# Optimizing Parameters for Bio-Hydrogen Production from Mixed Culture and Food Wastewater

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**ABSTRACT:** *In this research, Rice Boiling Wastewater (RBW) has been used as inexpensive food wastewater for bio-hydrogen production by dark fermentation. The effective parameters such as temperature, pH, time reaction, and substrate concentration have been optimized. The $2^{k-1}$ factorial design method and response surface methodology were used to optimize the best reaction conditions. According to the results, under optimal conditions, pH (6.240), temperature (35.090°C), sugar concentration (5.400 g/l), and fermentation time (36 h), the highest hydrogen production in the proposed theoretical model was obtained equal to 3.30 mol $H_2$/mol glucose. The proposed theoretical model demonstrated a good agreement with the obtained experimental data.*

**KEYWORDS***: Bio-hydrogen; Optimal conditions; sludge; Rice boiling wastewater; Dark fermentation.*

## INTRODUCTION

In recent decades, environmental pollution has increased because of discharging industrial wastewater and releasing pollutants into the atmosphere from various sources[1]. One of the challenges facing humankind in today's era is to achieve a new source of renewable energy that can be an appropriate alternative to fossil fuel [2]. The massive usage of fossil fuels has led to many adverse effects such as global warming, acid rains, increased greenhouse gases, etc [3]. Renewable energies are environmentally friendly with low greenhouse gas emissions. Among renewable energies, biomass as an important energy source has attracted much interest [4]. One of the most popular methods of using biomass energy is the production of bioethanol and biogas containing hydrogen [5]. Hydrogen is recognized as a greenhouse gas alternative[6]. This is because burning hydrogen does not

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emit gasses like CO, SOx, NOx, and CO2. Hydrogen is a carbon-free fuel and the final by-product of its combustion is water [7]. However, hydrogen is not readily available in nature like fossil fuels and it requires chemical or biological processes to produce it [8]. There are different methods of biological hydrogen production; the most popular ones are bio-photolysis of water by algae, photo-fermentation, dark fermentation of organic materials, and also continuous dark and photo-fermentation operations. The various renewable resources can be converted into hydrogen by Microorganisms[9]. Research has proved that there are significant advantages to producing hydrogen with biological methods[10]. It sustainably creates clean H2 by using simple technological devices, so it looks like a more attractive alternative in terms of cost and sophistication than the current chemical production of $H_2$[11]. Additionally, carbon in the process of hydrogen production can be originated from different feedstock such as waste materials which are mostly low-cost or even free [12]. Photo and dark fermentation are two methods that are mostly used and are more favorable by scientists[13]. Photo-fermentation only accomplishes in the presence of light[14]. Hydrogen production by purple non-sulfur bacteria was due to the presence of nitrogenase under oxygen-deficient conditions which are using reduced compounds (organic acids) and light energy[15].

Photosynthetic bacteria undergo anoxygenic photosynthesis with organic compounds or reduced sulfur compounds as electron donors[16]. Some non-sulfur photosynthetic bacteria are powerful hydrogen producers, employing organic acids for instance, lactic, succinic, and butyric acids or alcohols as electron givers[17]. In the dark fermentation method, carbohydrates directly convert to $H_2$, $CO_2$, and organic acids like butyrate and acetate, without external energy[18]. Also, this method is significantly under the influence of other factors such as substrate, metallic ions, reducing agents, temperature, environmental conditions of microbe culture, etc[10]. Studies show the environment's pH directly affects the activities of micro-organisms, and it affects the activity rate of the enzymes with a metabolic process. Moreover, temperature[19], as well, influences the physiological activities of micro-organisms and their fermentation production rate.[20] The Dark-Fermentation of hydrogen takes place in different temperature ranges. Substrate concentration as a carbon source is also influential in hydrogen production[21]. In some studies, the rate of hydrogen production increased with increasing the concentration of the substrate, and in some other cases, it was the opposite[22]. One of the biggest challenges of fermenting hydrogen is the cost-effectiveness of its supplies. Using inexpensive and renewable materials such as lignocelluloses (corn, wheat starch, sugarcane, sugar beets) or biomass like agricultural residues as well as industrial waste and wastewater for production, can reduce these costs[15]. Accordingly, in recent decades, the use of industrial waste, as well as carbon-based sources has been studied for the production of biological hydrogen[23]. If the carbon-based sources, which are used as the substrate have been achieved using food waste the cost of hydrogen production decreases dramatically[24]. One of the food wastewaters that is daily discharged into the sewage is “Rice Boiling Wastewater” (RBW). This wastewater can be used as a cheap and proper alternative for the production of hydrogen gas due to its high organic matter. In this study, the $2^{k-1}$ Factorial Design Method and Response Surface Methodology were used for choosing the best reaction temperature, test duration, substrate concentration, and pH to produce the maximum amount of bio-hydrogen using municipal sewage and RBW.

