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Bioconversion of waste materials to hydrogen via dark fermentation using *Enterobacter aerogenes*

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ABSTRACT:

Hydrogen can be obtained via dark fermentation with the use of anaerobic *Enterobacter aerogenes*. The efficiency of hydrogen production by fermentation techniques is strongly dependent on the conditions used i.e. the pH range, temperature, composition of fermentation broths, oxygen content, or even the presence of substances with potentially inhibitory effects on the microbiological culture [1-4]. The paper describes the study of dark fermentation in four parallel thermostatic glass bioreactors with a working capacity of 50 mL each. The research concerned differences in hydrogen productivity depending on the type of carbon source used. Obtained exemplary results allowed to evaluate the effectiveness of the process [6] in relation to the production of hydrogen from various types of raw materials, i.e. pure glucose, waste glycerol and alkaline meadow grass hydrolysates, obtained according to the procedure given in [5].

Biokonwersja materiałów odpadowych do wodoru na drodze fermentacji ciemnej z wykorzystaniem *Enterobacter aerogenes*

STRESZCZENIE:

W drodze fermentacji ciemnej z wykorzystaniem względnych beztlenowców z gatunku *Enterobacter aerogenes* można otrzymać wodór. Wydajność wytwarzania wodoru technikami fermentacyjnymi jest silnie zależna od zastosowanych warunków w zakresie pH, temperatury, składu brzeczek fermentacyjnych, zawartości tlenu czy nawet obecności substancji o potencjalnie inhibitującym wpływie na hodowlę mikrobiologiczną [1-4]. W pracy opisano badania przebiegu fermentacji ciemnej w czterech wspólnie termostatowanych szklanych bioreaktorach o pojemności roboczej 50 mL. Badania dotyczyły różnic w produktywności wodoru w zależności od rodzaju zastosowanego źródła węgla. Uzyskane przykładowe rezultaty pozwoliły na ocenę efektywności procesu w odniesieniu do wytwarzania wodoru z różnego rodzaju surowców, tj. czystej glukozy, gliceryny odpadowej i hydrolizatów alkalicznych trawy polnej uzyskanych zgodnie z procedurą opisaną przez autorów w pracy [5].
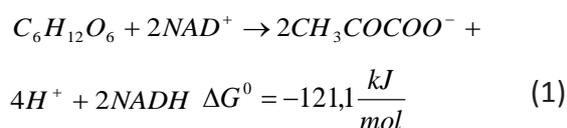
1. INTRODUCTION

Bioreactors or biofermentators are reaction vessels in which chemical and biochemical reactions take place to transform the substrate into the desired final product. They are used in the production of drugs and biomass, biotransformation of a particular compound or degradation of waste [7]. Reactions may occur in the bioreactor due to the presence of biocatalysts, i.e. enzymes isolated from microorganisms, microorganisms themselves, animal or plant cells, or their parts. A bioreactor also provides the appropriate reaction environment under aseptic conditions (especially using microorganisms sensitive to other cultures) and allows to control other environmental conditions such as oxygen content, pH and temperature. Bioreactors can be used for wastewater treatment in breweries, dairies, in the production of juices or in distilleries, for the treatment of municipal sewage, for obtaining enzymes, organic acids or antibiotics, for the production of therapeutic proteins and for the production of secondary metabolites in plant biotechnology [7-9]. Minibioreactors sets are used in laboratory scale to provide full functionality and to create configurations for any application or to preserve the scalability of the process. Due to the reduced scale of the breeding, the costs of research and the time needed to prepare the system for work, minibioreactors provide repeatability and safety of reaction or bioconversion, while saving time.

Cultivation of microorganisms in small-scale cell cultures is often used for screening purposes [10]. Bacteria, yeasts and other cultures may be screened as periodic cultures with or without nutrition for optimization of nutrient media and culture conditions.

In order to produce hydrogen via dark fermentation using cultures of *Enterobacter aerogenes* a set of quadruplicate minibioreactors with the working volume of 50 mL was constructed. The process conditions characteristic for the breeding were to be screened depending on the broth composition. Quadruplicates allow to perform a control fermentation parallel with triplicate breeding culture in exactly equal conditions, which is crucial for statistical purposes. The small size of bioreactors greatly facilitates and accelerates the preparation of vessels and their sterilization reducing the amount of medium needed to run a process which reduces the plant and operating costs [11].

