TWO-STAGE ANAEROBIC DIGESTION OF WHEAT STRAW USING IMMOBILIZED MICROBIAL CONSORTIA

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Abstract: The serious energy and environmental problems associated with the use of fossil fuels necessitate the search for alternative energy sources. One of the modern approaches is the anaerobic degradation of organic waste from agricultural wastes. The hydrogen and methane thus obtained are sources of environmentally friendly energy, which reduces carbon dioxide emissions from fossil fuels, as well as gaseous emissions resulting from natural degradation processes in the disposal of waste materials. The described two-stage anaerobic digestion (TPAD) system with an immobilized microbial consortium represents an innovative biotechnological approach that seeks to obtain an increased energy yield and raised degree of processing of waste materials. Some additional raw materials which represent waste materials from other industrial scale processes can be successfully applied and support higher biohydrogen production from wheat straw. The temperature regime suitable for wheat straw biodegradation is 55°C resulting in 2.5 time more biohydrogen production. The VFAs obtained from BR-1 are suitable substrate for the immobilized microbial consortia which is formed for nearly twenty days of bioreactor maintenance.

Keywords: biohydrogen, biomethane, two-stage anaerobic digestion, immobilized microbial consortia, wheat straw

INTRODUCTION

Globally, more than 1.3 billion tons of organic waste materials per year are produced from agricultural production, during processing and transportation, in distribution and consumption. The lignocellulosic materials are some of the most common organic wastes. Wheat straw, as an abundant and sustainable source, is considered a feedstock with high potential for biogas production [18]. Agricultural waste digestion has a complex nature and is accomplished by successive degradation pathways and syntrophic microbial associations. Although the process separated organization favours the degree of control and process management a further optimization in terms of nutrients additives spectrum is necessary. Single agricultural waste degradation could cause fermentation instability and even process disruption [16]. Moreover, the drawback associated with the lignocellulose rigidity and burdened enzymatic hydrolysis is still a subject of improvement, notably at mesophilic conditions. In lignocellulosic biomass digestion, hydrolysis is considered the rate-limiting step [25]. The hydrogen yield is also crucial for biogas production. Different types of hydrogen metabolism occur in the microbial world. Diverse microorganisms have the capacity for hydrogen production via dark fermentation in light and oxygen-free conditions. Hydrogen production by anaerobic fermentation proceeds in two stage – an acidogenic phase where high hydrogen production along with acetic and butyric acid is observed and is associated with rapid microbial growth. The second phase is called solventogenic and is described by low hydrogen production and slower microbial growth [13]. Different approaches have been utilized to improve the hydrogen production process and to enable sustainable fermentation. However further investigations at a molecular level are still in demand [20]. One of a number of technologies that can be used to reduce the quantity of agricultural wastes, to decrease global warming and waste management problems is anaerobic digestion. The process involves metabolic reactions such as hydrolysis, acidogenesis and methanogenesis [22]. Under controlled conditions, anaerobic digestion has the potential to contribute to useful products such as biofuels and organic additives (soil improval) [6]. Physical separation in two- stage digestion makes it possible to overcome the problem of differences in the optimal conditions for microorganisms. This separation allows to optimize conditions that are favorable for the growth of each group of microorganisms in each reactor [11].

Two-stage anaerobic digestion (TPAD) is concerned with optimizing each step of digestion. The separation of the biological chain of acetogenesis and methanogenesis into two different bioreactors is not a new approach to anaerobic digestion. This idea was first suggested in 1984 [5]. Separation of the natural ecology and metabolism of an anaerobic bacteria consortium into different classes: H_2-producing bacteria (*Clostrium*) and H_2-consuming methanogens (*Archaea*) underlie the two-stage bioreaction [24]. In the first acidic step, pH conditions are selected in order to favor the production of H_2. The liquid metabolites produced at this stage consist of volatile fatty acids (VFA) such as acetic and butyric, and alcohols which are readily metabolized by methanogens. The second stage under conditions of