## EXPERIMENTAL SECTION

### *sample Preparation*

The samples for the current study were gathered and prepared from the activated sludge of the municipal wastewater. The active waste sludge has been included 2.03% TS , 1.58% VS , 3334 mg/L TC , 0.599 µmol/L ATP , 12735 mg/L TCOD , and 1723 mg/l SCOD . To dwindle the quantity of the hydrogen-consuming microorganisms, each of the samples was openly encountered with a thermal shock of 70 °C for a length of 60 minutes31. All sets of the experimentation were conducted in a 500 ml glass bottle. In addition, 300 ml of active sludge was also applied in each of the tests(Genus of bacteria identified in activated sludge are Clostridium, Methanogens, Neisseria, Bacillus, and Staphylococcus). In the meantime, Mineral salt was added to all the samples for enhancing the proliferation and activity of bacteria. Their concentrations were $CoCl_2.6H_2O$ 0.004, $NaHCO_3$ 0.8, $K_2HPO_4$ 0.1, $CaSO_4.5H_2O$ 0.01, $MgCl_2.6H_2O$ 0.017, $MnSO_4.7H_2O$ 0.03, $NiSO_4$ 0.02, and finally $FeSO_4.7H_2O$ 0.02 (g/L). The pH of the fermentation medium was set and adapted

***Table 1: Coded and non-coded degrees and scope of variables schemed by $2^{k-1}$ factorial method***

| Level | Coded level | Non-coded level | | | |
|---|---|---|---|---|---|
| | | Temperature (C° ) | pH (-) | Time (hr ) | Substrate ( g/l glucose) |
| High | +1 | 25 | 6 | 12 | 5.4 |
| Centre | 0 | 35 | 7 | 24 | 7.6 |
| Low | -1 | 45 | 8 | 36 | 10 |
| Coded formula: $\frac{x-\frac{x(high)+x(low)}{2}}{\frac{x(high)-x(low)}{2}}$ , x: -ω …, -3, -2, -1, 0, 1, 2, 3, +ω | | | | | |

