ensure biogene removal by microalgae biomass [35].

3.2. Production of T. Subcordiformis Biomass

In the present study, the mineral forms of the biogenes, i.e., nitrates and orthophosphates, turned out to prevail in the wastewater, regardless of the experimental series (Table 2). The highest efficiency of *T. subcordiformis* biomass production was determined in the control variant with a control medium made of deionized water supplemented with chemical reagents. The biomass growth rate reached 321 ± 21 $mg_{o.d.m.}/dm^3 \cdot d$, and the final microalgae concentration was 3410 ± 162 $mg_{o.d.m.}/dm^3$ (Figures 5 and 6). Feeding wastewater to photobioreactors significantly suppressed microalgal biomass growth. In variant 2, wherein wastewater accounted for 50% of the photobioreactor's volume, the final biomass concentration reached 2240 ± 206 $mg_{o.d.m.}/dm^3$ (Figures 5 and 6), which constituted only 66% of the value obtained in variant 1. The biomass growth rate reached 204 ± 19 $mg_{o.d.m.}/dm^3 \cdot d$. Increasing the wastewater content to 100% of the PBR's volume in variant 3 had no significant effect on the growth rate of the microalgae population and the final concentration of *T. subcordiformis* biomass, which reached 190 ± 32 $mg_{o.d.m.}/dm^3 \cdot d$ and 2110 ± 273 $mg_{o.d.m.}/dm^3$, respectively (Figures 5 and 6). In all of the experimental variants, the lag growth phase spanned for 3 days. It was followed by the exponential growth phase, which after 9 days, shifted into the stationary growth phase (Figure 5).

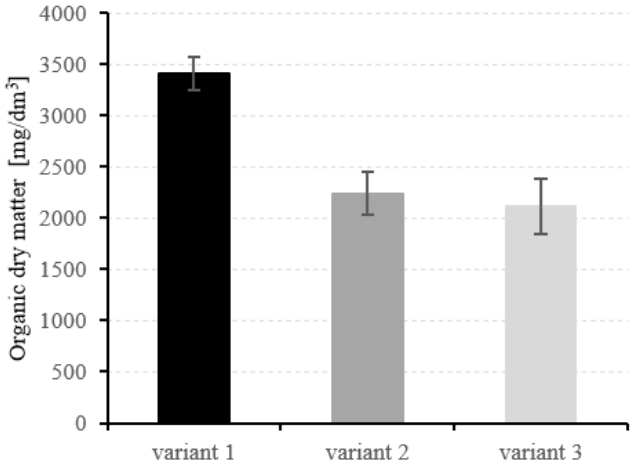

Figure 5. Production of *T. subcordiformis* biomass in particualr experimental variants.

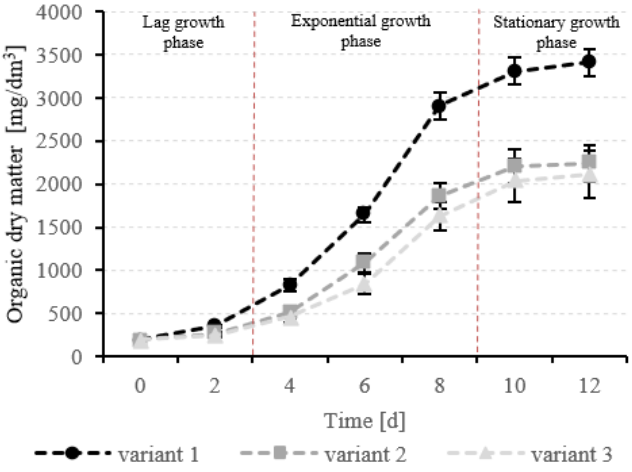

Figure 6. Final concentration of *T. subcordiformis* biomass in particualr experimental variants.

