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Mass-energy balance analysis for estimation of light energy conversion in an integrated system of biological H₂ production

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HIGHLIGHTS

- The conversion of light energy into H₂ was examined in an integrated three-stage scheme.
- Mass-energy balance regularities were applied to estimate energy conversion efficiencies at different stages.
- This three-stage scheme was found counterproductive for light energy bioconversion to H₂.

GRAPHICAL ABSTRACT

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ABSTRACT

The present study investigated an integrated system of biological H₂ production, which includes the accumulation of biomass of autotrophic microalgae, dark fermentation of biomass, and photofermentation of the dark fermentation effluent. Particular emphasis was placed on the estimation of the conversion efficiency of light into hydrogen energy at each stage of this system. For this purpose, the mass and energy balance regularities were applied. The efficiency of the energy transformation from light into the microalgal biomass did not exceed 5%. The efficiency of the energy transformation from biomass to biological H₂ during the dark fermentation stage stood at about 0.3%. The photofermentation stage using the model fermentation effluent could improve this estimation to 11%, resulting in an overall efficiency of 0.55%. Evidently, this scheme is counterproductive for light energy bioconversion due to numerous intermediate steps even if the best published data would be taken into account.

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Abbreviations	
Bchl	Bacteriochlorophyll
Chl	Chlorophyll
FE	Fermentation effluent
DW	Dry weight
HS	High salt
PhBR	Photobioreactor
TAP	Tris-Acetate-Phosphate

1. Introduction

Our dependence on fossil fuels correlates with the increasing level of carbon dioxide concentration in the atmosphere. To avoid this problem, new alternative energy sources should be introduced into the practice.

Microalgae are unicellular organisms capable of converting light into chemical energy. They grow faster than plants and do not compete with plants for the land. That is why microalgal biomass is considered as a valuable alternative energy source. Different approaches of microalgal biomass usage as an energy source are under investigations including direct digestion into methane (González-Fernández et al., 2012), biodiesel (Sheehan et al., 1998; Verma et al., 2010) and ethanol (Miranda et al., 2012). Complex utilization of microalgal biomass is also being pursued (Rizwan et al., 2015).

Another possibility of microalgal biomass usage is a three-stage integrated system for H₂ production in which the first stage is microalgal biomass production followed by the dark fermentation of the algal biomass as the second stage, and finally the photofermentation of the dark fermentation products (Fig. 1).

Fig.1. An integrated scheme for the light energy accumulation by microalgae, dark fermentation of algae biomass and photofermentation of the dark fermentation effluent.

This three-stage H₂ production has been studied by several research teams (Ike et al., 1998; Ike et al., 2001; Kim et al., 2006). The dark fermentation of microalgae biomass with H₂ production has been most successful if biomass is rich in starch. Kim et al. (2006) claimed that the highest H₂ yield achieved at this stage was 2.58 mol mol⁻¹ glucose, calculated on the basis of accumulated starch. They also reported that during the photofermentation of the dark fermentation effluent (FE), the H₂ yield increased to 5.7 mol mol⁻¹ glucose, and the total H₂ yield amounted to 8.3 mol mol⁻¹ glucose (Kim et al., 2006). However, it is worth noting that by taking into account the fact that microalgal biomass contains other organics besides starch, the H₂ yields may be over-estimated.

In a different study, Kawaguchi et al. (2001) argued that they successfully produced hydrogen from the starch fraction of the biomass using the mixed culture of *Lactobacillus amylovorus* and *Rhodobium marinum* A-501. In their proposed system, *L. amylovorus*, which possesses amylase activity, utilized algal starch for lactic acid production, and *R. marinum* A-501 produced

hydrogen in the presence of light using lactic acid as an electron donor. In the described experiments, the biomass of *Dunaliella* and *Chlamydomonas* was freeze-thawed (Kawaguchi et al., 2001). This procedure disrupts most of the cells, simplifying biomass processing in the dark fermentation reactor. In some cases, the processing of microalgal biomass was not described. Though these results are promising in terms of high H₂ yield and with respect to algal starch, but the efficiency of the light energy transformation in the system was not considered. In better words, the particular elements of these systems have been explored thoroughly but there is no estimation of the total efficiency of the system, i.e., the ratio of the energy of the H₂ obtained to the energy of the incident light. It is worth mentioning that these systems theoretically require only sunlight and are neutral in terms of CO₂ production and are therefore, considered very promising.

On such basis, the aim of the present work was to realize the three-step integrated system for H₂ production using microalgae, dark fermentative, and purple bacteria, and to estimate the overall efficiency of energy conversion using mass-energy balance regularities.