Hydrogen is often considered as the fuel of the future due to very high energy efficiency equal to 10-13 $MJ*m^{-3}$ and due to its ecological values. Several methods of producing hydrogen on an industrial scale are applied, i.e. electrolysis of water, catalytic reforming of natural gas and conversion of carbon monoxide with water vapor [12-15]. Different media for broth composition can be applied, however the investigations for the hydrogen productivity for each of them need to be carried.
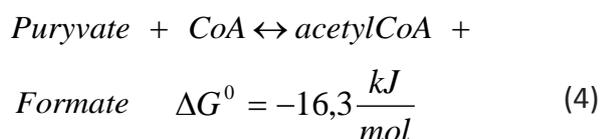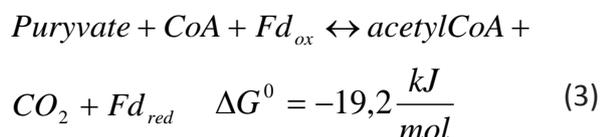
The use of waste glycerin and biomass hydrolysates, i.e. meadow grass hydrolysates was proposed in order to examine the possibility to use the designed quadruplicate bioreactors set. Hydrogen production is natural for many prokaryotes and the process of hydrogen production by microorganisms has been known for many years. However, only glucose is the basic raw material for energy transformation in the cell, as it enters the cycle of glycolysis providing ATP. The cost of glucose oxidation is generated in the energy cell and consumed in anabolic processes [16, 17]. The first step of metabolic pathways in *Enterobacter aerogenes* is the metabolism of glucose towards pyruvate, according to reaction (1):

$$C_6H_{12}O_6 + 2NAD^+ \rightarrow 2CH_3COCOO^- +$$

$$4H^+ + 2NADH \quad \Delta G^0 = -121{,}1\frac{kJ}{mol} \quad (1)$$

Reaction (1) may be described as the source of hydrogen which is generated during the subsequent regeneration of produced *NADH* in reaction (2):

$$NADH + H^+ \rightarrow NAD^+ + H_2 \quad (2)$$

Enterobacter aerogenes uses ferredoxin oxidoreductase Fd_{ox} for *acetyl-CoA* production (3) and pyruvate – formate lyase to generate *acetylCoA (4)*:

$$Puryvate + CoA + Fd_{ox} \leftrightarrow acetylCoA +$$

$$CO_2 + Fd_{red} \quad \Delta G^0 = -19{,}2\frac{kJ}{mol} \quad (3)$$

$$Puryvate + CoA \leftrightarrow acetylCoA +$$

$$Formate \quad \Delta G^0 = -16{,}3\frac{kJ}{mol} \quad (4)$$

There are a number of bacteria that are able to produce hydrogen from glycerine, but there is no technology developed so far on the industrial scale, because of certain problems related to the optimisation of fermentation procedure, occurring even in laboratory scale [18]. Glycerol is a substrate for gluconeogenesis, i.e. the production of glucose from non-sugar substances. This pathway clearly indicates the differences between mentioned sole carbon sources, because glycolysis (the conversion of glucose into

pyruvate) is thermodynamically conditioned (ΔG = -20 kcal*mol^{-1}) [1]. Gluconeogenesis cannot be combined with glycolysis at the same time, because the activities of the respective enzymes of both paths exclude cooperation. Moreover, gluconeogenesis is not exactly an inversion of pyruvate production from glucose. The process of glucose generation from non-sugar substrates, i.e. glycerol is thermodynamically favorable although it has a lower driving force (ΔG = -9 kcal*mol^1) [19]. The possibility of cost-effective generation of hydrogen from waste glycerine could help to solve both the problems of excess glycerin and the production of eco-friendly and cheaper energy [20-22]. Also lignocellulosic biomass has great potential for hydrogen production due to its large-scale availability, low cost and low greenhouse gases emissivity [23, 24]. The idea of hydrogen production from lignocellulosic biomass (abr. BMLC) arises, as in the case of mentioned material no food versus fuel debate needs to be considered.

