Studii şi Cercetări Ştiinţifice
Chimie şi Inginerie Chimică, Biotehnologii, Industrie Alimentară
2023, **24** (1), pp. 025 – 040

Scientific Study & Research
Chemistry & Chemical Engineering, Biotechnology, Food Industry *ISSN 1582-540X*

ORIGINAL RESEARCH PAPER

ENHANCED BIOHYDROGEN PRODUCTION THROUGH REPEATED-BATCH FERMENTATION OF RICE-BRAN

Ebenezer O. Dada[1,2], Abass O. Alade[1*], Ademola T. Adeniji[3], Tinuade J. Afolabi[1]

[1]Ladoke Akintola University of Technology (LAUTECH), Department of Chemical Engineering, PMB 4000, Ogbomoso Nigeria
[2]Univerisiti Kebangsaan Malaysia, Bangi, Department of Chemical and Process Engineering, 43600Bangi, Selangor, Malaysia
[3]University Paris-Saclay, Laboratory of Process Engineering and Materials (LGPM), Centralesupelec, Plateau de Moulon 3 rue Joliot-Curie F-91192 Gif-sur-Yvette Cedex Paris, France

*Corresponding author: aoalade@lautech.edu.ng

Received: March, 31, 2022
Accepted: March, 27, 2023

Abstract: Repeated-batch fermentation experiments were carried out to determine the best switching time, stability, and consistency of the process for enhanced biohydrogen production from Rice-bran. After the initial batch process, one repeated Cycle was made for three 'runs' to determine the best switching time, the 'runs' were terminated after 45 h, 60 h and 75 h, respectively. The effect of switching time on cell growth, hydrogen yield and productivity was investigated. The pattern of cell growth suggested that switching from batch mode to repeated batch mode at the 60th h led to the production of the highest amount of hydrogen. The lowest *p*H value (5.22) was observed at the end of the first fermentation Cycle. Cycle 2 has the highest amount of cumulative hydrogen production (mL 1280.3 L^{-1} medium) and the highest *p*H value (5.38). The profiles of the biomass concentration for all the Cycles were similar showing the stability and consistency of the process. There was an increment of 22 % in the overall Hydrogen gas production rate for the repeated batch process when compared to that of the ordinary batch process this result justified the use of the repeated batch system as a process that enhances biohydrogen production.

Keywords: *cell growth, Clostridium saccharoperbutylacetonicum N1-4, hydrogen energy, initial substrate concentration, switching time*

INTRODUCTION

Hydrogen fuel is widely regarded as an energy of the future due to its zero-carbon footprint. Interestingly, the product of hydrogen fuel combustion is water which makes it an ideal and greener source of energy [1]. Several processes such as water electrolysis, coal gasification, water thermal decomposition, natural gas reforming via steam process and microbial hydrogen production have been utilized for hydrogen production [2]. However, hydrogen production via the biological route has proved attractive due to the activities of fermentative microbes that are effective in hydrogen production with economical cost based on the prolonged periods of the microbial cells used [3]. Biomass valorization by microbes is an environmentally friendly process with promising sustainable biohydrogen production. Lignocellulosic waste biomass provides an abundant substrate source for continual biohydrogen production.

Agricultural wastes and residues as well as energy crops are the major sources of lignocellulosic materials that find application in green energy creation [4]. Rice-bran (RB) is an example of lignocellulosic material, which has great potential in hydrogen fuel production. RB consists of complex molecules such as hemicellulose, cellulose, and starch which are pivotal in biohydrogen production. RB contains naturally occurring components such as Ca^{2+}, Zn^{2+}, and Mg^{2+} that make it fit to be used as a substrate in hydrogen fuel production. *Clostridia* species are one of the most prominently used groups of bacteria for optimal biofuel production using the fermentation process [5]. *Clostridia* species belong to the obligate, gram-positive, and spore-forming groups of anaerobic bacteria. Their acidogenic nature makes them suitable for this purpose. *C. saccharoperbutylacetonicum N1-4* belongs to the *Clostridia* species that can feed on the organic molecule to produce ABE (Acetone, Butanol and Ethanol) products [6]. Previous works have reported the use of *C. saccharoperbutylacetonicum* in the production of biohydrogen from the fermentation of glucose [7].

Biohydrogen can be produced in three different ways: biophotolysis, photofermentation and dark fermentation [8, 9]. Of these distinct technologies, dark fermentation is very attractive as it could proceed in the absence of light for biohydrogen production. Dark fermentation may be carried out in different ways including batch, continuous, repeated-batch, and fed-batch operations [10]. Out of all these operations, the batch fermentation system has been most commonly studied [7, 8, 11]. In a batch fermentation system, the microorganism is grown in a fixed volume of the culture medium.

Repeated batch fermentation is the fermentative process in which a known quantity of the fermentation broth is withdrawn at time intervals, and the residual part of the broth is used as an inoculum for the next batch. The volume of the broth remains constant with time since the volume of the broth that is removed is replaced with an equal volume of the fresh medium. With the introduction of the fresh medium, the acidic accumulation in the old batch which is supposed to trigger the metabolic shift to solvent production is prolonged as the broth becomes diluted to allow for metabolic activities of the microorganism which is in favor of hydrogen production. This mode of reaction has been utilized to enhance the yields of many industrial–based bio-products such as citric acid [12], hydrogen [13], L-lactic acid [14, 15] and ethanol [16, 17]. The important factors affecting the production efficiency in the repeated-batch process are cell concentration, fermentation time and recycling volume [16, 17].

Repeated batch fermentation has some attributes that distinguish it from batch and other system operations. A repeated-batch operation involves the periodic withdrawal of a portion of the culture broth from the bioreactor at some stage of the fermentation and its replacement with an equal volume of fresh medium. The residual broth from the previous batch becomes the inoculum for the next batch. The unproductive downtime associated with the preparation of inoculum and washing of the bioreactor, in preparation for a new batch is minimized in a repeated-batch operation and additional time is available for the microbial action responsible for the production of fermentation products. A repeated-batch operation enables substantial savings of labor and time compared to a batch operation [18]. The consideration of repeated-batch fermentation process is a way of enhancing the yield of fermentative hydrogen production.
Switching time is a factor in the fermentation time. It is the actual time when the batch process is switched to the repeated-batch process in the first cycle of the repeated batch, and for the subsequent cycles. Specifically, the switching time is the period when the fermentation process ceases for a particular cycle and the broth is drained and replaced with a fresh medium. In this instance, the broth becomes more diluted - less inhibitory- and this allows the continuation of metabolic activities of the microorganism to further favor the production of hydrogen. This cycle will continue for as long as a fresh medium is added to the system. Switching time importance lies in the fact that the output of a cycle depends on when the cycle is terminated. It is to be noted that terminating a cycle when the cells are actively engaged in the production of hydrogen will reduce the productivity and yield of a particular cycle, thus the switching time is determined such as to allow maximum yield and productivity to be obtained from the cycle. This occurs mostly in the stationary phase of the process i.e., when the environmental condition within the broth no longer favors hydrogen production, this is largely caused by the accumulation of acid and since cells must ensure their continued survival, the tendency is for their activity to change to a metabolic pathway that will ensure their continued existence, thus there is a metabolic shift towards solvent production [16]. In this study, a repeated batch fermentation process was used to enhance biohydrogen production from rice bran, in addition, the best switching time that gave the highest biohydrogen yield and productivity was established. To the best of our knowledge, this is the first study to document the production of biohydrogen using *C. saccharoperbutylacetonicum N1-4* and rice-bran as a substrate under a repeated-batch fermentation process.