Previous investigations have suggested that the lower effectiveness of biomass growth in wastewater-based culture media is due to the complex character and the presence of often difficult-to-identify substances that inhibit microalgae growth [36]. Wastewaters are characterized by an excessively high concentration of organic compounds, low transparency, and high turbidity. Nutrients are present mainly in the complex form that are difficult to use directly by microalgae [6]. The high concentration of organic compounds may favor the development of harmful organisms, mainly bacteria, and turbidity and a dark color may limit the availability of light and inhibit photosynthesis [37]. Nutrients, such as nitrogen and phosphorus, are essential for the efficient growth of microalgae biomass [38]. High nitrogen concentrations in the culture medium usually stimulate and intensify the multiplication of microalgae biomass, and the limiting nature resulting from the presence of this element is observed in specific situations, e.g., in conditions of high ammonium concentration in the environment [39]. It has been proven that nitrogen deficiency significantly reduces the growth rate and the obtained cultivation efficiency. The technological efficiency of systems for the intensive production of microalgae biomass may also be significantly inhibited by limiting the availability of phosphorus compounds. It is assumed that 1 kg of this element makes it possible to produce about 50 kg of microalgae dry mass. The availability of organic carbon, in contrast to the availability of nitrogen and phosphorus, sporadically limits the growth of autotrophic microalgae. However, it may be a factor that limits the efficiency of cultivation in heterotrophic or mixotrophic systems [40]. In photobioreactors, a high concentration of organic carbon stimulates the growth of species of competing microorganisms, including bacteria, which can cause turbidity in the culture medium and reduce the efficiency of light transmission. It has a direct impact on the inhibition of the microalgae biomass growth process. Industrial wastewater or municipal sewage have been proven to contain high amounts of toxic compounds, such as antibiotics or heavy metals [41]. The presence of florfenicol and oxytetracycline in the culture medium has been proven to inhibit the growth of *Tetraselmis* sp. [42]. Naorbe and Serrano [43] proved the inhibiting effect of mercury and cadmium on *Tetraselmis tetrathele* population development. On the other hand, various types of industrial and municipal wastewater, as well as post-fermentation water and post-production waters from aquaculture, have been shown to be utile in the cultivation of *Tetraselmis* sp. [44]. For instance, the rate of *Tetraselmis* sp. biomass production in experiments testing municipal sewage as the culture medium reached 343 $\pm$ 53 mg/dm^3·d, whereas the rate of nitrogen and phosphorus removal was at 31.4 $\pm$ 0.4 mg/dm^3·d and 6.66 $\pm$ 1.57 mg/dm^3·d, respectively [25].

3.3. Removal of Biogenes by Tetraselmis sp.

The investigations conducted so far have proven the usability of many *Tetraselmis* sp. strains in wastewater treatment technologies, particularly in the removal of biogenic compounds [45]. This taxonomic group has been harnessed for the treatment of wastewater from the tanning industry [46]. However, the high effectiveness of biogene removal was observed upon the use of multi-species consortia of microalgae. Xiang et al. [47] applied synthetic industrial wastewater with a nitrogen concentration of 3500 mg/dm^3 for the culture of *Tetraselmis subcordiformis*. They proved the feasibility of the complete removal of this biogene in the technological system. The removal effectiveness of the biogenes was found to be closely related to the culture medium type [48]. Investigations conducted in systems characterized by the low effectiveness of nutrient consumption demonstrated significantly lower final biomass concentrations after completed culture [49].

In the present study, the highest effectiveness of nitrogen and phosphorus consumption by microalgal biomass was determined in variant 1, i.e., 98% and 94%, respectively. In variants 2 and 3, wherein wastewater served as the culture medium, the effectiveness of biogene removal was significantly lower. In variant 2, the concentration of nitrogen compounds determined at the end of the culture oscillated around 1.12 ± 0.9 mg N/dm^3, which corresponded to 91% total nitrogen consumption (Figures 7 and 8). In turn, the removal effectiveness of nitrogen compounds reached 75%, which corresponded to their final concentration of 2.0 ± 1.3 mg P/dm^3 in the effluent (Figures 9 and 10). In variant 3, the effectiveness of biogenes consumption was nearly 74% for nitrogen and 32% for phosphorus (Figures 8 and 10). At the end of biomass proliferation, the concentrations of biogenic compounds in the culture medium were at 6.1 ± 1.7 mg N/dm^3 and 9.8 ± 1.4 mg P/dm^3 (Figures 7 and 9). In the available literature, the lower effectiveness of biogene removal from wastewater is claimed to be due to the presence of factors suppressing microalgae biomass growth [50]. Usually, they include organic compounds that contribute to the development of competitive groups of microorganisms, including bacteria [51]. This phenomenon, coupled with naturally increased wastewater turbidity, reduces light penetration, which directly decreases the rate of photosynthesis and biomass growth [52]. This, in turn, exerts a direct influence on the effectiveness of nutrient consumption from the culture medium [53].