The general idea considering the approach from BMLC to biohydrogen, including reactions given by equations 1, 2, 3, 4 is presented in the Figure 1. Primary sources of BMLC are energy crops, hardwoods, softwoods, cereal processing waste and grasses [1, 25, 26].
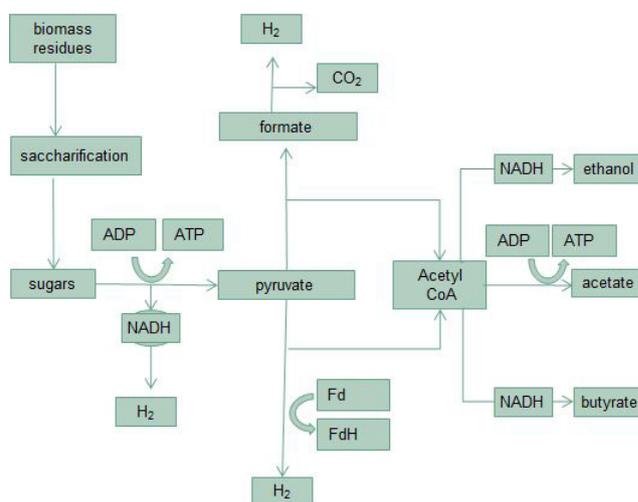

Figure 1 Possible paths and products in microbial systems during dark fermentation
(ATP – adenosine triphosphate, ADP – adenosine diphosphate, Fd – ferredoxin, FdH – reduced ferredoxin, NADH – reduced nicotinamide adenine dinucleotide)

The aim of the work is to develop equipment to carry out a bioconversion of waste materials, to hydrogen through dark fermentation in a laboratory scale. The possibility of conducting bioconversion processes in four jointly thermostated parallel bioreactors with a small working volume of 50 mL was considered. The experiment is planned to be conducted in small volumes due to the preliminary nature of the research. It is necessary to indicate the parameters significantly affecting the course of the process, and the use of small broth volumes allows reduction of both economic and ecological costs related to the carried processes. The identification of significant parameters for the process course may be a developing contribution for potential scaling up.

2. EXPERIMENTAL

Alkaline pre-treatment and enzymatic saccharification of lignocellulosic biomass was carried according to [27]. The procedure for preparation of lignocellulosic biomass hydrolysates, requires a multi-step procedure considering the fragmentation and convergence of the raw material, alkaline hydrolysis and enzymatic saccharification. The first stage in the treatment of lignocellulosic biomass was the fragmentation of the raw material. Biomass milling was done in two stages, initially using a garden crusher, and then a laboratory mill. It was found that the greatest possible fragmentation is the most advantageous from the point of view of further processing steps, however due to the high costs of the grinding process, the highest possible granulation value was determined experimentally, which allows obtaining results that are close to optimal in subsequent processing stages. The applied granulation at the level of 0.75 mm is possible to obtain after two-stage grinding. The next stage of treatment, was alkaline hydrolysis using monoethanolamine (MEA) catalyst for lignin and its derivatives removal. After the initial alkaline treatment, the solid and liquid fractions were separated by centrifugation. The solid residue was used in the next processing step, i.e. during enzymatic hydrolysis. Pre-treatment of lignocellulose material by alkaline hydrolysis is a key step in facilitating the release of simple sugars during enzymatic hydrolysis from cellulose and hemicellulose, as it improves the accessibility of the enzymes to polysaccharides, i.e. cellulose and hemicellulose. For the effective modification of the complex structure of lignocellulosic materials, it is necessary to determine the optimal, balanced combination of cellulolytic enzyme mixtures. Optimization of hydrolysis conditions was published considering different type of biomass, i.e. softwoods [24, 27] and then applied for meadow grass in this publication. It was found, that in the case of BMLC pre-treatment and saccharification steps are required in order to improve the process effectiveness [27, 28].