MATERIAL AND METHODOLOGY

Material Preparation

The materials used in this study were prepared according to methods described and reported in our earlier studies [4, 5].

Repeated-batch Fermentation

Two sets of experiments were carried out. The first set of repeated-batch experiments was carried out to determine the best switching time while the second set of experiments was carried out to determine the stability and consistency of the process. A fresh fermentation

process began as a batch process and it was later switched to a repeated-batch mode of operation. For the initial batch mode, a batch bioreactor culture experiment was carried out in a 250 mL Duran bottle. *C. saccharoperbutylacetonicum N1-4* and the fermentation media were prepared according to the description in Dada *et al*., (2013) [5] and the former was used as the inoculum. The anaerobic condition was created and maintained in the fermentation medium inside the batch bioreactor by passing nitrogen gas into the bioreactor through the inlet gate [5]. The initial substrate concentration was 100 $g \cdot L^{-1}$, while the cell concentration was 1.5 $g \cdot L^{-1}$ as earlier determined [5, 19]. The initial medium *p*H was 6 ± 0.2. The *p*H of the fermentation medium was not controlled during fermentation. Fermentation temperature was maintained at 30 ºC throughout the process using a Digital Precise Shaking Water-bath. The gas produced was collected in an inverted cylinder over acidified distilled water (*p*H ≤ 2) [5].

Different switching times of 45 h, 60 h and 75 h referred to as 'run A', 'run B' and 'run C' respectively, in the first set of experiments, were used to determine the best switching times. These three switching times were considered based on the observation from earlier work in this study that *C. saccharoperbutylacetonicum N1-4* attains an exponential growth stage between the 12^{th} to 18^{th} h of inoculation and remains very active for upward of 36 h, from the earlier reports in this study, it was observed that *C. saccharoperbutylacetonicum N1-4* reached the peak of hydrogen production at about 48h after which its activities wear out. It is thus intended to investigate the most active period in which the introduction of fresh medium will increase the productivity of the microorganism and thus increase the overall yield of hydrogen.

At the instance of the switch, 90 % of the volume of the fermentation broth was withdrawn, leaving 10% behind in the reactor, and the removed broth was replaced with an equal volume of a fresh medium. The implication of this is that the remaining 10 % in the reactor was used as the inoculum for the fresh (repeated) batch. Recall that the initial broth (batch fermentation mode) was inoculated with 10 % of the working volume, thus an equal volume of 10 % was used again as inoculum size in the broth. The volume exchange took place about 10 min before the start of the subsequent Cycle, this was to allow the component to homogenize completely and acclimatize to the environment before the process. After the initial batch process, one repeated Cycle was made for the three 'runs' to determine the best switching time and the 'runs' were terminated after 45 h, 60 h and 75 h, respectively. In the second set of experiments to determine the stability of the process, the same procedure as described above was followed, but a switching time of 60 h was used, and 4 repeated Cycles were made in addition to the initial batch process.

Repeated-batch Performance Evaluation

Performance of the repeated-batch mode was evaluated for the hydrogen yield on the substrate; $Y_{P/S}$, measured in mol $H_2 \cdot mol^{-1}$ glucose consumed, the substrate-specific hydrogen production rate; SHPR, measured in mL $H_2 \cdot g^{-1} \cdot h^{-1}$ glucose consumed)', the volume-specific hydrogen production rate; QP, measured in mL $H_2 \cdot L^{-1} \cdot h^{-1}$ medium', the volume-specific biomass production rate; QX, measured in g (dry cell weight, DCW)· $L^{-1} \cdot h^{-1}$ medium', the biomass yield on the substrate ($Y_{X/S}$), measured in g $DCW \cdot g^{-1}$ glucose consumed and the substrate conversion efficiency (CE) measured in %. Sen *et al*. (2008) [20] defined the specific hydrogen production rate (SHPR) as the ratio of the

amount of hydrogen produced to the product of the mass of substrate used and the time duration of production. Mathematically, it is expressed as:

$$SHPR = \frac{\Delta C_p}{\Delta C_t \times t} \quad (1)$$

Other performance criteria are evaluated using the following equations as defined by Sen *et al*. (2008):

$$Y_{X/S} = \frac{\Delta C_X}{\Delta C_s} \quad (2)$$

$$QP = \frac{\Delta CP}{\Delta t} \quad (3)$$

$$QX = \frac{\Delta CX}{\Delta t} \quad (4)$$

ΔCP is the cumulative H_2 production by time t, measured in mL $H_2 \cdot L^{-1}$ medium; ΔCx is the change in biomass concentration in time t, measured in DCW $g \cdot L^{-1}$ of the medium; ΔCS is the change in substrate concentration in time t, measured in $g \cdot L^{-1}$ medium. Flow rates of the gas mixture from the bioreactor and of the hydrogen from the absorber were calculated by dividing the measured volume by the time interval over which it had been collected [20]. Another particularly useful parameter for evaluating the performance of microbial hydrogen production is substrate conversion efficiency; CE, is the ratio of the actual amount of hydrogen evolved to the amount expected through stoichiometric conversion of the substrate [21]. It is expressed as:

$$CE = \frac{100 \times \eta_{actual}}{\eta_{theoretical} \times V \times M_o} \quad (5)$$

where η_{actual} is moles of hydrogen produced (in moles), $\eta_{theoretical}$ is the moles of hydrogen expected to be produced per mole of glucose utilized, V is the culture volume in L and Mo is the Molar concentration of the carbon source [21] (in this study glucose-simple sugar from rice-bran).

RESULTS AND DISCUSSION

Effect of switching time on cell growth, hydrogen yield and productivity

The result of the effects of the switching time on cell growth is shown in the experiments designated as A, B and C, the exchange of broth occurred at 45 h, 60 h and 75 h respectively, (Figure 1) from the commencement of the initial batch mode. The initial glucose concentration for the two Cycles, in the three cases (A, B and C), was equal since the same hydrolysates were used. The final biomass concentration for the two Cycles in each run was very similar, almost equal (Figure 1). It can also be seen that cell growth was still in the exponential stage at the termination of both Cycles in run A (at 45 h), thus the production of hydrogen was constrained in this run. This is confirmed by the continuous growth of the cell (beyond the 45^{th} h) in runs B and C. The growth at 45^{th} h in Cycle 1, (at the termination of run A) for Run B was 1.74 g DCW$\cdot L^{-1}$ medium (which was almost the same value as in run A), this growth continued in the exponential phase

for run B until 60th h (Figure 1) when the cell started manifesting signs of stationary growth.