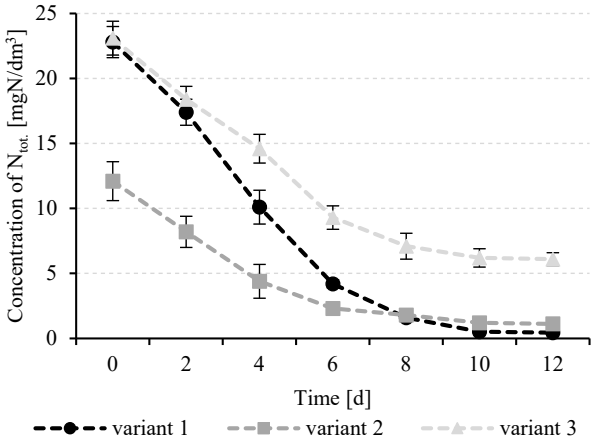

Figure 7. Changes in total nitrogen concentration ($N_{tot.}$) in the culture medium in particular experimental series.

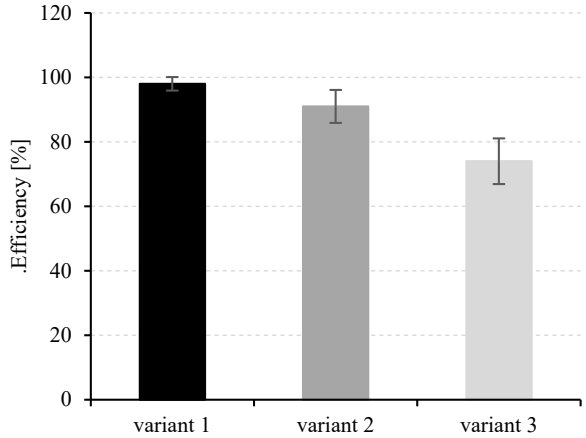

Figure 8. Efficiency of total nitrogen ($N_{tot.}$) removal in particular experimental variants.

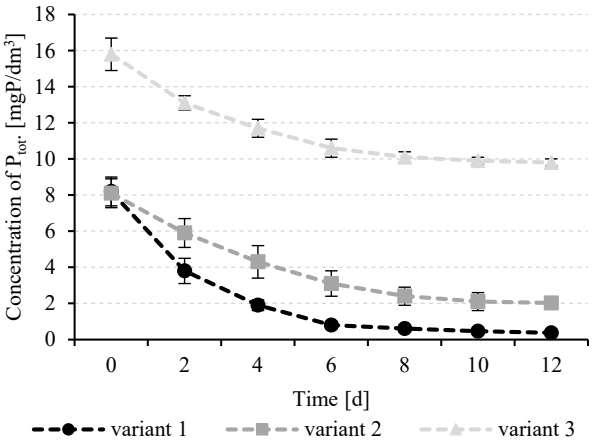

Figure 9. Changes in total phosphorus concentration ($P_{tot.}$) in the culture medium in particular experimental series.

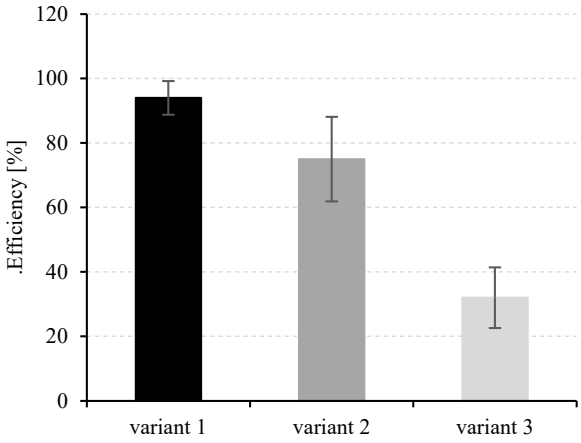

Figure 10. Efficiency of total phosphorus ($P_{tot.}$) removal in particular experimental variants.

