HYCELTEC 2022
VIII Symposium on Hydrogen, Fuel Cells and Advanced Batteries
Buenos Aires, July 11-14, 2022

8th Symposium on Hydrogen, Fuel Cells and Advanced Batteries, Buenos Aires, July 11th-14th, 2022

Design of experiments to optimize hydrogen production through dark fermentation of sugars (P-17H)

V. Martinez[*1**], A. Alfonso[1], R. García[2], M. Lavorante[1], M. Galvagno[4], M. Cassanello[3]

[1] *División Energías Renovables, Instituto de Investigaciones Científicas y Técnicas para la Defensa (CITEDEF), S.J.B. de La Salle 4793, B1603ALO, Vicente López, Buenos Aires, Argentina*

[2] *DIIV, ARA, Argentina*

[3] *LARSI e ITAPROQ, Dpto. Industrias, FCEyN, Universidad de Buenos Aires-CONICET, Ciudad Universitaria, C.A.B.A., Argentina*

[4] *Instituto de Micología y Botánica (INMIBO), CONICET—Universidad de Buenos Aires, Pabellon 2, Ciudad Universitaria, C.A.B.A., Argentina*

(*) Pres. author: vmartinez@citedef.gob.ar (**) Corresp. author: vmartinez@citedef.gob.ar

Keywords: Biohydrogen, Dark Fermentation, Bioprocess, Design Of Experiments

1. Introduction

Humanity is facing a very urgent problem in the present time that can be regarded as the most important in history[1]. Our future way of life, and maybe even survival, depends on its outcome. The problem is climate change and its consequences. The burst of the human population in the second half of the XX century was accompanied by a growth in human activities and an increase in the demands of food, energy, and physical space. This brought increased amounts of gas emissions to the atmosphere, pollutants in water courses and oceans, and reduction of natural environments to make place for crops and cities[2]. The nature of present-day economy has reached a limit as to the amount of harm that the Earth can absorbe and neutralize. This linear economy in which everything is exploited, produced, used, and discarded is exerting an irreversible damage to the ecosystem, directly affecting our lives. It is also causing an increase in the Earth's mean temperature with consequences that are difficult to predict, since it is something that has never happened before[3]. We are realizing that our lives need to become more sustainable, in tune with the planet's capacity of recycling elements and residues. Our economy needs to become circular, where products and materials can be reused and recycled, or absorbed by the ecosystem if thrown away[4].

Fossil fuels are seen in the XXI century as a source of contamination rather than progress, as it was in the XX century. Efforts are being made worldwide to lower its extensive use as the main source of energy and progressively replace them with renewable energy sources. Solar and wind energy have seen, in the past 10 years, a very important expansion, with many parks and farms built in most coutries[5].

In this sense, hydrogen (H_2) is seen as the fuel of the future, due to its high energy yield and lack of contaminating emissions. The downside of H_2 is its difficulty in production and storage. Hydrogen technologies are also witnessing an increased interest, with much research getting done on means of production, new materials, and safety[6].

In line with the hydrogen circular economy, this work is focuses on the production of H_2 using natural bacterial communities and renewable resources. The bioprocess by which H_2 producing bacteria work is called dark fermentation and it can be carried out in rather simple fermentation tanks. This process has the potential of working with direct carbon sources such as glucose ($C_6H_{12}O_6$) or sucrose ($C_{12}H_{22}O_{11}$), but also degrading second generation biomass, such as lignocellulosic litter from crops or animal residues from livestock, among others. Hydrogen producing bacteria can be collected from several natural sources, and selected to seed the tank. The conditions inside the tank must be optimized to promote bacterial growth, since H_2 is a byproduct of their metabolism, along with carbon dioxide (CO_2), and is simply released into the tank's headspace[7]. Dark fermentation is a stage in the natural degradation of organic material, which ends up in methane (CH_4) and CO_2, which are greenhouse gases. However, if the residues are pretreated before the dark fermentation, the only gases produced are H_2 and CO_2. The total amount of gas is less than the one obtained if CH_4 is produced. The rest of the carbon (that would either end up in more CO_2 or in CH_4) are part of short chain acids in the effluent, mostly acetic and butiryc acids.

This work centers on the optimizacion of conditions required to maximize H_2 production in a stirred tank, by means of design of experiments (DOE) methodology.

2. Experimental

2.1 H_2 Evolution Process

For the H_2 production process, a water sample was taken from a water treatment plant installed inside a ship that belongs to the Argentine Navy, the ARA Almirante Irízar. It was stored at room temperature until needed.

Firstly, a modified plate count agar medium[8] was prepared (PCA), replacing casein with meat peptone, and without the agar in order to keep it liquid. This modified PCA was nicknamed "growth medium". It was sterilized. Secondly, the bacterial consortium was pre-treated for 50 minutes at 75ºC, and centrifuged for a spin (1 minute, 2800 rpm). Thirdly, the growth medium was inoculated with 1 mL of consortium each 10 mL of modified PCA. The flasks were purged with nitrogen (N_2) to achieve anaerobic headspace and stored at 37ºC over the weekend (approximatly 65 hours).