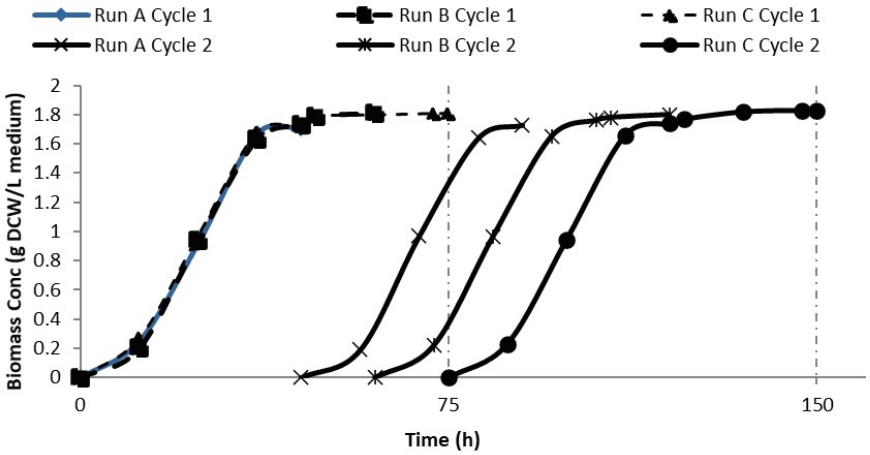

Figure 1. *Time profile of biomass concentration for cycles 1&2 of runs A, B and C*

It was considered that the best time to introduce fresh feed into the fermentation broth, to accomplish the enhancement of hydrogen production, was during the exponential phase just before the deceleration phase. In doing this, acidogenesis; which favors hydrogen production will be prolonged to increase hydrogen production while solventogenesis will be delayed. In addition, the introduction of fresh feed during acidogenesis will further dilute the broth and prevent the broth from attaining an acidic nature that may slow down cell growth. This is in line with the well-established procedure used by previous researchers that have utilized repeated batch processes [22]. Switching the mode of reaction at the 60th h resulted in the harvest of the probable maximum amount of hydrogen obtainable from the run since the process was still in the exponential phase and just about going into the stationary phase where production of hydrogen greatly reduced majorly due to accumulation of acids that will eventually cause a metabolic shift to solventogenesis. In the third run, (run C), where a switching time of 75th h was considered, although, the growth pattern followed that of run B, the stationary growth which was characterized by insignificant changes in the size of cell growth was very prominent after 60th h, cell growth and thus hydrogen production after this mark was barely significant as more accumulation of acid is believed to have favored the shift to solvent production, this observation was like switching time patterns reported in earlier investigations [23 – 25]. The maximum biomass concentration for all the runs was in the range of 1.73 - 1.83 g DCW·L^{-1} of the medium.

The pattern of cell growth suggested that switching from batch mode to repeated batch mode at the 60th h led to the production of the highest amount of hydrogen. The parameters were calculated from the start of their respective Cycles 1 to 90 h for run A, 120 h for run B and 150 h for run C (2 Cycles each of 45 h-run A, 60 h-run B and 75 h-run C respectively) (Table 1). The cumulative hydrogen produced in the two Cycles in the respective run was identical; however, the production of H_2 for the respective second Cycles was slightly higher than for Cycle 1. The values of specific hydrogen production rates for runs A and B are almost equal, this suggests that for run A if the Cycle was not terminated, production of hydrogen would still have continued since the production rate

suggests that production was still in the exponential phase. However, this is different from the situation in run C, as, at the termination of the Cycle at the 75th h, production was already decreasing; this suggests that production has entered the stationary phase where further production is neither significant nor noticeable. The values of the hydrogen yield on the substrate for the three switching times show that run B was the best with the highest values. The same pattern was noticed in terms of conversion efficiency and biomass yield on the substrate. These terms had earlier been defined. The implication of this is that run B is better than the two other runs and in conclusion, the switching time of 60 h seems to be the best of the three.

Table 1. *Effect of Switching Time Interval on Biohydrogen Production in Repeated batch Process*

Runs	**A**		**B**		**C**	
Switching Time [h]	45 (Cycle 1)	45 (Cycle 2)	60 (Cycle 1)	60 (Cycle 2)	75 (Cycle 1)	75 (Cycle 2)
ΔCs [g·L^{-1} medium]	24.31	24.31	24.87	24.87	28.22	28.22
Total Gas Produced [mL·L^{-1} medium]	1110	1120	1550	1556	1620	1635
ΔCp [mL H_2·L^{-1} medium]	916.86	925.12	1280.3	1285.26	1338.12	1350.51
mole of H_2 [mol]	0.0368	0.0372	0.0514	0.0516	0.0538	0.0543
moles of glucose used [mol]	0.0134	0.0134	0.0138	0.0138	0.0156	0.0156
$Y_{P/S}$ [mol H_2·mol^{-1} glucose consumed]	2.73	2.76	3.73	3.74	3.43	3.46
ΔCx [g DCW·L^{-1} medium]	1.71	1.73	1.81	1.8	1.83	1.83
$Y_{X/S}$ [g DCW·g^{-1} glucose consumed]	0.070	0.071	0.073	0.072	0.065	0.065
SHPR [mL H_2·g^{-1}·h^{-1} glucose consumed]	0.84	0.84	0.86	0.86	0.63	0.63
CE [%]	68.3	68.9	93.2	93.6	85.9	86.6
Standard Deviation of CE	0.9539	0.9643	0.9000	0.9165	0.9165	0.7549

Repeated-batch process stability

The switch time was 60 h from the initiation of the batch reaction till the reactor is drained, and a fresh medium was introduced at about 10 minutes to the end of each Cycle, for the experiments carried out under the repeated batch mode in this study. This is to allow for acclimatization of the organism to the system before the fermentation duration time commenced. For the fresh medium, 90 % of the broth was drained and replaced with 90 percent of fresh medium. The time profile of the volume-specific hydrogen production rate is shown in Figure 2. In the initial batch process, which is the first Cycle of the repeated-batch mode, 7 % of the total hydrogen was produced in the first 12 h i.e., during the acidogenesis phase (explained earlier-section 3.1). This might have been a result of the effect of the start-up and the lag-phase of the microorganism exhibited in the first 6 h, during which the bacteria are more interested in its growth rather than the production of metabolites, thus hydrogen production was extremely low (53 mL). This is agreed with an earlier report [26]. However, after 18 h, a slight increment was noticed in the production rate and this became obvious by the 24th h when 30 % of the total hydrogen had been produced.