Heo et al. [54] harnessed pre-treated wastewater from the food sector, having nitrogen and phosphorus concentrations of ca. 390 ± 14 mg/dm^3 and 438.3 ± 54.4 mg/dm^3, respectively, for *Tetraselmis* sp. cultivation in a photobioreactor with an active volume of 2.5 dm^3, at a temperature of 20 ± 1 °C for 14 days. At the end of the culture, the biomass concentration reached 2000 mg/dm^3, whereas the phosphorus concentration decreased by 52.3%, and the effectiveness of nitrogen removal from the culture medium reached 99%. It can, therefore, be posited that the pre-treatment effectiveness obtained in other experimental works was similar to that achieved in the present study. The treatment of social wastewater with the use of the *Tetraselmis indica* strain yielded the following efficiencies of removal of the monitored contaminants: 72.94% for COD, 73.17% for BOD_5, 60.93% for P, and 72.94% for N, and allowed producing 880 ± 40 mg/dm^3 of biomass [55].

3.4. Hydrogen Production

Hydrogen production effectiveness was found to depend on the concentration of microalgae achieved at the biomass proliferation stage, meaning—on the initial concentration of the *T. subcordiformis* population in respirometers. The highest total hydrogen production, reaching 162 ± 7 cm^3, was obtained in variant 1 (Figures 11 and 12). At this phase of experiments, the rate of H_2 production was 1.73 ± 0.31 cm^3/h (Table 3). Significantly lower values were determined in variant 2, i.e., 139 ± 8 cm^3 of hydrogen produced during incubation, and in variant 3, yielding 116 ± 3 cm^3 of hydrogen at the end of *T. subcordiformis* biomass incubation (Figures 11 and 12). The rate of hydrogen production determined in these variants reached 1.45 ± 0.37 cm^3/h and 1.22 ± 0.11 cm^3/h, respectively (Table 3). The percentage content of H_2 in the gaseous metabolites was comparable in all experimental

variants and ranged from 55.4 ± 2.2% in variant 3 to 57.2 ± 4.1% in variant 1 (Table 3). Given the differences in the initial biomass concentration in respirometers, i.e., 3.0 $g_{o.d.m.}/dm^3$ in variant 1 and 2.0 $g_{o.d.m.}/dm^3$ in variants 2 and 3, it seemed essential to determine the hydrogen production yield per biomass concentration unit. Significantly highest value of this parameter, reaching 69 ± 4.2 cm^3 $H_2/g_{o.d.m.}$, was determined in variant 2 (Table 3). In variant 1, its value reached 54 ± 2.1 $cm^3/g_{o.d.m.}$, whereas in variant 3—48 ± 1.9 $cm^3/g_{o.d.m.}$· of *T. subcordiformis* microalgae (Table 3).

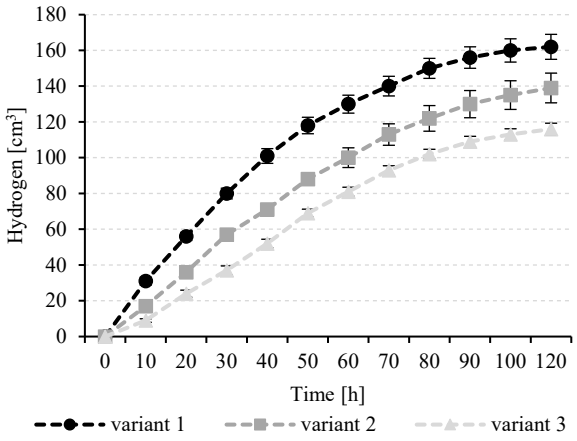

Figure 11. Hydrogen production dynamics in particular experimental variants.

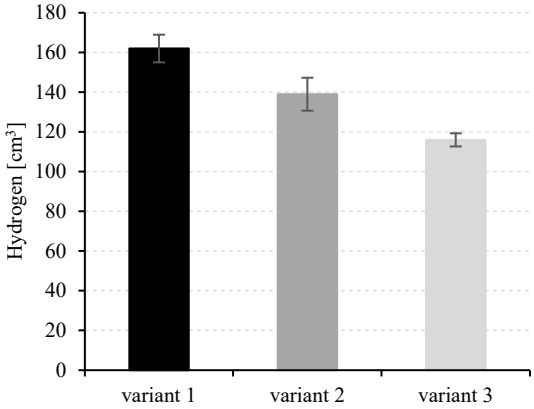

Figure 12. Final hydrogen volume produced in particular experimental variants.