After this long incubation, Kitasatos flasks were prepared in the following manner: 200 mL as a final volume was added to each flask containing the final concentration of micronutrients, ammonia, sucrose and bacterial biomass (inoculum) determined by the Design of Experiments chart (Table 1). In

addition, sodium acetate (CH_3COONa) was added to all the flasks to a final concentration of 0.5 M. The micronutrients solution corresponded to a modified Logan medium studied in García et al.[9]

2.2 Design of Experiments layout

The Excell macro "Essential Experimental Design", Version 2.216, by R.P. Yeater, D.D. Steppan and J. Werner was used to design an optimization Box-Behnken strategy with four (4) factors. The factors to optimize were micronutrient concentration (x), varying from 20 to 200 mg/L, amonia concentration (y), in the form of ammonium bicarbonate (NH_4HCO_3), varying from 0.5 to 4 g/L, sucrose (z), in the range of 5 to 25 g/L, and a quantitative biomass reference value (h) calculated as an optical density magnitude per each 100 mL (see next section for protocol). The design resulted in 28 conditions, 4 of which were centerpoints (Table 1).

Exp #	Micronut. mg/L	NH_4 g/L	Suc. g/L	Biom. i DO/100 mL	H_2 tot mL
1	110	2.25	15	12.5	467.8
2	20	0.5	15	12.5	0
3	20	2.25	15	20	3.75
4	20	4	15	12.5	1
5	110	2.25	5	20	73.8
6	200	2.25	5	12.5	63.75
7	110	0.5	5	12.5	184.6
8	110	2.25	25	20	700
9	200	0.5	15	12.5	195
10	110	4	25	12.5	25.2
11	110	2.25	5	5	0
12	110	4	5	12.5	15
13	20	2.25	15	5	6
14	110	4	15	20	312.9
15	110	0.5	15	20	199.38
16	20	2.25	25	12.5	195.6
17	110	0.5	25	12.5	274.8
18	200	2.25	25	12.5	912.2
19	20	2.25	5	12.5	0
20	110	2.25	25	5	260
21	200	4	15	12.5	415.5
22	110	2.25	15	12.5	498.1
23	200	2.25	15	20	547
24	110	2.25	15	12.5	590.8
25	110	4	15	5	8
26	110	2.25	15	12.5	484
27	110	0.5	15	5	381
28	200	2.25	15	5	455.2

Table 1. Four factor, four centerpoint Box-Behnken design.

The centerpoints are the checkpoints of the design and validate the experiments, since they are the only 4 same reactors with all 4 factors at their center value (110 mg/L of micronutrients solution, 2.25 g/L of ammonia, 15 g/L of sucrose and 12.5 DO/100 mL).

2.3 Stock solutions and biomass estimation

A concentrated micronutrients solution was prepared to use as stock for all experiments. Contents were monopotassium phosphate (KH_2PO_4), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$), calcium chloride dehydrate ($CaCl_2.2H_2O$), manganese sulfate heptahydrate ($MnSO_4.7H_2O$) and ferrous sulfate heptahydrate ($FeSO_4.7H_2O$)[2]. An allicuot of this solution was added to each reactor according to Table 1 defined final concentration. The same was done for ammonium with a concentrated solution of ammonium bicarbonate. Solid sucrose was added directly in the reactor according to Table 1.

As for biomass, as mentioned in section 2.1, the natural bacterial consortium was obtained from the treatment plant of an army ship, which makes this source rather "dirty". Impurities were discarded by an initial centrifuge spin, performed after temperature selection, this supernatant was cultured in a modified liquid anaerobic PCA medium, and after 72 hours a sample was taken and read at 600 nm in a spectrophotometer. Growth medium with no inoculum was taken as blank. If required, dilutions were made until absorbance fits the readable scale. When the amount of biomass was enough to perform experiments, all bacterial mass in the growth medium was concentrated by centrifuging 15 minutes at 5000 rpm. Then, it was resuspended with distilled water and immediately used. The optical density (DO) was determined again after cells were resuspended in the water, and the allicuots with the DOE value of initial biomass were introduced as seed in the reactors. This procedure was followed to ensure that all the reactors seeding allicuots came from the same mother solution.

3. Results

3.1 Significant Factors and Interactions

The volume of H_2 produced for each condition has been included in Table 1. The ANOVA analysis of this data is listed in Table 2.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	14	1450187	103585	5.18	0.003
Linear	4	877995	219499	10.97	0.000
x	1	472944	472944	23.63	0.000
y	1	17416	17416	0.87	0.368
z	1	343637	343637	17.17	0.001
h	1	43999	43999	2.20	0.162
Square	4	357085	89271	4.46	0.017
x*x	1	91670	91670	4.58	0.052
y*y	1	294206	294206	14.7	0.002
z*z	1	109130	109130	5.45	0.036
h*h	1	65657	65657	3.28	0.093
2-Way Interaction	6	215106	35851	1.79	0.178
x*y	1	12047	12047	0.60	0.452
x*z	1	106545	106545	5.32	0.038
x*h	1	2211	2211	0.11	0.745
y*z	1	1600	1600	0.08	0.782
y*h	1	59177	59177	2.96	0.109
z*h	1	33526	33526	1.68	0.218
Error	13	260184	20014		
Lack-of-Fit	10	251052	25105	8.25	0.055
Pure Error	3	9131	3044		
Total	27	1710370			

Table 2. ANOVA

P value of less than 0.05 indicates a significant factor or interaction. In this analysis the significant linear factors are micronutrients and sucrose concentration. These two (2) factors along with ammonium concentration are significant in quadratic form, and there is a significant positive interaction of micronutrients with sucrose (Table 2).