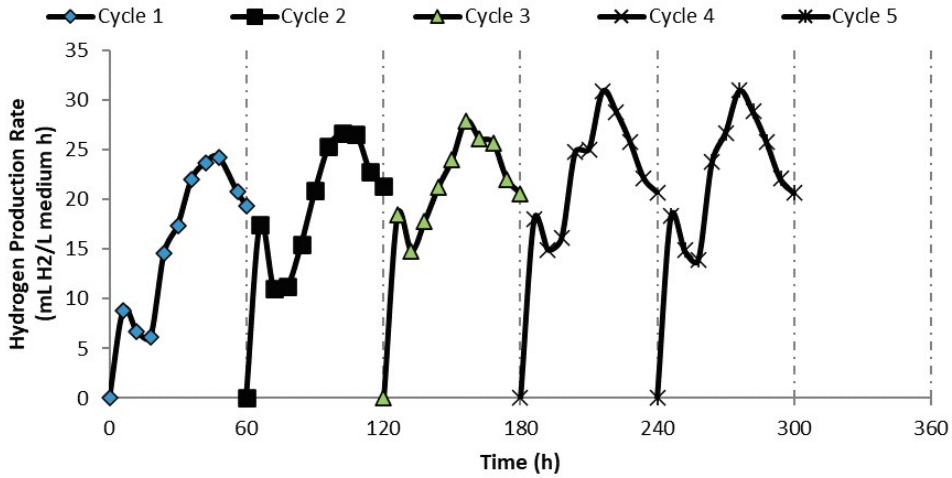

Figure 2. *Volume-specific hydrogen production rate* (Q_p)

This indicates the transition to the exponential phase, and it is evidenced in the increased rate of hydrogen production. There was a steady increment in the production rate (exponential phase) till the 48th h (24.21 mL $H_2 \cdot L^{-1}$ medium h) after which a decline in both production rate and cumulative hydrogen was observed. During the exponential phase (between the 18th to 48th h), almost 91 % of the total hydrogen production was recorded. The highest cumulative hydrogen volume recorded in this Cycle was 1164 mL $H_2 \cdot L^{-1}$ of medium (Figure 2). In addition, within the first 18 h, the flow rate was not uniform, but shortly after this, the flow rate stabilized and rose (exponential phase) until the 48th h when the system entered the stationary phase. The hydrogen production rate can be influenced by factors such as the nature of the organism, the presence of inhibitors and the non-conducive environment.

An acidic environment may trigger off inactivity on the part of the microorganism such that it can go into sporulation and remain unproductive. In a normal fermentation system, there are two distinct stages: acidogenic and solventogenesis. However, in the cell growth profile, during the exponential phase, i.e., when the cell is undergoing exponential growth, the products formed are majorly acids (acetate and butyrate). As the acids accumulate, cell growth ceases and some of the acids in the hydrolysate are converted to solvents (acetone, butanol, and ethanol), this is the second major stage in ABE fermentation; solventogenesis. In the former stage, actively growing cells have been established to be rod-like in shape while they are more fattened and cigar-shaped in the latter stage [11, 27], during this stage, they were observed as accumulating granulose and eventually the cells sporulate [28].

Sporulation is a process that microorganisms (especially bacteria) go through with the result that they produce an endospore. Endospores are dormant bodies that are formed by some types of bacteria in response to hostile conditions. The purpose of sporulation is to enable bacteria to survive through difficult living conditions until the unfriendly conditions change and become more hospitable to the bacteria. A similar observation had earlier been reported in an earlier study [29]. The presence of inhibitors such as furan and its derivatives, 5-hydroxymethylfurfural, weak acids and to a less extent, phenolics [30, 31] may change the metabolic pathway of the microorganism and thus cease the production of hydrogen which is the product in focus. In Cycle 2, the same trend was

observed for production rate and cumulative Hydrogen volume. However, there were some minor variations. The lag phase did not last for as long as it was in the first Cycle and the hydrogen production rate doubled the corresponding rate in Cycle 1. This implied that the system has overcome the start-up inertia and has thus attained a more stabilized state. For instance, after 6 h of production, almost double the amount of cumulative hydrogen produced in Cycle 1 has been produced in Cycle 2 (Figure 3) indicating that the transition to the exponential phase was faster than in Cycle 1 and this enabled production of more hydrogen.

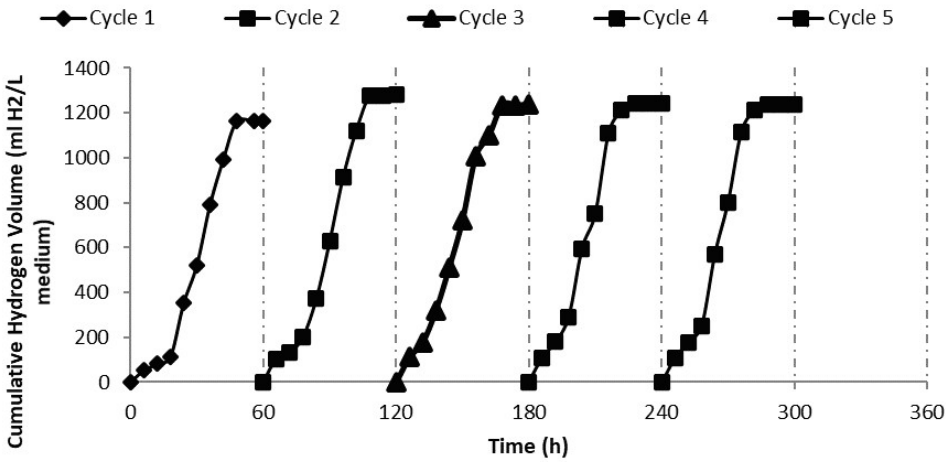

***Figure 3**. Cumulative hydrogen volume of five cycles*

However, the highest production rate (26.68 mL $H_2 \cdot L^{-1}$ medium h) was observed at the 42nd h. The highest cumulative hydrogen volume recorded for this Cycle was 1280.3 mL $H_2 \cdot L^{-1}$ medium. Cycles 3-5 followed the same pattern as Cycle 2 except that the lag phase became smaller, the flow rate increased tremendously, and this reduced greatly the duration of their respective exponential phases. The highest production rates for the three Cycles were recorded at the 36th h. Although, the production of hydrogen gas continued but the production rate reduced greatly and this set the tone for the early transition to the stationary phase and the subsequent metabolic shift to solventogenesis. The extremely high production rate might have led to the early depletion of the carbon source. In addition, the high rate of metabolism might have led to the production of intermediate products which catalyzed the metabolic shift that ended the production of hydrogen. The highest cumulative hydrogen volume for Cycles 3-5 were 1236.52 mL $H_2 \cdot L^{-1}$ medium, 1242.3 mL $H_2 \cdot L^{-1}$ medium, and 1239.8 mL $H_2 \cdot L^{-1}$ medium, respectively.