Table 3. Percentage composition of biogas produced and indicators of hydrogen production dynamics.

Variant	Biogas Composition [%]			H_2 Production Per Biomass Concentration Unit	Rate of H_2 Production (r)	Production Rate Constant (k)
	H_2	CO_2	O_2			
	[%]			[$cm^3/g_{o.d.m.}$]	[cm^3/h]	[1/d]
1	57.2 ± 4.1	41.1 ± 3.4	1.7 ± 0.9	54 ± 2.1	1.73 ± 0.31	0.17
2	55.7 ± 5.6	42.3 ± 4.5	2.0 ± 1.1	69 ± 4.2	1.45 ± 0.37	0.13
3	55.4 ± 2.2	43.2 ± 1.8	1.4 ± 0.6	48 ± 1.9	1.22 ± 0.11	0.12

Other investigations corroborated the feasibility of applying natural waters in the culture and effective hydrogen production by *T. subcordiformis*. The rate of biomass growth reached 317.6 ± 42.3 mg d.m.^{3}·d and ensured a biomass concentration of 3493 ± 465 mg d.m./dm^3. The percentage content of hydrogen in the biogas produced was 63.2 ± 1.4%, and the rate of its production ranged from 0.53 ± 0.05 cm^3/h to 0.70 ± 0.01 cm^3/h [20]. The study also analyzed the impact of the culture medium on biomass growth and hydrogen production by *T. subcordiformis*. When cultured in the optimized medium, the

population of microalgae needed only 6–8 days to reach the highest culture concentration, reaching $2.00 \pm 0.18 \times 10^6$ cells/cm^3. When the culture medium was composed of wastewater, the needed time was extended to 18–22 days, and the ultimate value reached $1.85 \pm 0.20 \times 10^6$ cells/cm^3. The volume of the biogas produced was reported to range from 330 cm^3 to 570 cm^3, depending on the experimental variant [56]. It was also proved that the efficiency of hydrogen production by *T. subcordiformis* was substantially affected by the method of proliferated biomass separation and its transfer to the culture medium devoid of sulfur and oxygen [57]. These conditions shall be met to activate hydrogenase, i.e., an enzyme responsible for the direct biophotolysis of a water molecule and hydrogen production [58]. A study by Dudek et al. [59] proved the viability of applying vacuum membrane filtration, which allowed for the production of 156.3 ± 11.0 cm^3 H_2 with a mean rate of $r = 1.38 \pm 0.1$ cm^3/h in the most effective variant, compared to the respective values of 138.3 ± 12.8 cm^3 H_2 and $r = 1.09 \pm 0.09$ cm^3/h achieved after centrifugation. The same technological solution—vacuum membrane filtration—was deployed in the present study.

4. Conclusions

The quality of the dairy wastewater treated in a hybrid bioreactor was found suitable for consideration as a component of the culture medium used to produce *T. subcordiformis* biomass and to ensure effective and hydrogen-yielding biophotolysis. The study proved that the process of microalgae cultivation with dairy wastewater was nearly 35% less effective compared to the process with a culture medium based on pure chemical reagents, which is a beneficial effect. Nitrogen and phosphorus compounds present in wastewater were found to represent an available source of nutrients for the *T. subcordiformis* population, as indicated by the high efficiencies of their removal. Hydrogen production efficiency was found to depend on the concentration of microalgae achieved at the biomass proliferation stage and, by these means—on the initial concentration of the *T. subcordiformis* population in respirometers. The highest total hydrogen production, reaching 162 ± 7 cm^3, was obtained in variant 1. The percentage of the H_2 content in the gaseous metabolites ranged from $55.4 \pm 2.2\%$ to $57.2 \pm 4.1\%$. A significantly higher hydrogen unit yield, reaching 69 ± 4.2 cm^3/g$_{o.d.m.}$, was determined in the variant with wastewater accounting for 50% of the culture medium. The respective value noted in the control respirometer was 54 ± 2.1 cm^3/g$_{o.d.m.}$

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