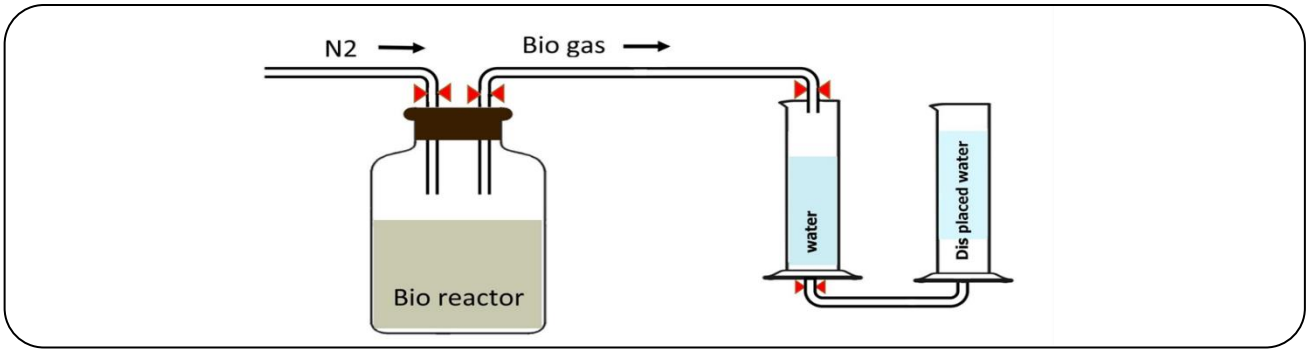


***Fig. 1: Schematic illustration of the designed bio-reactor.***

using 2N hydrochloric acid and 1N sodium hydroxide. To prepare the substrate, 200 g of rice was mixed with 1 L of water, and then the mixture was heated at 100 °C for 30 minutes. After half an hour, the rice was separated from the water by a strainer, and the rice juice was finally used as the substrate.

### *Setup of fermentation*

Since all of the experiments are performed in a glass bottle, after each preparation of the samples, the glass bottle was closed using a lid in which two pipes were installed and sealed. One tube was used to anaerobic the fermentation medium and the other was used to transfer the produced gas to the calibrated cylinder. To reach anaerobic atmospheres and conditions, nitrogen gas was injected into the medium for 5 min. The biogas created throughout the fermentation process was collected through the water displacement method, and then it was analyzed (Fig. 1). The fermentation container was situated within the incubator at the designated temperatures. At different intervals, both the volume and the pressure of the generated gas in conjunction with the hydrogen content were all measured inside the biogas mixture.

### *Test design*

To lower the number of experiments and also the systematic review of the process to reach optimal efficiency, the factorial $2^{k-1}$ design is utilized to inspect all the elements influencing the generation of the bio-hydrogen. Based on the previous studies, temperature (B), pH (A), time (D), and substrate concentration (sugar per glucose) (C) were conceived as the effectual determinants at three levels (Table 1). By the design matrix, 18 experiments were chosen and extracted at random to examine the production of the bio-hydrogen. The results of the tests were achieved after considering hydrogen percentage inside the gas mixture and concentration of substrate sugar (mole glucose/mole $H_2$).

### *Analysis methods*

*Sugar measurement*

To decompose the sugars, a high-performance ion-exchange chromatography device (HPAWC-PAD, manufactured in the U.S.A.) was employed. The materials and contents, with flow rate (1 ml/min) were transferred from a 250-1 CARB column with the dimensions (4.6 mm x 250 mm) and temperature (30°C). Right upon depicting

a calibration curve, the sugar concentration of the samples was determined per glucose[25].

*Biogas measurement*

The water displacement mechanism was utilized to calculate the volume of the Biogas. The pressure of the generated gas was also estimated by measuring the difference in water level in the two cylinders. By considering the biogas mixture as an ideal gas, the mole number of biogas was determined.

*Hydrogen measurement*

In the meantime, Hydrogen concentration was calculated using a gas chromatograph (Varian CM 3500), which was supplied with a Thermal Conductivity Detector (TCD) stainless steel column (2m×3mm), packed with molecular sieve 5A, using $N_2$ as the carrier gas. In parallel, the operational temperatures of the column, the injector, and the detector were maintained at 50 °C, 80 °C, and 90°C, respectively [26].