2. Materials and methods

2.1. Microalgae and purple bacteria

Stock cultures of *Chlamydomonas reinhardtii*, strain Dang cc124, and *Chlorella pyrenoidosa* 82T were maintained on agar plates with a standard Tris-Acetate-Phosphate (TAP) medium (pH 6.9) at 28 °C under illumination (36 μE m⁻²s⁻¹). Single colonies were transferred into 10 ml of TAP medium and incubated for 2 d under the same conditions. Then, the cultures were grown autotrophically on the High-Salt (HS) medium (Sueoka et al., 1967) in 500 ml Erlenmeyer flasks which were bubbled with 2% CO₂ in air, filtered through 0.2 mm pore-size membrane filters (Acro 37 TF, Gelman Sciences, Inc., Ann Arbor, MI). The CO₂ content in the airflow was analyzed with a DX6100-01 gas analyzer (RMT Ltd., Russia) and maintained using a TRM1 microprocessor system (Oven, Russia).

A microbial consortium (with *Clostridia* predominated) obtained from silo pit liquid (Belokopytov et al., 2009) was used during the dark fermentation stage. The inoculum was grown anaerobically using the medium recommended for biogas-producing microbial communities (Tzavkelova et al., 2012), and was then adapted for starch hydrolysis at 37 °C (Belokopytov et al., 2009).

The purple bacterium *Rhodobacter sphaeroides* N7 (Khusnutdinova et al., 2012) was used at the photofermentative stage. The inoculum was grown 4-5 d on the Ormerod medium (Ormerod et al., 1961) with 10 mM (NH₄)₂SO₄ and 20 mM lactate at 28 °C, 60 W m⁻².

2.2. Microalgae cultivation

To study the efficiency of light energy conversion and to produce microalgal biomass, *C. reinhardtii* was cultivated on the HS medium in a 1.5 L photobioreactor (PhBR) consisting of glass coaxial cylinders (Tsygankov et al., 1994). The thickness of the culture layer was 13 mm. The computerized system was designed to maintain the turbidostat mode (OD 0.05, pH 7.0, and 28.0±0.2 °C). The inoculum was added at 5-10%. The culture was bubbled with 3% CO₂ in air at 137 ml min⁻¹. The PhBR was illuminated with cool-white fluorescent lamps with the light intensity varying from 36.7 to 256.6 μE m⁻² s⁻¹ PAR. The illuminated area of the culture was 0.083 m². The outflow culture was collected in 10 L vessels during 7 d. Then, the biomass was harvested by centrifugation (4500 rpm, 15 min) and stored at -10 °C. The microalgae *Chl. pyrenoidosa* was grown in a similar manner in the same medium.

2.3. Hydrolysis of *C. reinhardtii* biomass

Our preliminary experiments showed that microbial consortium from silo pit liquid did not digest intact cells of *C. reinhardtii* even during 1 month. That is why the pretreatment of biomass appeared to be necessary. The biomass of *C. reinhardtii* was hydrolyzed as follows: 187.5 g of the

thawed biomass (corresponding to 15 g of dry weight, $DW_{C,r}$) was incubated in 112.5 mL of 3.5N H_2SO_4 at 120 °C, 1.2 atm, 30 min.

2.4. Anaerobic dark fermentation of microalgae biomass

Before fermentation, the pH of the hydrolysate was adjusted to 6.8. Moreover, the hydrolysate was supplied with Mg, Ca, microelements, and phosphates according to the medium composition described by Tzavkelova et al. (2012). Fermentation was performed anaerobically at 37 °C in 500 mL vessels using 4 mL of the microbial consortium as inoculum. At the end of the fermentation, the culture (450 mL) was neutralized to pH 7.0 and harvested by centrifugation (4500 rpm, 15 min). The supernatant was autoclaved, centrifuged again, and was used as fermentation effluent (FE).

2.5. Utilization of FE for cultivation of purple bacteria (photofermentation)

To cultivate the purple bacteria, i.e., *R. sphaeroides* N7, the FE was used in non-diluted or diluted form with distilled water, as specified. When indicated some nutrients were added ($mg L^{-1}$): YE, 100; $FeSO_4 \cdot 7H_2O$, 10; EDTA, 20. Experiments were made in Hungate tubes (16 mL) with 8 mL of the medium with 2% of inoculum, under Ar. Tubes were incubated at 28 °C under illumination; 60 $W m^{-2}$ (incandescent lamps).