Effect of biomass concentration on sugar consumption and *p*H

The microorganism grew perfectly well in the medium during Cycle 1, within the first 12 h, an increase of 0.35 g DCW·L^{-1} medium was noticed in the biomass concentration, at 48th h, when hydrogen gas was at its maximum production, and the biomass concentration reached its peak; 1.97 g DCW·L^{-1} medium, the highest in all the Cycles and after this time, a decline was noticed in the pattern of the growth until the end of the Cycle. At the initiation of the process, there was a sudden decrease in the *p*H value of the broth as

evidenced by a shift in the *p*H value initially from 6.0 ± 0.2 to 5.0 ± 0.33 within the first 24 h (Figure 4). During this stage (believed to be the acidogenic phase), there was tremendous production of hydrogen gas and acids.

The metabolic activities of the microorganisms reached a maximum level and continued until the stationary phase whereas as a result of the accumulation of acids, the environment became non-conducive for the microorganisms, and since the microorganism had to survive, a change in the metabolic pathway of the microorganism was triggered. This resulted in a *p*H shift from 5.0 ± 0.33 to 5.0 ± 0.51) and eventually to solventogenesis. From Figure 4, it is observed that for all the Cycles, the lowest *p*H value (acidic condition) corresponded with the highest biomass concentration for all Cycles (Figure 4). This implies that the microorganism grew well in the broth until the environment could not sustain its continued survival, there was a decline in the biomass concentration after this value and this triggered the solventogenesis that increased the *p*H values to make the medium habitable for the microorganism. They agreed with earlier observations [21, 32]. The optimum *p*H range for hydrogen production is between the range of 5 - 6 [33].

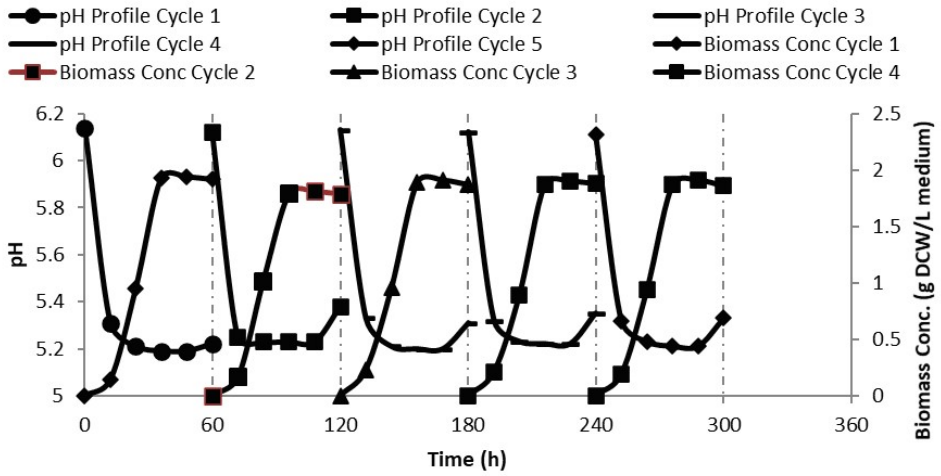

Figure 4. *pH profile and biomass concentration*

The results of sugar consumption and acid production are shown in Table 2. Sugar consumption was uniform in the 5 Cycles. There were few variations in the amount of final cumulative hydrogen produced. This difference might have occurred because of the metabolic activities of the microorganism. Careful observation and comparison of the data in Table 2 and the *p*H profile (Figure 4) show that Cycle 1 has the lowest *p*H value at the end of the fermentation process. *p*H is a function of the acidity of its medium, the highest concentration of the total acid at the end of Cycles was recorded for Cycle 1 and Cycle 1 has the least amount of cumulative hydrogen production. It is suspected that excessive production of acid product inhibited the formation of hydrogen by switching to the solventogenesis phase and that was why Cycle 1 with the highest concentration of accumulated acid characterized by the lowest *p*H value (*p*H 5.22 at 60 h - Figure 4) produced the least amount of cumulative hydrogen.

Table 2. *Sugar Utility and Acid Production*

CYCLE	Initial Sugar [g·L^{-1}]	Final Sugar [g·L^{-1}]	Sugar Consumed [g·L^{-1}]	Acetic Acid [g·L^{-1}]	Butyric Acid [g·L^{-1}]	Total Acid [g·L^{-1}]	Total Hydrogen produced [mL·L^{-1} medium]
1	27.59	2.65	24.85	2.86	0.84	3.7	1164.66±2.22
2	30.89	6.02	24.87	0.84	0.16	1.00	1280.3±2.86
3	24.26	0.082	24.178	2.56	0.94	3.5	1236.52±2.36
4	32.5	8.35	24.15	0.84	0.6	1.44	1242.30±1.69
5	28.59	4.28	24.31	1.83	0.8	2.63	1239.83±2.28

Whereas the opposite is the case for Cycle 2. With the least accumulation of acid product characterized by the highest *p*H value at the end of the hydrogen production phase (*p*H 5.38 at 60 h-Figure 4), Cycle 2 has the highest amount of cumulative hydrogen production (1280.3 mL·L^{-1} medium) (Table 2). This occurrence agreed with previous observations from Akao [14] and Jones [27]. Generally, it can be seen that the *p*H value at the end of a particular cycle is a function of the acid concentration at that instant in the Cycle (Figure 4) and this greatly influences the amount of cumulative hydrogen that would be obtained from such a Cycle. Similar trends were observed for Cycles 3-5. In addition, at the termination of a Cycle, just before the replacement of the old broth with a new one, the *p*H of the medium was always lower than the *p*H at the beginning of the Cycle (Figure 4). The introduction of a new broth brings about a dilution action in the old broth thus increasing the *p*H and making the medium environment more conducive to increased metabolic activities of the organisms [34, 35]. At the termination of all Cycles, the *p*H was between 5.22 - 5.38. After the fresh medium had been added, the *p*H rose to 6.11 - 6.13 (Figure 4). Therefore, throughout the various runs, the *p*H was closer to the generally recommended initial *p*H of 7 for anaerobic fermentation as suggested by various studies. The highest biomass concentration (1.97 g DCW·L^{-1}) was recorded at 48th h in Cycle 1. Other Cycles also had their highest biomass concentration at the equivalent of 48 h. The maximum biomass concentration for all Cycles was in the range of 1.81 - 1.94 g DCW·L^{-1}. The profiles of the biomass concentration for all the Cycles were similar showing the stability and consistency of the process.