Analysis concerning gas and liquid phase samples during the process were carried according to [24]. The presence and the concentration of reducing monosugars and disugars (glucose, xylose, arabinose, mannose, galactose and cellobiose) were determined using HPLC. Sugar fraction was filtered through a cationic as well as anionic ion exchange column followed by vigorous shaking (30 min). Ion exchange procedure was completed by filtration through a syringe filter. Then, the eluent was evaporated in a stream of nitrogen. Sugar fraction was dissolved in 200 µL of water and directed to HPLC analysis (injection volume 50 µL). HPLC analysis of sugars: HPLC-RID (Knauer Smartline RID 2300, Berlin, Germany); temperature of separation: 60°C; column: Rezex Pb^{2+} column (300 × 7.8 mm, 8 µm) (Phenomenex, Torrance, CA, USA); eluent: water; flow: 0.6 mL/min.

Changes in the gas composition during fermentation were measured using gas chromatography. Gaseous products of fermentation (H_2 and CO_2) were analyzed using a gas chromatograph (AutoSystem XL, Perkin-Elmer, Norwalk, CT, USA) equipped with a Porapak Q column (Sigma-Aldrich, Merck, Darmstadt, Germany), (column details: lenght 6.5 m, diameter 1/8", pores 100-120 mesh) and a thermal conductivity detector (TCD). Oven temperature was set at 60°C. Turbochrom software was used for recording and processing of chromatograms. Gaseous samples were taken from the reactor at the lag, exponential and decline phases of culture growth. The growth of *E. aerogenes* culture was monitored by measuring of optical density of culture at λ = 600 nm [24]. For comparative purposes, an installation for parallel dark fermentations in quadruplicates was equipped in measuring system for the volume of the evolved gas (Fig. 2).

Dark fermentation was carried in sterile 80 mL glass bioreactors with initial working volume equal to 50 mL with the use of *Enterobacter aerogenes* (Selectrol TCS, Biosciences Ltd.). The initial

fermentation broth was composed of Buffered Peptone Water (Biomaxima) and carbon sources i.e. glucose, meadow grass hydrolysates and glycerol (concentration of carbon source equal to 10 g per L). The initial pH was equal to 7.00 (adjustment with 1 M NaOH). Bioreactors were inoculated with 5 mL of *Enterobacter aerogenes* culture in liquid breeding at OD_{600} = 2.40 ±0.13. No agitation was applied, temperature set point was equal to 37°C. Fermentation time of 60 h was applied, as it corresponds to the late logarithmic death phase of *Enterobacter aerogenes* culture in periodical conditions [24, 29]. The composition of fermentation broth for dark fermentation processes with respect to glucose, meadow grass hydrolysate and glycerol is given in Table 1.

Gas obtained during fermentation was collected in the bottom up rotated water-filled 1000 mL cylinders. The gas outflow was carried out with a teflon tube under the surface of the water to the cylinder as shown in the Figure 2. The volume is measured with an accuracy of 5 mL. Figure 2 presents the dark fermentation control equipment in the 4 × 50 mL system. The thermostated (C) glass bioreactors (1-4) contain 50 mL of fermentation broth. In bioreactors, anaerobic conditions are established using the D-line, which is connected to the nitrogen from the network and to the pipes supplying each bioreactor. With the progress of fermentation, the bacteria grow.

Thanks to the small size, the bioreactor occupies little space in the laboratory, small scale breeding allows for a low cost of a large selection of fermentation media.