2.6. Other methods

Chlorophyll (Chl) *a+b* content was assayed spectrophotometrically in 95% ethanol extract (Harris, 1989). Bacteriochlorophyll (Bchl) *a* concentration was measured spectrophotometrically at 772 nm after extraction in 7:2 (v/v) acetone:methanol (Clayton, 1966). Gas production was measured manometrically, and the H_2 percentage was analyzed by gas chromatography. The concentration of acetate was determined by gas chromatography as described earlier (Belokopytov et al., 2009). Lactate concentration was assayed by enzymatic method and monitored as NAD reduction at 340 nm (Asatiani, 1969). Glucose concentration was measured by using the Glucose GOD FS kit (DiaSys, Germany). Starch accumulated in the cells was determined as glucose (see above) after enzymatic hydrolysis, according to the method described by Gfeller and Gibbs (1984). The total content of soluble monosaccharides and polysaccharides (which could be hydrolyzed by sulfuric acid) was assayed using anthrone reagent and expressed as glucose equivalents (Hanson and Phillips, 1984). Protein concentration was estimated according to the classical Lowry method. The ammonium content was analyzed by the microdiffusion method (Lyubimov et al., 1968). Light intensity was measured in the 400-900 nm region using quantummeter (Quantum Meter QMSW-SS) and pyranometer (CM3; Kipp&Zonen, Delft, The Netherlands). During measurements, infrared light with a wavelength more than 850 nm was cut off by filter SZS24. Carbon, hydrogen, and nitrogen (CHN) content in biomass was measured using a CHN analyzer.

3. Calculations of energetic efficiency

Calculations were made using the mass and energy balance regularities (Erickson et al., 1978). The energy content of dry algae biomass (Q_b) was calculated using the Equation 1:

$$Q_b (kJ g^{-1}) = 112.8 \times \gamma / M_b \quad (1)$$

where 112.8 is the heat released during the combustion of biomass, which contains 1 g-atom of carbon, with the degree of reduction γ . M_b is the calculated molecular mass of biomass, equal to 25.564 and 25.532 $g mol^{-1}$ for *C. reinhardtii* and *Chl. pyrenoidosa*, correspondingly. The biomass elemental composition is given below.

To calculate γ , the CHN content was measured. The O content was calculated assuming that biomass contains 95% CHNO. According to our measurements, the empirical elemental composition of *C. reinhardtii* and *Chl. pyrenoidosa* was $CH_{0.128}N_{0.178}O_{0.684}$ and $CH_{0.132}N_{0.192}O_{0.666}$, correspondingly. The biomass degree of reduction (γ) was calculated based on the Equation 2:

$$\gamma = 4+x+3y-2z \quad (2)$$

where x, y, z represent the numbers of H, N, O atoms, correspondingly, based on biomass composition. Consequently, γ is 3.294 and 3.376, and energy content of biomass is 14.5 and 14.9 $kJ g^{-1}$ for *C. reinhardtii* and *Chl. pyrenoidosa*, respectively.

The efficiency of light energy conversion to energy accumulated in biomass (η) was calculated as shown in the Equation 3:

$$\eta (\%) = 100E_b/E_{ii} \quad (3)$$

where E_b is the energy of heat combustion of biomass produced by the PhBR during 1 h, E_{ii} is the incident light energy to the PhBR during 1 h.

The energy of heat combustion of biomass produced by the PhBR during 1 h (E_b) was calculated as follows (Eq. 4):

$$E_b (kJ h^{-1}) = 112.8 \times \mu \times C \times \gamma \times V \quad (4)$$

where μ is specific growth rate (h^{-1}), C is the steady-state biomass concentration in the PhBR measured as the number of moles of carbon in biomass per 1 L of culture ($mol L^{-1}$), V is the volume of the culture and is equal to 1.125 and 1.5 L for *C. reinhardtii* and *Chl. pyrenoidosa*, correspondingly.

The incident light energy per 1 h was calculated as follows (Eq. 5):

$$E_{ii} (kJ h^{-1}) = 3600 \times I_o \times S / 1000 \quad (5)$$

where I_o ($W m^{-2}$) is the incident light intensity, S (m^2) is the illuminated surface of the culture equaling 0.083 and 0.095 m^2 for *C. reinhardtii* and *Chl. pyrenoidosa*, respectively.

The specific energy of substrate combustion (Q_s) was calculated for acetate, lactate, and glucose based on their CHO formula:

$$Q_s (kJ g^{-1}) = 112.8 \times \gamma / M_s \quad (6)$$

where γ is 4 and M_s is 30 (formula of a common type CH_2O). Thus, the specific energy of acetate, lactate, and glucose was similar and amounted to 15.04 $kJ g^{-1}$.

The specific energy of hydrogen combustion is 143.1 $kJ g^{-1}$.

4. Results and discussion

4.1. Production of microalgae biomass and efficiency of light energy conversion

Production of microalgae biomass was studied using *C. reinhardtii* and *Chl. pyrenoidosa* under turbidostat cultivation. The growth rate of *C. reinhardtii* increased with the increase of the light intensity and saturated at 38.5 $W m^{-2}$ reaching the maximal value of 0.115 h^{-1} (Table 1).

Table 1. Influence of incident light intensity on the growth parameters and efficiency of light energy conversion in *C. reinhardtii*.