Kinetic Parameters of the System

The repeated-batch fermentation experiments were carried out for five consecutive Cycles to evaluate the stability of the repeated-batch process for biohydrogen production. The first Cycle started as a batch mode operation and was switched to a repeated-batch process after 60 h. At the commencement of Cycles 2-5, 90 % of the volume of the broth was drained and replaced with a fresh medium of equal volume. The key kinetic parameters for Cycles 1-5 are presented in Table 2. The hydrogen gas yield on the substrate ($Y_{P/S}$) was evaluated using Equation 2 and the procedure described in section 2.2. The values of $Y_{P/S}$ were in the range of 3.70 - 3.73 mol H_2·mol^{-1} substrate consumed. The $Y_{P/S}$ values are higher than 3 probably due to the assumptions that in this work, the sugars consumed during the fermentation process were monosaccharides obtained after the pretreatment and hydrolysis of the complex sugars in the lignocellulosic RB structure, and thus the molecular weight of the sugar consumed was assumed to be that of glucose [36].

It was also assumed that the composition of the total biogas produced during the fermentation process was hydrogen and carbon dioxide only. Any oxygen detected is assumed to be from the atmosphere, while the nitrogen is suspected to be from the nitrogen gas used to sparge the system to make it to be anaerobic. The values of $Y_{P/S}$ were calculated based on these assumptions. The conversion efficiency (CE) and other kinetic parameters were evaluated using the equations defined in section 2.2 In addition, it has been reported that a repeated batch process enables microorganisms to become conditioned or adapted to the fermentation process through recycling and this increased the process yield [37]. Their cumulative hydrogen volume ranged between 1236.52 to 1280.3 mL $H_2 \cdot L^{-1}$ (Table 3). The *p*H profiles for all the Cycles were similar (Figure 4) and at the termination of a Cycle, the *p*H always increased a bit above the value in the stationary phase this indicated that a metabolic shift to solventogenesis had taken place. Cycles 2-5 were very similar in every manner.

The amount of sugar consumed was also similar for all the Cycles, this is further proof of consistency [24]. The values of $Y_{P/S}$ were in the range of 3.70 - 3.73 mol $H_2 \cdot mol^{-1}$ substrate consumed while the values of $Y_{X/S}$ were in the range of 0.072 - 0.078 g DCW$\cdot g^{-1}$ substrate consumed. Thus, the Cycles were highly consistent and the repeated batch production of biohydrogen could be considered very stable. The hydrogen production efficiency of the various Cycles ranged from 84.9 to 93.3 %. This was higher than what had been previously reported [38], although, Tanisho [39] had earlier reported that a conversion efficiency of greater than 10 % is considered economically viable for conventional fuels [38].

Many investigations have been carried out on the production of hydrogen by a dark-fermentation batch process, as observed earlier in this study, but information on the production of hydrogen using Clostridium species from lignocellulosic materials is limited [24]. In this work, the production of biohydrogen via a repeated batch process was investigated. The result demonstrated that a system operated in the repeated mode could obtain higher efficiency for hydrogen production. When RB was used as the sole carbon source, significant microbial growth and hydrogen production, (Figures 4 and 5) were observed, indicating that the concentration of RB was the factor limiting the growth rate of bacteria and hydrogen production. Excessive feeding of RB concentration may cause substrate inhibition on hydrogen production. This might be attributed to the phenomena of carbon repression encountered during the complex anaerobic metabolism [33]. Therefore, initial substrate concentration plays an important role in hydrogen production. The *p*H was observed to have increased slightly (Figure 4), after the termination of a Cycle and on the introduction of fresh broth, hence hydrogen production ability was increased, and this is in contrast to a batch process where there was no introduction of a fresh batch. This indicated that *p*H is another factor that can be manipulated in the production of hydrogen of higher yield as supported by previous researchers [40]. It is apparent that the change in *p*H significantly affected the cumulative hydrogen volume and biomass (Figure 4).

This result further supported the observation earlier reported elsewhere [32, 41]. In a repeated batch hydrogen production process with five Cycles, the conversion efficiency of substrate showed an increasing trend from the first phase of 84.9 to 92.5 % (Table 3). After the lag phase, the biomass increased rapidly and reached a higher level, and RB was also consumed effectively within 60 h (Figure 3), this seems to imply that most of the hydrolysate might mainly contribute to cell growth and then hydrogen yield

increased rapidly. In addition, in the exponential phase, the hydrolysate was more used for hydrogen production rather than for the growth of bacteria cells, thereby hydrogen yield was enhanced. At the decelerating stage, bacterial growth began to decrease due to limited substrate and this was when the fresh broth was introduced leading to the renewed activity of the bacteria hence the enhanced hydrogen production which is the hallmark of this repeated batch process.

This result corroborated what Kim *et al.* (1980) [42] had earlier reported. After 300 h of operation, the cumulative hydrogen production of the five Cycles was 6163.62 mL $H_2 \cdot L^{-1}$ medium. However, the actual time for the production of this cumulative total hydrogen was 260 h because the 10 min used for acclimatization in each Cycle except the last Cycle makes the total time of production to be 260 h. The overall production rate for the repeated batch is 6163.22/260 which is 23.7 $mL \cdot h^{-1}$ compared with the batch process, wherein 60 h, 1164.66 mL was obtained. However, the overall production rate for the batch process is 1164.66/60 which is 19.4 $mL \cdot h^{-1}$. This shows that there is an increment of 22 % in the production rate of hydrogen when the repeated batch was used, and this result justified the use of the repeated batch system as a process that enhances biohydrogen production. Earlier work on a two-stage sequential dark and photofermentation of starch in a repeated-batch operation had reported a hydrogen production rate of 37.5 $mL \cdot L^{-1} \cdot h^{-1}$ in the dark stage and 20.8 $mL \cdot L^{-1} \cdot h^{-1}$ in the photofermentation stage [43]. Yokoi *et al.* (2001) [44] reported a value of ≈ 20 $mL \cdot L^{-1} \cdot h^{-1}$ for a repeated batch photo-fermentation using unknown species of the genus *Rhodobacter*.