The generated gases are discharged from the bioreactors to the measuring system (A) thanks to the pipes (b1-b4) – in this way, the total volume of gases formed can be read off. A three-way valve (B) is placed on the gas line, which enables gas sampling using a gas-tight syringe (Fig. 3a), the single bioreactor and its gas collecting system is presented in Figure 3b.
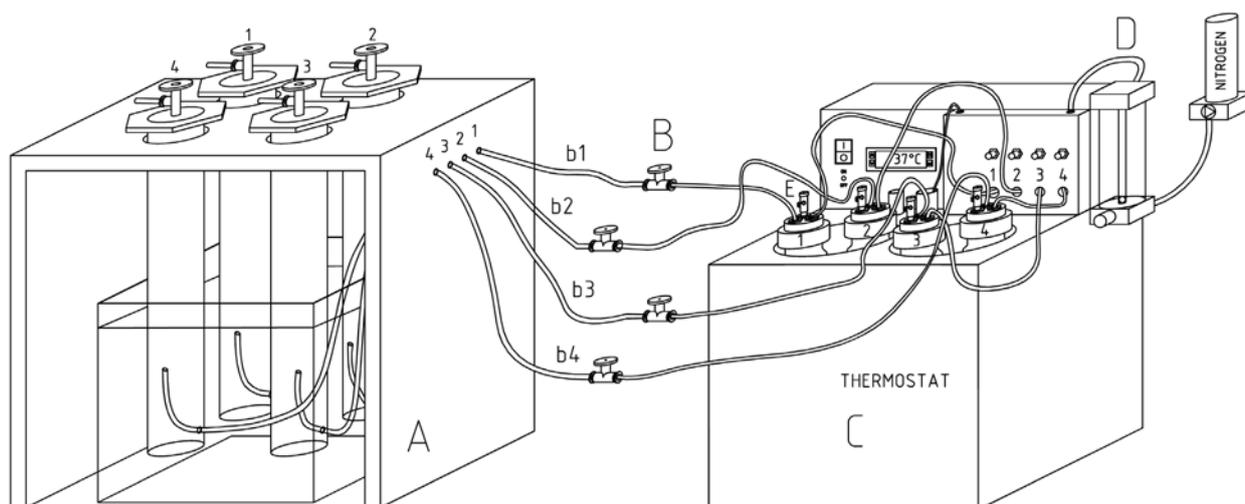

Figure 2 Quadruplicate bioreactor set for dark fermentation of differentiated broths combined with gas accretion volume measurement (A – gas accretion measuring system, B – tree-way valve, C – thermostat, D – nitrogen line, E – bioreactors 4 × 50 mL)

Table 1 Composition of fermentation broths for dark fermentation processes

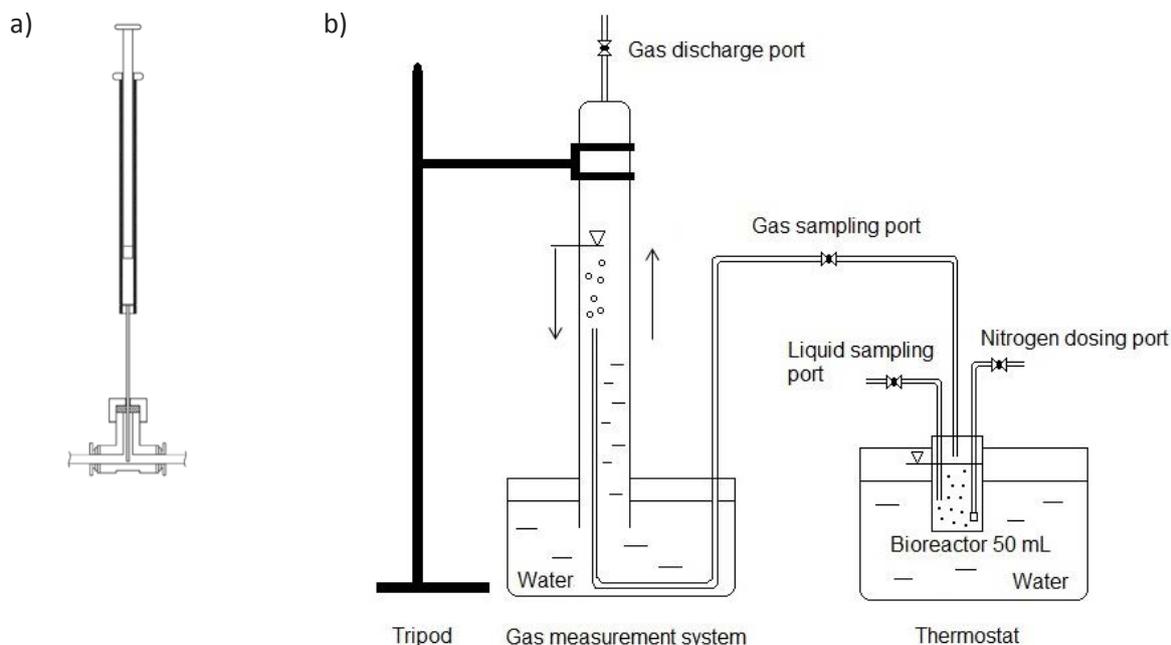| Component	Content in fermentation broth, mL		
	A	B	C
Buffered peptone water	40 mL (concentration = 20 g/L)		
Inoculum – *E. aerogenes* ATCC 13048 at OD_{600} = 2.40 ±0.13	5 mL		
Sole carbon source	Glucose, 10 g/L	Meadow grass hydrolysate 10 g/L	Glycerol, 10 g/L
Water	Up to 50 mL		