doi.org/10.32006/eeep.2022.1.3544

neutral pH provides conditions for methanogenesis with CH_4 production [8]. The two-stage anaerobic digestion process, which produces hydrogen gas in the first phase, followed by methane production in the second phase, has many advantages. However, the process of biohydrogen production is much faster than biomethane formation, which requires selection of the respective volumes of the two bioreactors. A possible approach for reducing the difference in the hydraulic retention time of the two processes and hence the reduction of the volume of the second bioreactor is the use of immobilized microbial cells in the second stage of the integrated system.

The use of immobilized organisms in modern biocatalysis allows the acquisition of many positive effects. Anaerobic sludge cells are capable of self-immobilization via formation of granules and biofilms. The fixed-film system has some advantages such as: 1. It's easier to operate with it; 2. Deals with shock loads associated with increasing the concentration of incoming contaminant; 3. Less solid sludge wastes; 4. Requires less energy to work [1, 9].

The purpose of this study was to select the conditions for starting and investigate the efficiency of the two-stage anaerobic digestion bioreactor system for producing hydrogen and methane from straw wastes, as well as application of immobilized microbial community in the second stage.

MATERIALS AND METHODS

Raw materials

Wheat straw was used as main substrate to be digested. It was mechanically chopped using hammer mill followed by additional milling using knife mill until final particle size of 1-2 mm were reached.

ADM® Corn Steep Liquor 104 (Amylum Bulgaria EAD) were used as an additive.

Inoculum

The inoculum was obtained from a working on wheat straw anaerobic digestion process with methane production. The digestate from this process was taken and sieved trough 1 mm coarse sieve in order to remove all residual straw particles. For removal of methanogens the liquid fraction was thermally treated at 75ºC as it is previously described [10] and was added to each flask at concentration of 10 % (v/v).

Experimental set-up

In the present study two types of experimental set-ups were used. First one consisted of five Erlenmeyer flasks 500/1000 ml. Flasks were sealed using butyl rubber stoppers each one supplied with two holes for biogas evacuation and for sampling. They were cultivated on a water bath rotary shaker at 37ºC, 30 rpm, for 96 h.

For experiments with feeding a cascade integrated system of two bioreactors was used (Fig. 1). The hydrolysis and acidogenesis accompanied by hydrogen accumulation in the resulting biogas are maintained in bioreactor 1 (BR-1). It works as typical continuous stirring tank reactor (CSTR) with automatic control of temperature and the stirrer speed and work volume of 2.2 dm^3. In bioreactor 2 (BR-2) there are concentrically arranged steel mesh cylinders. Biofilm is formed on them [8]. BR-2 also works as CSTR at work volume of 1.2 dm^3 and has the same control options as BR-1. Thus BR-2 works using immobilized microbial consortia to process the liquid acid products from the first bioreactor and to transform them into methane.

Fig. 1. Two-stage anaerobic digestion with biohydrogen and biomethane production set-up.

Analyses

The **biogas volume** was measured using graduated glass cylinders. The released biogas was collected using water displacement method.

Biogas content was estimated with a device model "Gasboard 3100P" (Cubic Sensor and Instrument Co., Ltd, Wuhan), equipped with infrared sensors for measuring the relative content of CO_2 and H_2 (in % by volume) or by Dräger X-am 7000 device equipped with infrared sensors for CH_4, CO_2 and a catalytic sensor for H_2S (in ppm) content measurement.

Cellulose was determined by the spectrophotometric method [23]. Cellulose-containing materials are released from impurities such as lignin, hemicellulose, xylosans and others low molecular weight compounds by extraction with an acetate-nitrite mixture. The purified cellulose was dissolved in 67% H_2SO_4, followed by a color reaction with an anthrone reagent. The cellulose concentration was determined after measuring the absorbance at 620 nm.