Table 3. *Performance of Repeated-batch (5-Cycles) operation for biohydrogen production*

Cycle	**1**	**2**	**3**	**4**	**5**
Time [h]	60	60	60	60	60
ΔCp [mL $H_2 \cdot L^{-1}$ medium]	1164.66	1280.3	1236.52	1242.30	1239.83
ΔCx [g DCW$\cdot L^{-1}$ medium]	1.97	1.81	1.91	1.9	1.91
ΔCs [g$\cdot L^{-1}$ medium]	24.85	24.87	24.18	24.15	24.31
mole of H_2 [mol]	0.047	0.052	0.049	0.049	0.049
moles of Glucose used [mol]	0.0137	0.0138	0.0134	0.0134	0.0134
Yp/s [mol $H_2 \cdot mol^{-1}$ substrate consumed]	3.39	3.73	3.71	3.73	3.7
Yx/s [g DCW$\cdot g^{-1}$ substrate consumed]	0.079	0.072	0.078	0.078	0.078
Qp [mL $H_2 \cdot L^{-1}$ medium$\cdot h^{-1}$]	19.41	21.34	20.61	20.71	20.68
Qx [g DCW$\cdot L^{-1} \cdot h^{-1}$ medium]	0.033	0.031	0.031	0.031	0.031
SHPR [mL $H_2 \cdot g^{-1}$ substrate consumed$\cdot h^{-1}$]	0.78	0.85	0.85	0.85	0.85
CE [%]	84.9±0.61	93.3±0.85	92.6±0.80	93.2±0.78	92.5±0.82

CONCLUSION

The study successfully investigated the effect of switching time on cell growth; hydrogen yield and productivity; repeated-batch process stability; the effect of biomass concentration on sugar consumption and *p*H; as well as kinetics parameters. For enhanced hydrogen production, it was observed that the best time to introduce fresh feed into the fermentation broth was during the exponential phase, just before the deceleration phases. Doing this, it will help dilute the broth and prevent it from attaining an acidic nature that may slow down cell growth. In addition, the hydrogen production rate can be influenced by factors such as the nature of the organism, the presence of inhibitors and a non-conducive environment. An acidic environment may trigger off inactivity on the part of the microorganism such that it can go into sporulation and remain unproductive. In addition, the *p*H value at the end of a particular fermentation Cycle is a function of the acid concentration at that instant in the Cycle and this greatly influences the amount of cumulative hydrogen that would be obtained from such a Cycle. The introduction of a new broth brings about a dilution action in the old broth thus increasing the *p*H and making the medium more conducive for increased metabolic activities of the organisms. Finally, this study, to the authors' best knowledge, serves as one of the pioneering works in the investigation of the repeated–batch fermentation process of rice bran using *C. saccharoperbutylacetonicum N1-4* as inoculum in biohydrogen production.

REFERENCES

1. https://katalog.ub.tu-braunschweig.de/vufind/Search2Record/DOAJ061097802 Hydrogen energy vision 2060: Hydrogen as energy Carrier in Malaysian primary energy mix – Developing P2G case, Accessed on October 12, **2021**;
2. Zhang, B., Zhang, S., Yao, R., Wu, Y., Qiu, R.: Progress and prospects of hydrogen production: Opportunities and challenges, *Journal of Electronic Science and Technology*, **2021**, **19** (2), 97-118;
3. Bhatia, S.K., Jagtap, S.S., Bedekar, A.A., Bhatia, R.K., Rajendran, K., Pugazhendhi, A., Rao, C. V., Atabani, A.E., Kumar, G., Yang, Y.H.: Renewable biohydrogen production from lignocellulosic biomass using fermentation and integration of systems with other energy generation technologies, *Science of the Total Environment*, **2021**, **765** (144429);
4. Dada, E.O., Alade, A., Adeniji, A., Afolabi, T.J.: Effect of Rice-Bran Pretreatment in Biohydrogen Production, *Current Journal of Applied Science and Technology*, **2021**, **40** (16), 12-19;
5. Dada, E.O., Yusoff, W., Kalil, M.: Biohydrogen production from rice-bran using *Clostridium saccharoperbutylacetonicum* N1-4, *International Journal of Hydrogen Energy*, **2013**, **38**, 15063-15073;
6. Liberato, V., Benevenuti, C., Coelho, F., Botelho, A., Amaral, P., Pereira, N., Ferreira, T.: Clostridium sp. as Bio-Catalyst for Fuels and Chemicals Production in a Biorefinery Context, *Catalysts*, **2019**, **9** (11), 962;
7. Alalayah, W., Kalil, M., Kadhum, A., Jahim, J., Alauj, N.: Hydrogen production using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564), *International Journal of Hydrogen Energy*, **2008**, **33** 7392-7396;
8. Levin, D.: Biohydrogen production: prospects and limitations to practical application, *International Journal of Hydrogen Energy*, **2004**, **29**, 173-185;
9. Miyake, J., Matsunaga, T., San Pietro, A.: *Biohydrogen* ii, Pergamon, **2001**, 63-67;
10. Dasgupta, C., Jose Gilbert, J., Lindblad, P., Heidorn, T., Borgvang, S., Skjanes, K., Das, D.: Recent trends on the development of photobiological processes and photobioreactors for the improvement of hydrogen production, *International Journal of Hydrogen Energy*, **2010**, **35**, 10218-10238;