I_o ($W m^{-2}$) [*]	E_{ii} ($kJ h^{-1}$)	Growth rate, μ (h^{-1})	Biomass concentration ($DW_{C,r}$)		E_b ($kJ h^{-1}$)	η (%)
			$g L^{-1}$	$mol L^{-1}$		
9.7	2.9	0.011	0.22	0.009	0.04	1.4
14.1	4.2	0.046	0.27	0.011	0.20	4.8
18.7	5.6	0.057	0.27	0.011	0.26	4.6
29.5	8.8	0.079	0.24	0.009	0.30	3.4
38.5	11.5	0.110	0.24	0.009	0.41	3.6
64.1	19.2	0.115	0.27	0.011	0.53	2.8

* The incident light intensity 9.7 - 64.1 ($W m^{-2}$) corresponded to 36.7 - 256.6 $\mu E m^{-2} s^{-1}$

The steady-state biomass concentration was about $0.25 \pm 0.02 \text{ g L}^{-1}$. Similarly, the energy of heat combustion of biomass produced by the PhBR during 1 h (E_b) (Section 3, Eq. 4) increased with light intensity up to 0.49 kJ h^{-1} . The incident light energy per 1 h (E_{in}) increased in proportion to incident light intensity (Section 3, Eq. 5). However, the efficiency of light energy conversion to energy accumulated in biomass (η ; Section 3, Eq. 3) was maximal (4.8 – 4.6%) at rather low light intensities of $14.1 - 18.7 \text{ W m}^{-2}$.

Thus, the optimal conditions for the growth rate (and biomass production) distinctly differed from those for efficiency of the light energy conversion. The most efficient light energy conversion took place when the growth rate was only 40% of the maximum value.

Similar results were obtained using the turbidostat culture of *Chl. pyrenoidosa*, while somewhat higher biomass concentration and lower growth rate were obtained (Table 2). The highest light energy conversion efficiency of 5.5% was observed at low light intensity (18.7 W m^{-2}) when the growth rate was 58% of the maximum value. Since the regularities were the same, *C. reinhardtii* was used in the subsequent experiments.

Table 2. Influence of incident light intensity on the growth parameters and efficiency of light energy conversion in *Chl. pyrenoidosa*.

I_0 (W m^{-2})	E_{in} (kJ h^{-1})	Growth rate, μ (h^{-1})	Biomass concentration ($DW_{Chl.pyr.}$)		E_b (kJ h^{-1})	η (%)
			g L^{-1}	mol L^{-1}		
9.7	3.3	0.016	0.26	0.010	0.09	2.7
18.7	6.4	0.056	0.29	0.011	0.35	5.5
38.5	13.2	0.093	0.28	0.011	0.58	4.4
64.1	21.9	0.097	0.35	0.014	0.78	3.6

These findings were in agreement with the published data on the efficiency of the light energy conversion of 0.2-5.0% (Klass, 1998). It should be noted that this parameter was often calculated in relation to the absorbed light energy bearing in mind that non-absorbed (transmitted) light may be further utilized in some other light-dependent processes. However, it should be emphasized that the intensity of transmitted light is much lower as compared to incident light (10-12 % in the present study). Furthermore, transmitted light is diffused light with modified spectral composition, hence, its utilization is counterproductive. Therefore, the energy of the transmitted light was neglected herein and calculations were made based on the incident light energy.

4.2. Pretreatment of the raw microalgae biomass

Our preliminary fermentation experiments using raw algae biomass (after freeze-thawing) failed and only insignificant production of methane-containing gas was observed (data not shown). Therefore, to improve the fermentation and the availability of carbohydrates to the microorganisms some disruption methods were necessary. Various pretreatment methods have been investigated to produce fermentable sugars from algae biomass ranging from simple heating or freezing-thawing to thermo-acidic or thermo-alkaline hydrolysis (Yang et al., 2011; Liu et al., 2012), ultrasonic disintegration (Jeon et al., 2013; Yun et al., 2013), osmotic shock (Lee et al., 2010), enzymatic pretreatment (Choi et al., 2010). Moreover, different combinations of grinding, enzymatic hydrolysis, hydrogenogens domestication, ultrasonication, microwave-assisted acid heating have also been tested (Cheng et al., 2012). In fact, pretreatment methods are chosen depending on the particular properties of a certain microalgae, especially of their cell wall. Efremenko et al. (2012) applied thermo-acidic pretreatment method for various microalgae and achieved extremely different H_2 production rates.