Soluble **proteins** were determined by a spectrophotometric method using Coomassie Brilliant Blue G-250 reagent [4]. Soluble **reducing sugars** were determined by the Somogy-Nelson method [21]. The concentration of **volatile fatty acids** (VFAs) was determined by a Thermo Scientific gas chromatograph (Focus GC model) equipped with a Split / Splitless injector, column: TG-WAXMS A, (length 30 m, diameter 0.25 mm, film thickness 0.25 µm) and flame ionization detector (FID). **TS** and **VS** were measured using standard procedure [2]. **pH** was measured using Microprocessor pH-meter, Model: pH210 (HANNA Instruments).

RESULTS AND DISCUSION

Batch biohydrogen production evaluation

The lignocellulose structure is very rigid and hence difficult to be accessed by microorganisms or microbial enzymes. For this reason, usually lignocellulose biomass is applied in anaerobic biodegradation processes after pretreatment [17]. In previous study it was assumed that after preliminary adaptation of the anaerobic microbial community in the bioreactor it is possible to carry out AD of wheat straw without pre- treatment. The authors obtained obtaining good results concerning quantity and quality of the resulting biogas and the degree of biodegradation of the cellulose [15]. We assumed that not only the complex composition of wheat straw, but also the small amount of soluble substances also contributes to the weaker development of the microbial community and there and lower yields of biogas and the target product – hydrogen. The aim of these experiments was to analyze the possibilities for biohydrogen production increase from wheat straw during batch cultivation at 37°C with some additives as process modulators. Each variant contains the following substrates: wheat straw (WS) only; WS +yeast extract (YE); WS + corn steep liquor (CSL); WS + milk whey (MW); WS + glycerol (GLY).

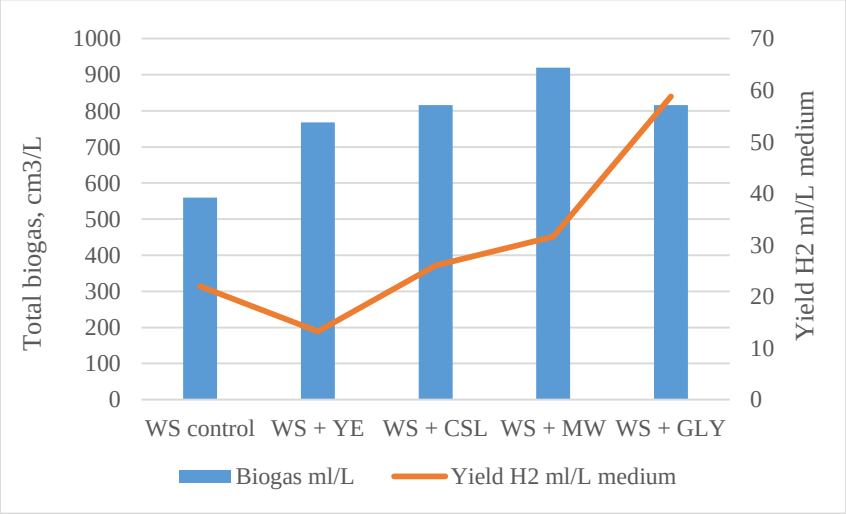

Fig. 2. Yield of biohydrogen related to total biogas yield.

The YE was chosen as it is one of most used components of microbiological cultivation media which was added as a source of a wide variety of vitamins and co-factors. It serves as "positive control" during the experiment and as "negative control" when only wheat straw was used. The other components were selected, each representing a by-product/ production waste. Thus, glycerol is a waste from the production of biodiesel, milk whey from the cheese production, SCL from the processing of corn grains. Wheat straw was in a non-treated form and it was added in concentration of 30 g/L. Every other component as well as YE was added in the ratio to wheat straw equal to 1:10 (i.e. 3 g of supplemented component to 30 g wheat straw).