Figure 3 a) A three-way valve and a gas tight syringe for gas sampling during dark fermentation, b) Schematic diagram of a single bioreactor and gas collecting system connection

The taken gas sample is subjected to gas chromatography (abr. GC) analysis. Fermentation bioreactors are also equipped with a valve for sampling liquid broth (E), which are subjected to high performance liquid chromatography (abr. HPLC) analysis to identify and determine the loss of reducing sugars and the resulting fermentation inhibitors.

3. RESULTS AND DISCUSSION

Anaerobic digestion is a multi-step process carried out by *Enterobacter aerogenes* that requires strictly anaerobic conditions. The use of 4 × 50 mL set allows to carry the experiment in quadruplicates (for control, glucose – control and two different broths or in triplicates for one type of broth and one control process). Composition of meadow grass and its hydrolysate is presented in Table 2.

Table 2 presents the exact composition of meadow grass hydrolysates. It is clearly visible that in addition to glucose, there are also other monosaccharides that can affect the performance of dark fermentation. In order to compare the hydrogen productivity results from broths containing glucose and other sole carbon sources dark fermentation processes were performed and the broth compositions were given by Tables 1 and 2. Exemplary results for the first type of investigation i.e. for control, glucose – control is presented in the Figure 4. The use of three different broth allowed to observe differences in the fermentation efficiency potential of different sole carbon sources. As expected, the broth containing glucose (A), distinguishes the highest efficiency of obtained gases. Fermentation gas yield decreases when distilled glycerin (C) is used as the medium. Lignocellulosic biomass hydrolysate i.e. meadow grass hydrolysate, presents the curve with the lowest gas yield (B), which corresponds to the relatively low glucose content in this material. The composition of fermentation gas, regarding the hydrogen concentration is given in Table 4.

Table 2 Composition of meadow grass and meadow grass hydrolysate

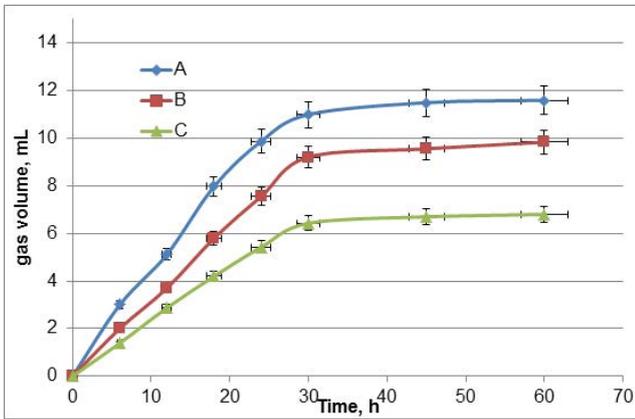| Biomass	Cellulose	Hemicellulose	Lignin content	Ash and extractives (ethanol)	Glucose	Xylose	Galactose	Mannose and Arabinose	Cellobiose
	%				mg/g biomass				
MG	27.1	22.6	16.8	33.5	376	81	48	13	20

Figure 4 Changes in accretion of total gas volume during dark fermentation (A – glucose, B – meadow grass hydrolysates, C – glycerin)

The obtained results are positively correlated with the growth of microorganisms, in accordance with the values presented in Table 3 and with the volume of hydrogen generated in dark fermentation, in accordance with Table 4.