In the present study, the thermo-acidic pretreatment method was used (Section 2.3). This resulted in an increase in the total carbohydrates (measured with anthrone) in the supernatant fraction from 44.8 to $229.7 \text{ mg g}^{-1} DW_{C.r.}$. Glucose concentration (measured with glucose oxidase) also increased from 0.09 to $4.3 \text{ mg g}^{-1} DW_{C.r.}$

4.3. Dark anaerobic fermentation of microalgae hydrolysate

The utilization of algae biomass for H_2 production by using various microorganisms e.g., *C. butyricum* (Kim et al., 2006; Liu et al., 2012), immobilized *Clostridium acetobutylicum* (Efremenko et al., 2012), and anaerobic sewage sludge microflora (Park et al., 2009), through dark fermentation has been reported. For instance, high H_2 production of $81-92 \text{ mL g}^{-1} DW$ was demonstrated for *Arthrospira platensis* and *Chl. vulgaris* ESP6 hydrolysate obtained through ultrasonic-acid or thermo-acidic pretreatment, correspondingly (Cheng et al., 2012; Liu et al., 2012).

The advantages of monocultures and mixed culture (consortia) have also been widely discussed. Application of pure cultures appears to be very useful approach for experimental estimation of H_2 production rates and yields as well as organic acids production. However, in practice, pure culture is not applicable due to high cost of waste sterilization and inability to use wide spectrum of organics (Tekucheva and Tsygankov, 2012).

Even though the dark fermentation of different wastes was studied intensively, this process, however, was not considered in the context of overall efficiency of energy conversion. Hence, the dark anaerobic fermentation of *C. reinhardtii* hydrolysate was performed using a *Clostridia*-predominated consortium as described in Section 2.4. Carbohydrates (glucose) were consumed while acetate (20 mM) and lactate (35 mM) were produced. Gas production started after 24 d and continued during the 56 d-experiment. The total H_2 production amounted to 5.8 mM per 1 L FE (Table 3), thus, the H_2 yield was as low as $0.15 \text{ mol mol}^{-1}$ glucose. Evidently, the heterolactic acid fermentation took place, but the low H_2 production signified probably that H_2 consumers were available in this microbial consortium. Other researchers also reported that low quantities of H_2 were produced from *Chl. vulgaris* and *Dunaliella tertiolecta* biomass fermented by anaerobic enrichment cultures derived from digester sludge, and that H_2 was subsequently consumed (Lakaniemi et al., 2011).

Table 3. The products of dark anaerobic fermentation of *C. reinhardtii* hydrolysate.

Substrate/ Products	Concentration of substrate/ products		Total energetic value**	
	mmol L of FE*	mg g ⁻¹ of DW _{C.r.}	kJ g ⁻¹ of DW _{C.r.}	kJ 100 kJ ⁻¹ of biomass
Carbohydrates (glucose):				
Initial	42.5	230.00	3.46	23.86
Final	3.1	18.00	0.27	1.86
H_2	5.8	0.34	0.05	0.34
CO_2	4.5	14.00	-	-
Acetate	20.0	36.00	0.54	3.72
Lactate	35.0	99.00	1.49	10.28
Total	-	-	2.35	16.21

* 1 L of FE corresponded to initial 33.3 g of $DW_{C.r.}$

** The specific energy of substrate combustion is given in Section 3.

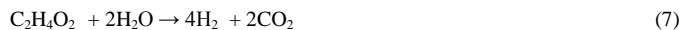
The carbon recovery in the products was close to 65.3%, which probably means that some additional fermentation products were not detected in the hydrolysate or, alternatively, a significant part of microbial biomass was not digested.

Energy content of the glucose consumed as well as the content of the dark fermentation products were calculated based on Equation 6 (Section 3). The results obtained showed that the energy content in the glucose available in the hydrolysate was 3.46 kJ g^{-1} (Table 3). Furthermore, the energy content of consumed glucose during the fermentation was 3.19 kJ g^{-1} . On the other hand, the total energy content of all the measured fermentation products (acetate, lactate, hydrogen, and residual glucose) stood at 2.35 kJ g^{-1} . Thus, the energy conversion efficiency of the consumed glucose to all of the measured products during the dark fermentation was 68% (probably underestimated because some products were not measured). While the energy conversion efficiency of the consumed glucose to H_2 was only 1.6%.

Moreover, by taking into consideration the initial energy content of dry algae biomass amounted to 14.5 kJ g⁻¹ of DW_{C.r.} (Section 3, **Eqs. 1** and **2**), the energy conversion efficiency of the consumed glucose during the dark fermentation into products and H₂ was recalculated at 16.2 and 0.3%, respectively.

4.4. Cultivation of purple bacteria using the FE after dark fermentation (photofermentation)

Theoretically, the VFAs available in the FE could be used for photofermentation by purple bacteria and H₂ could be produced according to the **Equations 7-9**:



Thus, using 1 L FE of a known composition (**Table 3**), one could obtain 40 mmol of hydrogen from acetate, 105 mmol from lactate and 18.6 mmol from glucose, in total 327.2 mmol H₂ per 1 L of FE. Nevertheless, the cultivation of *R. sphaeroides* N7 on non-diluted and diluted FE (25-50%) did not result in H₂ production (**Table 4**). This absence was evidently due to the high level of ammonium content (22.4 mM) in the FE, which was detrimental to the nitrogenase-mediated H₂ photoproduction. In fact, this is a common problem

for photofermentation stage when using FEs or different wastes with inappropriate C/N ratios. Different ways have been suggested to overcome this problem including chemical methods of ammonium removal (**Cheng et al., 2012**) and application of ammonium insensitive mutants (**Heiniger et al., 2012; Ryu et al., 2014**).