The complete regression equation to calculate the volume of H_2 produced is expressed by:

$$H_{2\,tot}(mL) = -299 + 1.62x + 167y + 24.7z + 11.6h - 0.01526x^2 - 72.3y^2 - 1.349z^2 - 1.86h^2 + 0.348x * y + 0.1813x * z + 0.035x * h - 1.14y * z + 9.27y * h + 1.221z * h$$

Where the R^2 value is 84.79%.

3.2 *Optimized parameters*

The plots for the optimized parameters, obtained from the model by maximizing the amount of H_2 produced, can be seen in Figure 1.

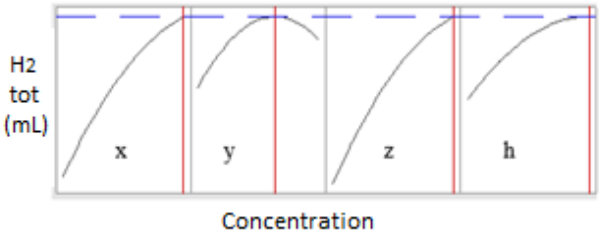

Fig. 1. Optimized concentrations according to the model: x= 200 mg/L, y= 2.73 g/L, z= 25 g/L, h= 20 DO/100 mL. Maximum amount of H_2 predicted= 869.5 mL

Optimal concentrations of micronutrients, sucrose and biomass are the maximum values within the range explored, indicating no inhibition of these factors. In contrast, the optimal ammonium concentration is 2.73 g/L, larger values lead to a negative effect on hydrogen generation.

4. Discussion

4.1 *Hydrogen production process*

The process for biohydrogen production in a fermentation tank is complex and comprises many variables. It is desirable to be able to predict the outcome of a certain setup, and to know the optimal concentrations in order to predict the best setup.

Four factors were chosen, being important for this process: micronutrients, ammonium ion and sucrose concentration, and also the amount of initial bacterial biomass necessary to obtain the most efficient growth. With the design of experiments protocol presented here, it is possible to obtain a mathematical model that approximates the outcome very well, with a fit of over 84%.

Design of experiments also enables obtaining the most optimal concentration of the selected factors to achieve the best desired result. For this process, the best result is the greatest volume of H_2.

5. Conclusions

Following a DOE protocol, an equation for biological hydrogen production using an anaerobic local bacterial consortium was determined. This model predicts hydrogen production with an 84% fit. It was used to estimate the optimal concentration of micronutrients, ammonium ion and sucrose in the production medium, and the amount of biomass necessary to seed a reactor.

6. Acknowledgements

The authors would like to thank the Scientific and Technical Institute for Defense (CITEDEF) and the Ministry of Defense for the funding and physical laboratories where this research was conducted.

References

[1] D. Introcaso, Climate Change Is The Greatest Threat To Human Health In History, *Health Affairs Forefront*, (2018). DOI: 10.1377/hblog20181218.278288

[2] J. Van Bavel, The world population explosion: causes, backgrounds and projections for the future, *Facts Views Vis Obgyn*, 5:4 (2013) 281–291.

[3] UN News, IPCC report: `Code red' for human driven global heating, warns UN chief, *Climate and Environment*, (2021).

[4] S. Jørgensen, L.J.T. Pedersen, The Circular Rather than the Linear Economy. *RESTART Sustainable Business Model Innovation*. Palgrave Studies in Sustainable Business In Association with Future Earth. Palgrave Macmillan, Cham. (2018).

[5] H. Ritchie, How have the world's energy sources changed over the last two centuries?, *Our World in Data*, (2021).

[6] US Department of Energy, Hydrogen Strategy: Enabling A Low-Carbon Economy, (2020).

[7] V.L. Martinez, R. Garcia, G. Curutchet, A. Sanguinetti, H. Fasoli, J.I. Franco, Demonstration of the possibility to power a fuel cell with hydrogen derived from the fermentation of sugar, *International Journal of Hydrogen Energy*, 37(2012) 14920-14925.

[8] Cambridge: Royal Society of Chemistry, Chapter P2. Plate Count Agar (PCA), *Handbook of Culture Media for Food and Water Microbiology*, (2011) 870–872, retrieved 2021-12-10.

[9] R.E. García, V.L. Martínez, J.I. Franco, G. Curutchet, Selection of natural bacterial communities for the biological production of hydrogen, *International Journal of Hydrogen Energy*, Volume 37, 13 (2012) 10095-10100.