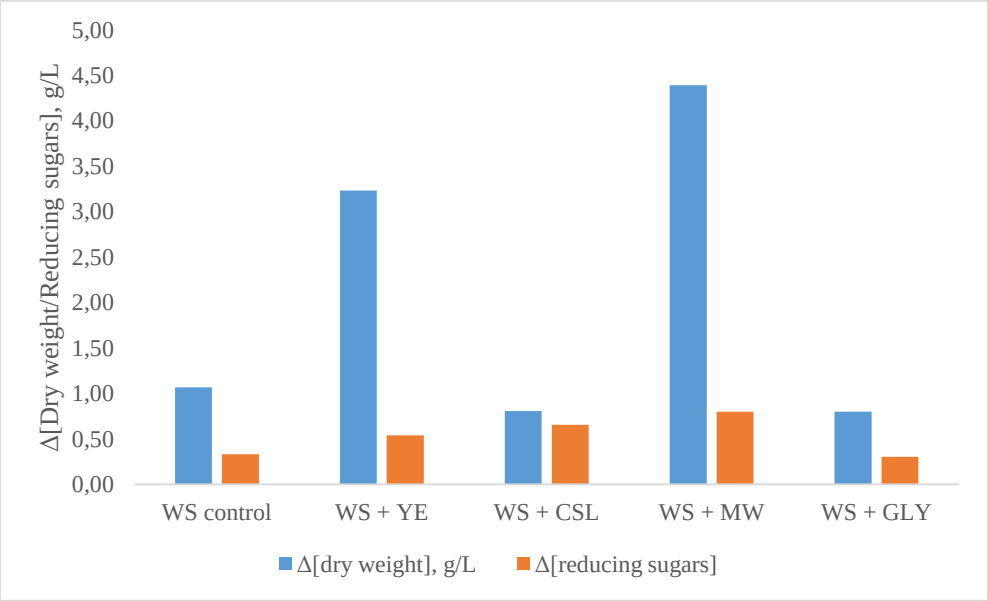

Fig. 3. Δ [dry weight] and Δ [reducing sugars]. Δ means the difference between the starting value of the parameter and the measured value at the end of the process.

The criteria for selecting a suitable supplement include not only maximum hydrogen yield, but also a high degree of biodegradation, as well as the accumulation of mainly acetate, as it serves as a primary substrate for the second stage of the integrated two-stage process – methanogenesis. In this regard although the GLY gave highest hydrogen production of 58.8 mL H_2/L cultural liquid (Fig. 2), CSL and MW became more attractive candidates. The results for the residual amounts of reducing sugars are comparable as their increase in the variants with CSL and MW is probably due to the higher baseline values for this indicator (Fig. 3). However, reducing sugars are another readily available substrate for the processes of methanogenesis and their presence in the feeding liquid is not undesirable. On the other hand, the variant with added MW shows a lower decrease in dry matter. Since our system seeks to maximize the solids transformation to liquid soluble metabolites during the first phase of hydrogen production, the addition of MW would not give one of the desired effects. The last of our criteria – the acetate production as a metabolic product show that CSL is produced in a satisfactory level (Table 1). Of all the variants, only in the case of anaerobic biodegradation of untreated wheat straw (without any additional compounds), the presence of only propionate and the complete absence of butyrate in the VFAs profile is observed. As propionate has a more pronounced inhibitory effect on the methanogenic process [12], the presence of this acid would lead to some difficulties in the second phase of the process, especially in a quasi-continuous mode of cultivation (with daily feeding). In the case when GLY was used as supplemented raw material, the total VFAs quantity is lower than other variants with additives but the main metabolic product is shown to be butyrate or at least the butyrate has a concentration close to this of acetate.

Table 1. Volatile fatty acids concentration in the end of the process.