Table 4.

Final characteristics of *R. sphaeroides* N7 culture grown on the FE (diluted with distilled water) after the dark fermentation.

FE	Bchl, (mg L ⁻¹)	Protein (mg mL ⁻¹)	NH ₄ ⁺ (mM)	Lactate (mM)	Acetate (mM)	Glucose (mM)	H ₂
50%	61.0±1.4	2.1±0.1	14.1±1.2	3.0±0.3	0	1.9±0.04	0
50%*	66.0±2.7	1.9±0.2	16.9±1.4	2.4±0.2	0	2.8±0.50	0

* In this case, YE, FeSO₄·7H₂O, and EDTA were added (Section 2.5).

The results obtained by using *R. sphaeroides* N7 cultivation on 50% FE are presented in **Table 4**. No significant differences were observed in response to dilution of the FE with distilled water or to the addition of some nutrients. It should be noted that the consumption of acetate was about 100% and that of lactate was above 80%, while the low glucose content did not change. The final concentration of bacterial cells (Bchl)

Fig.2. The counter-productiveness of the three-stage hydrogen production scheme.

was rather high. Thus, it could be concluded that the medium used was suitable for the growth of the purple bacteria even though the H₂ production did not occur.

Nevertheless, to estimate the potential H₂ production from the substrates available in the FE (Table 3) at non-inhibiting ammonium concentration, the synthetic 50% FE was used where the acetate and lactate concentration were identical to those in the 50% FE but the ammonium content was limited to 3.3 mM. In this case, 187.6 mmol H₂ per 1 L FE, i.e., 51.5% of theoretical value was obtained. In another word, the overall H₂ production stood at 142.3 mL g⁻¹ DW (i.e., 138 mL g⁻¹ DW during the photofermentation and 4.3 mL g⁻¹ DW during the dark fermentation stage).

The energy of the produced hydrogen was estimated as 1.6 kJ g⁻¹ DW_{Cr}. The energy content of the algae biomass was 14.5 kJ g⁻¹ DW_{Cr}. (Section 3). Thus, the energy conversion efficiency of the algae biomass to hydrogen was approximately 11%. As demonstrated in Section 4.1, the efficiency of the light energy conversion into the energy of algae biomass was about 5%. This indicates that the overall efficiency of the light energy conversion to the H₂ energy (through biomass synthesis and fermentation stages) was not more than 0.55% (Fig. 2). Moreover, it should be mentioned that we did not take into account all the additional energy expenditures at various stages (even the light energy consumed by the purple bacteria), which would decrease the efficiency dramatically.

To estimate the potential efficiency of the light energy conversion into H₂ using a three-stage scheme, the results obtained in the present study concerning the 1st stage, i.e., microalgae biomass production, could be combined with the most promising results reported previously on the subsequent stages, i.e., dark fermentation and photofermentation. On such basis, the maximal H₂ yield reported during dark fermentation and photofermentation was as high as 337 mL g⁻¹ DW_{Ar} using *Arthrospira* biomass (Cheng et al., 2012). In their study, the *Arthrospira* biomass was treated by microwave-assisted acid heating, enzymatic hydrolysis, and zeolite to remove ammonium (energy expenditure equivalent of 3.9 kJ g⁻¹ DW_{Ar}). Assuming that the energy content of *Arthrospira* and *C. reinhardtii* biomass was the same, i.e., 14.5 kJ g⁻¹ and that the efficiency of light energy conversion into the energy of algae biomass was about 5%, the efficiency of biomass energy conversion into H₂ energy will be 27% and the total energy conversion efficiency of initial light energy into the H₂ energy will not exceed 1.4%. Admittedly, the input of the light energy during the 3rd stage was not taken into account, and therefore, the accurate results will be much lower.

5. Conclusions

The application of mass and energy balance regularities appeared to be useful for the estimation of the efficiency of light energy conversion into the hydrogen energy at each stage of the three-stage integrated system. Accordingly, the three-stage system was found to possess rather low efficiency of light energy bioconversion even by taking into account the best results available in the literature. Therefore, it could be concluded that this scheme is unproductive for light energy bioconversion due to the numerous intermediate steps. Alternatively, direct light-dependent production of biofuels (ethanol, lipids, or hydrogen) by microalgae as elaborated by Sarsekeyeva et al. (2015) and Tsygankov and Abdullatypov (2015) might be more profitable.