## RESULTS AND DISCUSSIONS

### *Influence of effective parameters on the level of bio-hydrogen production*

Based on the equations of dark-fermentation bio-hydrogen production if the acetic acid forms as a volatile fatty acid, the maximum amount of 4 moles of $H_2$ can be produced per 1 mole of glucose. But in practice, because a part of the glucose substance is used for growth and maintenance, the rate of production will decrease. If butyric acid forms in the dark-fermentation method 2 moles of $H_2$, per each glucose mole, has been used for growth and maintenance therefore theoretically 2.5 moles of $H_2$ have been produced.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (1)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2 \quad (2)$$

So, to analyze, the result of the mol $H_2$/mol glucose ratio measuring has been shown in table 2. We can see that the different pH levels influence the productivity rate of bacteria and the metabolism of bio-hydrogen fermentation production, and an increase at its levels will decrease the ability of the micro-organisms to produce $H_2$. Therefore a pH = 6 will be the optimal option. Due to the effect of temperature on the growth rate of micro-organisms involved in bio-hydrogen production, by changing the temperature from 25℃ to 45℃ the rate of production will increase. On the other hand, because the hydrogen-producing micro-organisms can live longer than the methane-producing micro-organisms, by increasing the time of the test to 36 hours, the production rate of $H_2$ has been more than any other test duration. Moreover, due to organic load inhibition, the best production rate has been shown in a substrate concentration of 5.4 and the concentration increasing of the carbon substrate will not change production efficiency.

### *Dispersion experiments*

The dispersion of experiments based on the results of the $2^{k-1}$ factorial method has been shown in Fig. 2. As it can be seen for all forms of these plots, such as Normal Probability Plot, Versus Fits, Histogram and Versus order both positive and negative levels are equal (9 experiments done twice), consequently, it can be ensured that the experiments are done randomly and that different levels of the experiment are normally distributed.

### *Analysis of variance*

The analysis of variance has been used to examine experimental parameters on $H_2$ production. According to the results shown in table 3, experimental parameters A (temperature), B (pH), C (time), and D (Substrate) have a significant impact on the productivity rate due to P-value has been equal to zero (value α has been set to 0.05 in the software by default). Also, the results of the analysis of variance show that interactions between parameters AB, AC, BC, and BD have a great influence on the rate of bio-hydrogen productivity.

Fig. 3 shows the Pareto Plot and the schematics of parameter effects. The Pareto Plot confirms that these parameters and their interactions have a great influence on the results so the analysis of variance is working properly. In fact, the effects of the experimental parameters A (temperature), B (pH), C (time), and D (Substrate) are slightly different from each other, which is due to different amounts of ***F*** value for each parameter (please see Table 3).

### *Parameters Optimization:*

Based on the regression equation, $2^{k-1}$ factorial design for producing bio-hydrogen from active sludge the result

***Table 2: Results of the production rate of bio-hydrogen using a $2^{k-1}$ factorial method affected by experimental parameters.***

| Sample | Std order | Center Pt | A (C° ) | B (-) | C ( hr) | D (g/l) | REP | PSD (mol $H_2$/mol glucose) |
|---|---|---|---|---|---|---|---|---|
| A | 1 | 1 | -1 | -1 | -1 | -1 | 1 | 0.9284 |
| | | | | | | | 2 | 0.9127 |
| B | 7 | 1 | -1 | 1 | 1 | -1 | 1 | 0.5665 |
| | | | | | | | 2 | 0.5891 |
| C | 9 | 1 | 1 | 1 | -1 | -1 | 1 | 0.8135 |
| | | | | | | | 2 | 0.8437 |
| D | 5 | 1 | -1 | -1 | 1 | 1 | 1 | 0.9564 |
| | | | | | | | 2 | 0.9711 |
| E | 4 | 1 | -1 | 1 | -1 | 1 | 1 | 0.333 |
| | | | | | | | 2 | 0.3425 |
| F | 3 | 1 | 0 | 0 | 0 | 0 | 1 | 1.0884 |
| | | | | | | | 2 | 1.0995 |
| G | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 0.607 |
| | | | | | | | 2 | 0.6193 |
| H | 6 | 1 | 1 | -1 | 1 | -1 | 1 | 2.297 |
| | | | | | | | 2 | 2.3353 |
| I | 8 | 1 | 1 | -1 | -1 | 1 | 1 | 1.1003 |
| | | | | | | | 2 | 1.1089 |