Substrate	VFAs, g/L						
	Acetate	Propionate	i-Butirate	Butirate	i-Valerat	Valerat	tVFAs
WS control	1.00	0.3	0	0	0	0	1.30
WS + YE	1.30	0.1	0.1	0.60	0	0	2.10
WS + CSL	1.24	0	0	0.63	0.03	0	1.90
WS + MW	1.12	0	0	0.73	0	0	1.85
WS + GLY	0.68	0	0	0.80	0	0	1.48

Among other supplements CSL was chosen to be applied in our future experiments for biohydrogen and biomethane production in TPAD.

Selection of temperature regime in the first phase of TPAD

Choosing the right temperature regime is critical for the stable operation of the process as well as its speed. In the case of the use of untreated lignocellulosic materials, it would be even more important due to several factors. It is a known fact that the increased temperature leads to swelling of the structure of lignocellulosic materials and hence the possibility of improving their attack by microorganisms and their enzyme systems [3]. Despite the increased energy costs, the application of elevated temperature in the early stage of the process would lead, as paradoxical as it may sound, to some cost reduction of maintenance of the process. Thus, one of the well-known and used (including in our laboratory) methods for alkaline treatment involves the addition of an alkaline agent (NaOH) and at the same time the temperature of the reaction mixture must be maintained at 55 °C for 24 hours [14]. This pre-treatment procedure also requires a separate reaction vessel and appropriate equipment. Two different temperature regimes were studied in the first bioreactor: 35°C and 55°C. BR-2 works only at 35°C (Table 2).

Table 2. Experiments operational temperature and respective signature. TPAD mode of operation.

Experiment No.	*Signature*	*BR-1 operational temperature, °C*	*BR-2 operational temperature, °C*
1	M-M	35	35
2	T-M	55	35

The wheat straw was used as a substrate with addition of corn steep liquor as a nitrogen source. The concentration of wheat straw was 10 g/L and CSL was added in concentration 1 g/L in BR-1. The average composition of CSL is shown in Table 3. The highest concentration is of reducing sugars. This compound is very fast degradable and led to either ensuring fast source of carbon and energy but also to fast increase in VFAs content. Although the cellulose content is very small the cellulose is in accessible for microbial degradation form. Our expectations for higher protein content had not been justified but it might be due to the reason we measure only the soluble part of protein content. The feeding of BR-2 was realized using the liquid fraction containing primary liquefied metabolites produced in the BR-1. The residual solid mass consisted mainly of undegraded particles from wheat straw so it was withdrawn in order to prevent the biofilm bioreactor (BR-2) from blockage and deterioration of the hydrodynamic regime because of its constructive features. The additional experiments for determination of the possibility of using such kind of semi digested lignocellulose biomass are foreseen to be carried out.

Table 3. Corn steep liquor content per gram fresh weight.

Compound	*Concentration*
Total solids, %	50.5
Volatile solids, %	91.0
Cellulose, mg/mL	2.1
Reducing sugars, mg/mL	86.1
Proteins, mg/mL	1.7
tVFA, mg/mL	3.4
- Acetate, mg/mL	1.6
- Propionate, mg/mL	1.0
- Butirate, mg/mL	0.8

The biogas quantity reached during operation of BR-1 at 35°C (Fig. 4A and B) – 18,5 cm^3/L was accompanied by low hydrogen content (about 5 %). In comparison with the process carried out with temperature set at 55°C for the BR-1 the biogas quantity is ten times higher for the 55 °C. At the same time the hydrogen concentration in it is about twice higher (Fig. 6 A and B). Comparing the data for VFAs (Table 4 and 5) we might assume that lower hydrogen concentration is due to its capturing in the acidic compounds. The VFAs content at 35°C seemed to be favorable for biomethane production because of the preliminary acetate concentration, and the 42% of biomethane in the biogas from BR-2 is in accord with that, but with relatively low biogas quantity of about 181 cm^3/L in comparison with the tree times higher biogas production from the same bioreactor in the T-M mode of operation.