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References

[1] Asatiani, V., 1969. Enzymatic methods of analysis (Russ). Nauka, Moscow.

[2] Cheng, J., Xia, A.X., Liu, Y., Lin, R., Zhou, J., Cen, K., 2012. Combination of dark- and photo-fermentation to improve hydrogen production from *Arthrospira platensis* wet biomass with ammonium removal by zeolite. *Int. J. Hydrogen Energy*. 37, 13330-13337.

[3] Choi, S.P., Nguyen, M.T., Sim, S.J., 2010. Enzymatic pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production. *Bioresour. Technol.* 101, 5330-5336.

[4] Clayton, R.K., 1966. Spectroscopic analysis of bacteriochlorophylls in vitro and in vivo. *Photochem. Photobiol.* 5, 669-677.

[5] Efrementko, E.N., Nikolskaya, A.B., Lyagin, I.V., Senko, O.V., Makhliis, T.A., Stepanov, N.A. et al., 2012. Production of biofuels from pretreated microalgae biomass by anaerobic fermentation with immobilized *Clostridium acetobutylicum* cells. *Bioresour. Technol.* 114, 342-348.

[6] Erickson, L.E., Minkevich, I.G., Eroshin, V.K., 1978. Application of mass and energy balance regularities in fermentation. *Biotechnol. Bioeng.* 20, 1595-1621.

[7] Gfeller, R.P., Gibbs, M., 1984. Fermentative metabolism of *Chlamydomonas reinhardtii*. I: analysis of fermentative products from starch in dark and light. *Plant. Physiol.* 75, 212-218.

[8] González-Fernández, C., Sialve, B., Bernet, N., Steyer, J.P., 2012. Impact of microalgae characteristics on their conversion to biofuel. Part II: Focus on biomethane production. *Biofuels, Bioprod. Biorefin.* 6, 205-218.

[9] Hanson, R., Phillips, G., 1984. Chemical composition of bacterial cell, in: Gerhardt, P. et al., (Eds.), *Manual of Methods for General Bacteriology* (Russian translation). Mir, Moscow, pp. 283-375.

[10] Harris, E.H., 1989. *The Chlamydomonas Sourcebook: A comprehensive guide to biology and laboratory use*. Academic Press, San Diego, pp. 780.

[11] Heiniger, E.K., Oda, Y., Samanta, S.K., Harwood, C.S., 2012. How posttranslational modification of nitrogenase is circumvented in *Rhodospseudomonas palustris* strains that produce hydrogen gas constitutively. *Appl. Environ. Microbiol.* 78, 1023-1032.

[12] Ike, A., Toda, N., Murakawa, T., Hirata, K., Miyamoto, K., 1998. Hydrogen photoproduction from starch in CO₂-fixing microalgal biomass by a halotolerant bacterial community, in: Zaborsky, O.R. (Ed.), *Biohydrogen*. Plenum Press, NY, pp. 311-318.

[13] Ike, A., Kawaguchi, H., Hirata, K., Miyamoto, K., 2001. Hydrogen photoproduction from starch in algal biomass, in: Miyake, J., Matsunaga, T., San Pietro, A. (Eds.), *Biohydrogen II: an approach to environmentally acceptable technology*. Pergamon, Amsterdam, pp. 53-61.

[14] Jeon, B.H., Choi, J.A., Kim, H.C., Hwang, J.H., Abou-Shanab, R.A.I., Dempsey, B.A. et al., 2013. Ultrasonic disintegration of microalgal biomass and consequent improvement of bioaccessibility/bioavailability in microbial fermentation. *Biotechnol. Biofuels.* 6, 37-45.

[15] Kawaguchi, H., Hashimoto, K., Hirata, K., Miyamoto, K., 2001. H₂ production from algal biomass by mixed culture of *Rhodobium marinum* A-501 and *Lactobacillus amylovorus*. *J. Biosci. Bioeng.* 91, 277-282.

[16] Kim, M.S., Baek, J.S., Yun, Y.S., Sim, S.J., Park, S., Kim, S.-C., 2006. Hydrogen production from *Chlamydomonas reinhardtii* biomass using a two-step conversion process: Anaerobic conversion and photosynthetic fermentation. *Int. J. Hydrogen Energy*. 31, 812-316.

[17] Khusnutdinova, A.N., Hristova, A.P., Ovchenkova, E.P., Laurinavichene, T.V., Shastic, E.S., Liu, J. et al., 2012. New tolerant strains of purple nonsulfur bacteria for hydrogen production in a two-stage integrated system. *Int. J. Hydrogen Energy*. 37, 8820-8827.

[18] Klass, D. L., 1998. *Biomass for Renewable Energy, Fuels, and Chemicals*. Academic Press, San Diego, CA.