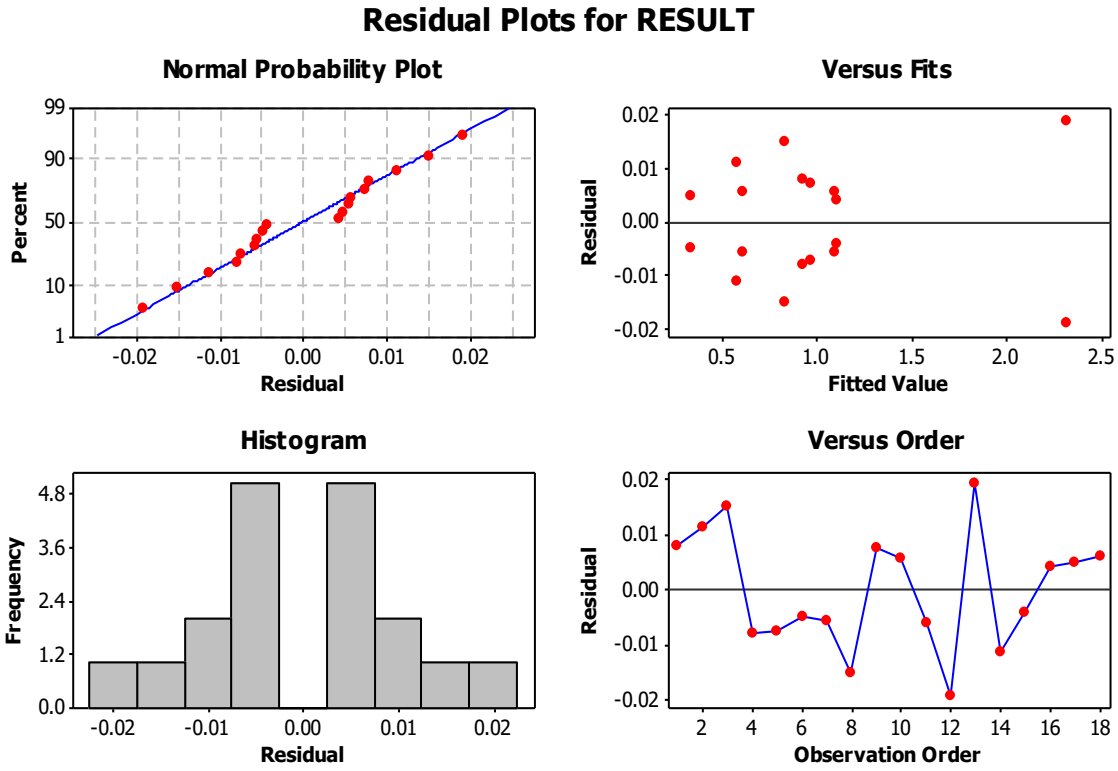


***Fig. 2: Probability distribution diagrams for the effective experimental parameters in the production of hydrogen.***

***Table 3: The ANOVA results of the effective parameters in the production of bio-hydrogen.***

| ANOVA for RESULT (coded units) | | | | | | |
|---|---|---|---|---|---|---|
| Source | DF | Seq SS | Adj SS | Adj MS | Fvalue | Pvalue |
| Main Effects | 4 | 4.30402 | 1.07600 | 1.07600 | 5130.19 | 0.000 |
| A | 1 | 1.06396 | 1.06396 | 1.06396 | 5072.77 | 0.000 |
| B | 1 | 2.17182 | 2.17182 | 2.17182 | 10354.82 | 0.000 |
| C | 1 | 0.40936 | 0.40936 | 0.40936 | 1951.76 | 0.000 |
| D | 1 | 0.65888 | 0.65888 | 0.65888 | 3141.41 | 0.000 |
| A*B | 1 | 0.25499 | 0.25499 | 0.25499 | 1215.74 | 0.000 |
| A*C | 1 | 0.12717 | 0.12717 | 0.12717 | 606.30 | 0.000 |
| A*D | 1 | 0.3365 | 0.33654 | 0.33654 | 1165.19 | 0.000 |
| B*C | 1 | 0.27879 | 0.27879 | 0.27879 | 871.67 | 0.000 |
| B*D | 1 | 0.12457 | 0.12457 | 0.12457 | 915.32 | 0.000 |
| C*D | 1 | 0.13478 | 0.13478 | 0.13478 | 1021.70 | 0.000 |