Biohydrogen production process is characterized by very dynamic pH changes and need of control and regulation. In our study pH drops in the first bioreactor after its start from 5.5 to about 5.2 for both operation conditions (Fig. 5 and Fig. 7) even for M-M experiments where tVFAs concentration was 2.9 g/L. Those VFAs are almost fully transformed and in the second stage of the system to 0.1-0.2 g/L.

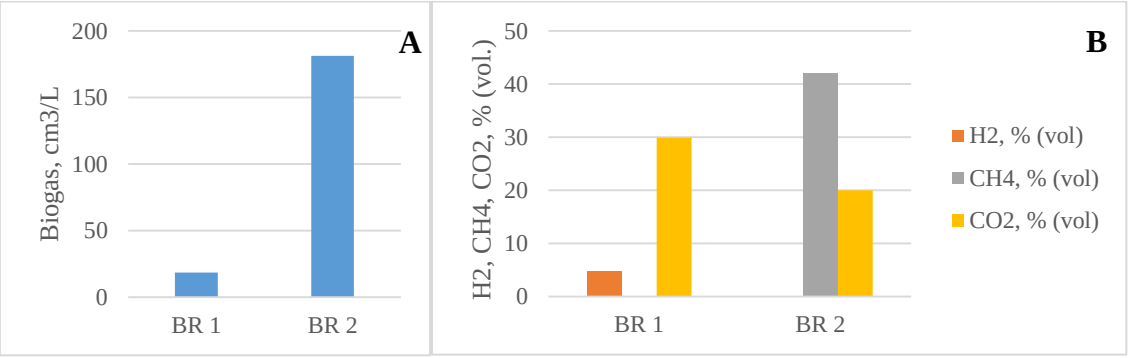

Fig. 4. Biogas production (A) and the biogas content (B) during anaerobic biodegradation at 35°C in both BR-1 and BR-2.

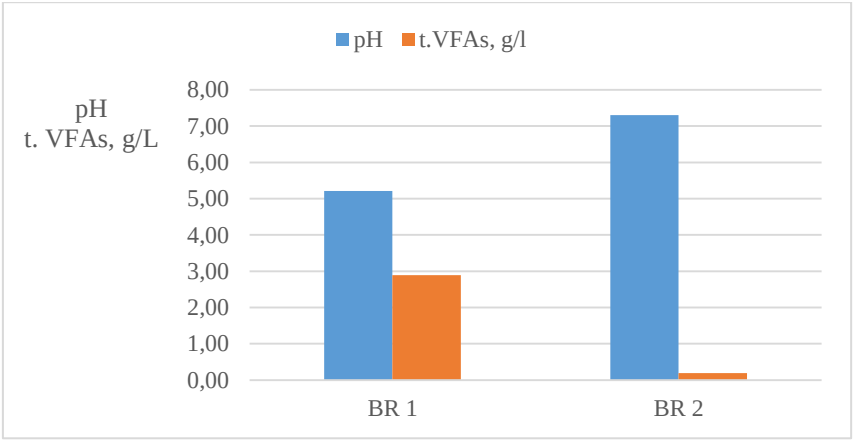

Fig. 5. pH value and the tVFAs quantity during anaerobic biodegradation at 35°C in both BR-1 and BR-2.

Table 4. VFAs content and concentration during anaerobic biodegradation at 35°C in both BR-1 and BR-2.

	Acetate	*Propionate*	*i-Butyrate*	*Butyrate*	*i-Valerate*	*Valerate*	*Caproate*
BR-1	1.67	0.12	0.13	0.65	0	0.08	0.24
BR-2	0.11	0	0	0.08	0	0	0