[19] Lakaniemi, A.M., Hulatt, C.H., Thomas, D.N., Tuovinen, O.H., Jaakko A Puhakka J.A., 2011. Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass. *Biotechnol. Biofuels.* 4, 34-46.

[20] Lee, J.; Yoo, C.; Jun, S.; Ahn, C., Oh, H., 2010. Comparison of several methods for effective lipid extraction from microalgae. *Bioresour. Technol.*, 101, 575-577.

[21] Liu, C.H., Chang, C.Y., Cheng, C.L., Lee, D.J., Chang, J.S., 2012. Fermentative hydrogen production by *Clostridium butyricum* CGS5 using carbohydrate-rich microalgal biomass as feedstock. *Int. J. Hydrogen Energy*. 37, 15458-15464.

- [22] Lyubimov, V.I., L'vov, N.P., Kirshteine, B.E., 1968. Modification of the microdiffusion method for ammonium determination. *Prikl. Biochim. Microbiol.* 4, 120-121.
- [23] Miranda, J.R., Passarinho, P.C., Gouveia, L., 2012. Bioethanol production from *Scenedesmus obliquus* sugars: the influence of photobioreactors and culture conditions on biomass production. *Appl. Microbiol. Biotechnol.* 96, 555-564.
- [24] Ormerod, J.G., Ormerod, S.K., Gest, H., 1961. Light-dependent utilization of organic compounds and photoproduction of hydrogen by photosynthetic bacteria. *Arch. Biochem. Biophys.* 64, 449-463.
- [25] Park, J-I., Lee, J., Sim, S.J., Lee, J-H., 2009. Production of hydrogen from marine macro-algae biomass using anaerobic sewage sludge microflora. *Biotechnol. Bioprocess Eng.* 14, 307-315.
- [26] Rizwan, M., Lee, J.H., Gani, R., 2015. Optimal design of microalgae-based biorefinery: Economics, opportunities and challenges. *Appl. Energy.* 150, 69-79.
- [27] Ryu, M.H., Hull, N.C., Gomelsky, M., 2014. Metabolic engineering of *Rhodobacter sphaeroides* for improved hydrogen production. *Int. J. Hydrogen Energy.* 39, 6384-6390.
- [28] Sarsekeyeva, F., Bolatkhan, K., Usserbaeva, Z.A., Bedbenov, V.S., Sinetova, M.A., Los, D.A., 2015. Cyanofuels: biofuels from cyanobacteria. Reality and perspectives. *Photosynth. Res.* 125, 329-340.
- [29] Sheehan, J., Dunahay, T., Benemann, J., Roessler, P., 1998. A look back at the U.S. Department of Energy's aquatic species program: biodiesel from algae. National Renewable Energy Laboratory, Report NREL/TP-580-24190.
- [30] Sueoka, N., Chiang, K.S., Kates, J.R., 1967. Deoxyribonucleic acid replication in meiosis of *Chlamydomonas reinhardtii*. I. Isotopic transfer experiments with a strain producing eight zoospores. *J. Mol. Biol.* 25, 47-66.
- [31] Tekucheva, D.N., Tsygankov, A.A., 2012. Coupled Biological Hydrogen-Producing Systems: A Review. *Prikl. Biochim. Microbiol.* 4, 357-375.
- [32] Tsygankov, A.A., Abdullatypov, A., 2015. Hydrogen Metabolism in Microalgae, in: Allakhverdiev, S.I. (Ed.), *Photosynthesis: New Approaches to the Molecular, Cellular, and Organismal Levels*, Wiley-Scrivener, Beverly, pp. 133-162.
- [33] Tsygankov, A.A., Laurinavichene, T.V., Gogotov, I.N., 1994. Laboratory scale photobioreactor. *Biotechnol. Techn.* 8, 575-578.
- [34] Tsavkelova, E.A., Egorova, M.A., Petrova, E.V., Netrusov, A.I. 2012. Biogas production by microbial communities via decomposition of cellulose and food waste. *Appl. Biochem. Microbiol.* 48(4), 377-384.
- [35] Verma, N.M., Mehrotra, S., Shukla, A., Mishra, B.N., 2010. Prospective of biodiesel production utilizing microalgae as the cell factories: A comprehensive discussion. *Afr. J. Biotechnol.* 9, 1402-1411.
- [36] Yang, Z., Guo, R., Xu, X., Fan, X., Li, X., 2011. Thermo-alkaline pretreatment of lipid-extracted microalgal biomass residues enhances hydrogen production. *J. Chem. Technol. Biotechnol.* 86, 454-460.
- [37] Yun, Y.M., Jung, K.W., Kim, D.H., Oh, Y.K., Cho, S.K., Shin, H.S., 2013. Optimization of dark fermentative H₂ production from microalgal biomass by combined (acid + ultrasonic) pretreatment. *Bioresour. Technol.* 141, 220-226.