11. Al-Shorgani, N., Kalil, M., Yusoff, W.: Fermentation of sago starch to biobutanol in a batch culture using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564), *Annals of Microbiology*, **2011**, **62**,1059-1070;
12. Anastassiadis, S., Rehm, H.: Citric acid production from glucose by yeast Candida oleophila ATCC 20177 under batch, continuous and repeated batch cultivation, *Electronic Journal of Biotechnology*, **2009**, **9**, 26-39;
13. Vázquez-Larios, A., Poggi-Varaldo, H., Solorza-Feria, O., Rinderknecht-Seijas, N.: Effect of type of inoculum on microbial fuel cell performance that used RuxMoySez as cathodic catalyst, *International Journal of Hydrogen Energy*, **2015**, **40**, 17402-17412;
14. Akao, S., Tsuno, H., Cheon, J.: Semi-continuous l-lactate fermentation of garbage without sterile condition and analysis of the microbial structure, *Water Research*, **2007**, **41**, 1774-1780;
15. Wu, X., Jiang, S., Liu, M., Pan, L., Zheng, Z., Luo, S.: Production of l-lactic acid by Rhizopus oryzae using semicontinuous fermentation in bioreactor, *Journal of Industrial Microbiology & Biotechnology*, **2010**, **38**, 565-571;
16. Choi, G., Kang, H., Moon, S.: Repeated-batch fermentation using flocculent hybrid, Saccharomyces cerevisiae CHFY0321 for efficient production of bioethanol, *Applied Microbiology and Biotechnology*, **2009**, **84**, 261-269;
17. Staniszewski, M., Kujawski, W., Lewandowska, M.: Semi-continuous ethanol production in bioreactor from whey with co-immobilized enzyme and yeast cells followed by pervaporative recovery of product – Kinetic model predictions considering glucose repression, *Journal of Food Engineering*, **2009**, **91**, 240-249;
18. Naritomi, T., Kouda, T., Yano, H., Yoshinaga, F., Shigematsu, T., Morimura, S., Kida. K.: Influence of broth exchange ratio on bacterial cellulose production by repeated-batch culture, *Process Biochemistry*, **2002**, **38**, 41-47;
19. Dada, E.O., Alade, A.O., Babatunde, K.A., Agbede, O.O.,: Effects of pretreatment on biobutanol yields from rice-bran and deoiled rice-bran processed with *Clostridium saccharoperbutylacetonicum N1-4, LAUTECH Journal of Engineering and Technology* (1), **2018**, **12**, 85-96;
20. Melis, A., Melnicki, M.: Integrated biological hydrogen production, *International Journal of Hydrogen Energy*, **2006**, **31**, 1563-1573;
21. Koku, H.: Kinetics of biological hydrogen production by the photosynthetic bacterium Rhodobacter sphaeroides O.U. 001, *International Journal of Hydrogen Energy*, **2003**, **28**, 381-388;
22. Cheng, Y., Edwards, J., Allison, G., Zhu, W., Theodorou, M.: Diversity and activity of enriched ruminal cultures of anaerobic fungi and methanogens grown together on lignocellulose in consecutive batch culture, *Bioresource Technology*, **2009**, **100**, 4821-4828;
23. Melnicki, M., Bianchi, L., Dephilippis, R., Melis, A.: Hydrogen production during stationary phase in purple photosynthetic bacteria, *International Journal of Hydrogen Energy*, **2008**, **33**, 6525-6534;
24. Pattanamanee, W., Choorit, W., Deesan, C., Sirisansaneeyakul, S., Chisti, Y.: Photofermentive production of biohydrogen from oil palm waste hydrolysate, *International Journal of Hydrogen Energy*, **2012**, **37**, 4077-4087;
25. Tao, H., Bingham, C., Strikwerda, A., Pilon, D., Shrekenhamer, D., Landy, N., Fan, K., Zhang, X., Padilla, W., Averitt, R.: Highly flexible wide angle of incidence terahertz metamaterial absorber: Design, fabrication, and characterization, *Physical Review B*, **2008**, **78** (24), 241103;
26. Krishna, R., Mohan, S., Swamy, A.: Evaluation of Kinetic Parameters for Bio Hydrogen Production by Anaerobic Suspended Growth Reactor Using Synthetic Feed and Up-Scaling Anaerobic Suspended Growth Reactor Using Complex Feed, *International Journal of Chemistry*, **2011**, **3** (2);
27. Jones, D., Woods, D.: Acetone-butanol fermentation revisited, *Microbiological Reviews*, **1986**, **50**, 484-524;
28. Schuster, K., Urlaub, E., Gapes, J.: Single-cell analysis of bacteria by Raman microscopy: spectral information on the chemical composition of cells and on the heterogeneity in a culture, *Journal of Microbiological Methods*, **2000**, **42**, 29-38;
29. Kumar, N., Das, D.: Enhancement of hydrogen production by Enterobacter cloacae IIT-BT 08, *Process Biochemistry*, **2000**, **35**, 589-593;
30. Olsson, L., Hahn-Hägerdal, B.: Fermentation of lignocellulosic hydrolysates for ethanol production, *Enzyme and Microbial Technology*, **1996**, **18**, 312-331;

31. Mussatto, S.: Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review, *Bioresource Technology*, **2004**, **93**, 1-10;
32. Al-Shorgani, N., Kalil, M., Yusoff, W., Hamid, A.: Impact of pH and butyric acid on butanol production during batch fermentation using a new local isolate of Clostridium acetobutylicum YM1, *Saudi Journal of Biological Sciences*, **2018**, **25**, 339-348;
33. Venkata, M., Vijaya, B., Sarma, P.: Biohydrogen production from chemical wastewater treatment in biofilm configured reactor operated in periodic discontinuous batch mode by selectively enriched anaerobic mixed consortia, *Water Research*, **2007**, **41**, 2652-2664;
34. Yoshida, A., Nishimura, T., Kawaguchi, H., Inui, M., Yukawa, H.: Enhanced Hydrogen Production from Formic Acid by Formate Hydrogen Lyase-Overexpressing Escherichia coli Strains, *Applied and Environmental Microbiology*, **2005**, **71**, 6762-6768;
35. Hwang, J., Choi, J.A., Abou-Shanab, R., Bhatnagar, A., Min, B., Song, H., Kumar, E., Choi, J., Lee, E.S., Kim, Y., Um, S., Lee, D., Jeon, B.: Effect of pH and sulfate concentration on hydrogen production using anaerobic mixed microflora, *International Journal of Hydrogen Energy*, **2009**, **34** (24), 9702-9710;
36. deVrije, T., Bakker, R., Budde, M., Lai, M., Mars, A., Claassen, P.: Efficient hydrogen production from the lignocellulosic energy crop Miscanthus by the extreme thermophilic bacteria Caldicellulosiruptor saccharolyticus and Thermotoga neapolitana, *Biotechnology for Biofuels*, **2009**, **2** 12;
37. Slininger, P., Thompson, S., Weber, S., Liu, Z., Moon, J.: Repression of xylose-specific enzymes by ethanol in Scheffersomyces (Pichia) stipitis and utility of repitching xylose-grown populations to eliminate diauxic lag, *Biotechnology and Bioengineering*, **2011**, **108**, 1801-1815;
38. Liu, B., Ren, N., Xing, D., Ding, J., Zheng, G., Guo, W., Xu, J., Xie, G.: Hydrogen production by immobilized R. faecalis RLD-53 using soluble metabolites from ethanol fermentation bacteria E. *harbinense* B49, *Bioresource Technology*, **2009**, **100** (10), 2719-2723;
39. Eker, S., Erkul, B.: Biohydrogen production by extracted fermentation from sugar beet, *International Journal of Hydrogen Energy*, **2018**, **43**, 10645-10654;
40. Ooshima, H., Takakuwa, S., Katsuda, T., Okuda, M., Shirasawa, T., Azuma, M., Kato, J.: Production of hydrogen by a hydrogenase-deficient mutant of *Rhodobacter capsulatus*, *Journal of Fermentation and Bioengineering*, **1998**, **85** (5), 470-475;
41. Liu, B., Li, Z.: A review: Hydrogen generation from borohydride hydrolysis reaction, *Journal of Power Sources*, **2009**, **187**, 527-534;
42. Kim, K., Fuchs, J., Woodward, C.: Hydrogen exchange identifies native-state motional domains important in protein folding, *Biochemistry*, **1993**, **32**, 9600-9608;
43. Laurinavichene, T., Belokopytov, B., Laurinavichius, K., Khusnutdinova, A., Seibert, M., Tsygankov, A.: Towards the integration of dark- and photo-fermentative waste treatment. 4. Repeated batch sequential dark- and photofermentation using starch as substrate, *International Journal of Hydrogen Energy*, **2012**, **37**, 8800-8810;
44. Yokoi, H., Saitsu, A., Uchida, H., Hirose, J., Hayashi, S., Takasaki, Y.: Microbial hydrogen production from sweet potato starch residue, *Journal of Bioscience and Bioengineering*, **2001**, **91**, 58-63.