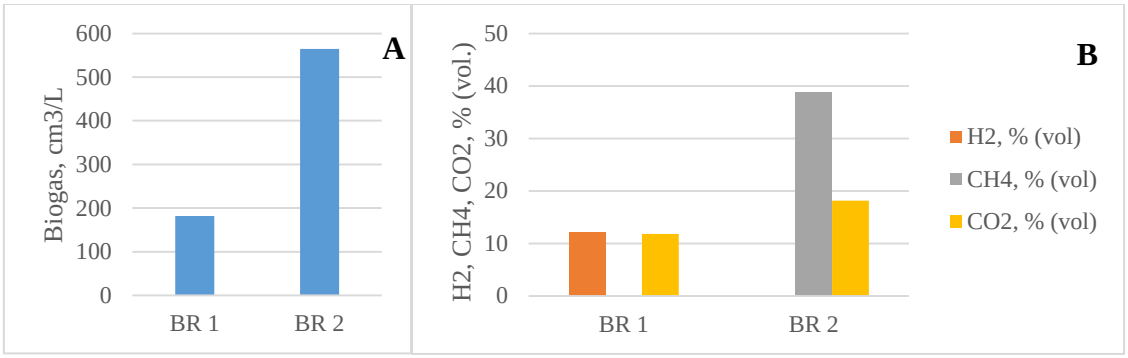

Fig. 6. Biogas production (A) and the biogas content (B) during anaerobic biodegradation at 55°C in BR-1 and 35°C in BR-2.

Table 5. VFAs content and concentration during anaerobic biodegradation at 55°C in BR-1 and 35°C in BR-2.

	Acetate	*Propionate*	*i-Butyrate*	*Butyrate*	*i-Valerate*	*Valerate*	*Caproate*
BR-1	0.43	0.08	0.09	0.51	0	0.07	0.13
BR-2	0	0.1	0	0	0	0	0

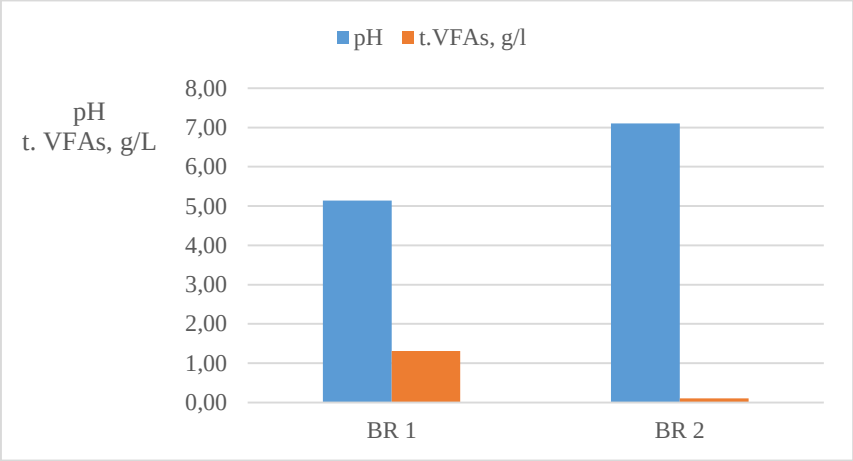

Fig. 7. pH value and the tVFAs quantity during anaerobic biodegradation at 55°C in BR-1 and 35°C in BR-2.

Biofilm formation in BR-2

For starting a stable TPAD system with immobilized microbial community in second stage inoculum from working bioreactor for biomethane production were used. The strategy we used for providing of contact between microorganisms and the fixed carrier include starting the bioreactor with liquid digestate from working bioreactor. In those experiments digestate withdrawn from our biogas pilot plant [19] was used and 300 mL were placed in the BR-2 and the stirrer rotation was set at 20 rpm. Anaerobic conditions were ensured purging bioreactor with nitrogen gas. Feeding of bioreactor was perform with 300 mL liquid phase from BR-1 digestate. Mode of operation for the TPAD system were chosen to be T-M (see Table 2). First twenty days of operation are characterized by very unstable biogas production. The fluctuations observed in the BR-2 are related with the dynamics of the biohydrogen production process, but also the due to the the way the bioreactor works. In the first days, the microorganisms are not yet attached to the stationary phase (carrier) and the reactor operates as a reactor with suspended biomass. The amplitudes of fluctuations in biogas production become smaller around the twentieth day after the start of the system. Their follow-up over the next ten days shows that the process is stabilizing and can be put into continuous operation. From the course of the graph it can be assumed that the time required for the initial attachment of the cells to the carrier (in our case a metal mesh made of stainless steel) takes approximately twenty days.