Table 3 Changes in optical density of bacterial cultures occurring during *E. aerogenes ATCC 13048* growth during the dark fermentation process for different feed materials, (A – glucose, B – meadow grass hydrolysates, C – glycerin)

Time [h]	Optical density, $\lambda = 600$ nm		
	Fermentation broth		
	A	B	C
0	0.000	0.000	0.000
6	0.584	0.487	0.463
12	2.200	1.987	1.215
18	2.614	2.164	1.624
24	2.635	2.214	2.226
30	1.542	1.487	1.827
45	1.120	1.021	1.126
60	1.095	0.998	0.954

Table 4 Changes in hydrogen concentration generated during dark fermentation for different feed materials (A – glucose, B – meadow grass hydrolysates, C – glycerin)

Time [h]	Hydrogen concentration mL H_2/mL gas		
	Fermentation broth		
	A	B	C
0	0.000	0.000	0.000
6	0.405	0.032	0.104
12	0.315	0.046	0.096
18	0.306	0.116	0.437
24	0.611	0.351	0.512
30	0.681	0.394	0.514
45	0.596	0.375	0.485
60	0.530	0.328	0.476

The average concentration of hydrogen generated during the dark fermentation carried by *Enterobacter aerogenes* differs for diversified broths. It can be assumed that the obtained results can be a good source of information for a voluptuous evaluation of the fermentative potential of various types of broths.

Understanding the changes occurring during the dark fermentation of selected broths materials (differing in the structure of sole carbon source) is crucial for understanding the relationship between the composition and hydrogen productivity in the process of dark anaerobic fermentation. The gas with the highest hydrogen content was obtained when glucose was used. It occurred, that applying meadow grass hydrolysates and glycerine results in lower hydrogen concentration, however this type of broth can also deliver hydrogen. In the currently available literature, there are few papers [30-33] that comprehensively investigated the effect of various factors on the course of hydrogen biomass fermentation taking into account the influence of process parameters such as pH, temperature, substrate concentration and composition of the medium for hydrogen production in the dark fermentation process. However, an universal method was not met, and despite the growing interest in this issue, the results of the published research do not provide an unambiguous answer as to how the structure and properties of the raw material affect the efficiency of a given method. There are no comprehensive studies on the influence of such factors or the presence and concentration of by-products produced on the rate of enzymatic degradation and the course of hydrogen fermentation. For this reason, hydrogen productivity related issues are still an object of interest due to possible directions of feed material preparation. The use of small volumes is an asset, because the experiment is designed to provide information about the meaningful directions for further exploration in the field of fermentation feeds. Low nutrient volumes, a comprehensive research kit and the possibility of ongoing comparisons during the experiment cause, that the set can be easily applied on a laboratory scale and is additionally characterized by making proxies in the field of waste generation with respect to waste broth defined in a very small scale, which is also a significant advantage. The laboratory scale cannot be the target method for conducting rese-

arch, however the development of the possibility of conducting parallel processes in repetitive conditions definitely facilitates the development of technology and process scaling up.

The dark fermentation residue can be methanized to exhaust the possibility of obtaining gas products, i.e. for biogas generation. During dark fermentation the efficiency can only be measured regarding hydrogen concentration, however for the purposes of broth management purposes also further processing needs to be considered. The liquid phase may also contain a rich matrix of chemical compounds with low concentrations that can be separated mainly using extraction, sorption, distillation, crystallization and membrane methods. Development of digestate on an industrial scale will undoubtedly become a challenge. The choice of parameters significantly affecting the course of the process is carried out on a laboratory scale also due to the minimization of the emerging waste streams and the possibility of refining the applied methods of technical analysis.

4. CONCLUSIONS

Concluding, the proposed quadruplicate bioreactors set allows to provide a precise comparison of the rate and efficiency of bioconversion processes in four parallel simultaneous anaerobic digestions. The fermentation process is monitored on the basis of precise measurement with respect to gas products liquid broth under strictly controlled growing temperature conditions and initial pH. Hydrogen production is stoichiometrically related to the transformation of the sole carbon source. Proposed set may be applied for the screening of microorganisms, for the optimization of fermentation set points and to define the statistically significant variables to be monitored during scale-up. Amongst the advantages of presented set the possibility of simultaneous running of four independent fermentation processes with the allowance to a precise control of the critical process parameters should be mentioned.

Author Contribution: Karolina Kucharska carried the experiments, Karolina Kucharska and Edyta Słupek carried the analysis, Karolina Kucharska and Edyta Słupek wrote the paper, Marian Kamiński provided materials/reagents/apparatus.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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