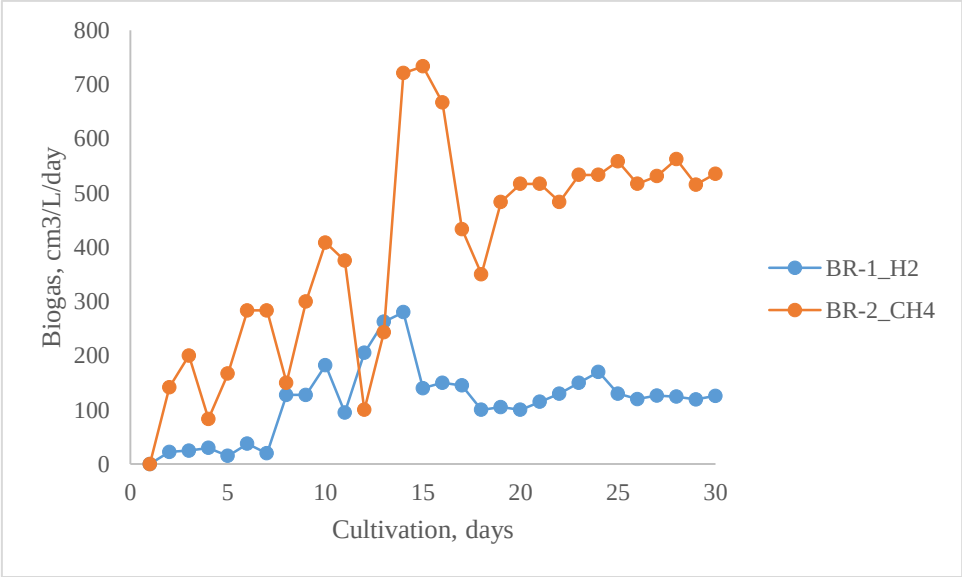

Fig. 8. Daily biogas production during the operation of two-stage anaerobic digestion.

After a month of operation from starting the TPAD system, the BR-2 was disassembled and the microbial attachment to the carrier were visually observed (Fig. 9). Additional experiments about the kinetics of immobilization as well as stability of the process over longer operation (3-6 months) are necessary to prove the ability for continuous mode of operation.

Fig. 9. The steel mesh carrier with immobilized microbial consortia (white arrow) after 30 days of operation.

CONCLUSIONS

Tree different additives represent waste materials from various industrialized processes were used for improving biohydrogen yield from wheat straw. All of them lead to increase of VFA production (substrates for the next biomethane producing step) biohydrogen as aim product. CSL was assumed as favorable because of the VFA content and concentration and satisfactory dry weight removal and reducing sugars release in the liquid phase. Maintaining the first bioreactor from the TPAD at 55°C led to biogas production near to 180 mL and increase of hydrogen content about 2.5 times in comparison with the same process at 35°C. Taking into account other parameters as pH, VFAs concentration as well as the BR-2 operation the T-M mode of operation can be assigned as preferable for biohydrogen and biomethane production in two-stage digestion process. Anaerobic immobilized mesophilic microorganisms can be attached to stainless steel carrier using very low speed of stirrer (25 rpm) and necessary nutrients. These process takes about twenty days for reaching stable biogas production rate.

***ACKNOWLEDGEMENTS**: This study is funded by the Bulgarian National Science Fund under contract КП-06-М 26/5 "Innovative two-stage system for production of hydrogen and methane using immobilized microbial anaerobic community".*

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