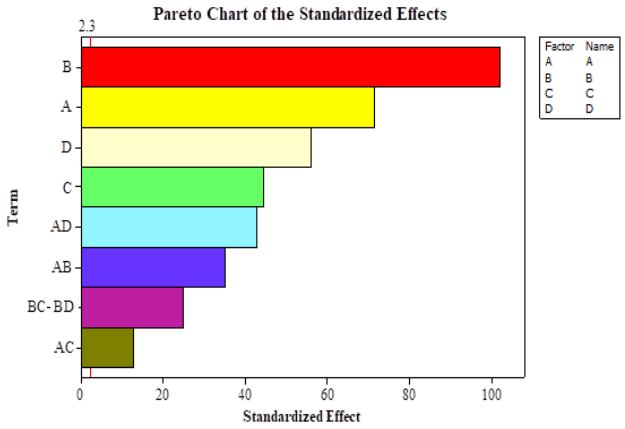


***Fig. 3: Analysis of variance and Pareto diagram of effective parameters in the production of bio-hydrogen.***

has been 0.973 + 0.258 A - 0.368 B + 0.160 C - 0.203 D - 0.126 AB + 0.0892 AC- 0.154 AD- 0.154 BC - 0.126 BD. According to this and by this formula, we can theoretically estimate the bio-hydrogen production in different substrate concentration, pH, temperature ,and time.

Fig. 4 shows a 3D diagram of relations between these experimental parameters and bio-hydrogen production based on a $2^{k-1}$ factorial method. By choosing a variable for any parameter the amount of $H_2$ production can be estimated. The interesting factor is that the results from this theoretical equation correspond to the experimental results in Table 2.

***Response Surface Methodology (RSM) and contour diagram***

Because this study aimed to produce bio-hydrogen with the highest concentration, we used RSM. Based on the output of the computer software, decoded results of this methodology have been demonstrated in Fig. 5 and Table 4. According to Table 4, the optimal result of 3.3 mol $H_2$/mol glucose has been obtained. To examine the compliance of the results of RMS, experiments have been conducted which show that the experimental results match with RMS results by a 7.83% error, and 3.06 mol $H_2$/ mol glucose was obtained. This amount of hydrogen production compared to non-optimal conditions that was around 0.29 mol $H_2$/mol glucose is significant. According to studies, the highest efficiency of biohydrogen production in pure culture medium and use of glucose substrate is 2.28 (mol $H_2$/mole glucose)[27], also the sweet potato bagasse in mixed-culture is 2.4 (mol $H_2$/mole glucose)[28], and food industry waste in mixed-culture is 2 (mol $H_2$/mole glucose) [29]. In other studies, and laboratory conditions, Lutpi et al. produced the highest amount of 2.8 mol $H_2$ per mole hexose with a pH of 5.5[30], and *Kim et al.* achieved 2.1 mol $H_2$/mole hexose using sewage sludge and food waste [31]. As can be seen in the laboratory condition and using different substrates, the values obtained in this study are significant, which is

***Table 4: The results of optimizing parameters affecting the hydrogen production based on software output.***

| Response | Goal | Lower | Target | Experimental parameters | | | | Desirability | Predict response value |
|---|---|---|---|---|---|---|---|---|---|
| | | | | A ($C^o$ ) | B | C (hr ) | D ( g/l glucose) | | |
| SSA(nm) | maximize | 2.5 | 3.3 | 35.09 | 6.24 | 36 | 5.4 | 1 | 3.32 |

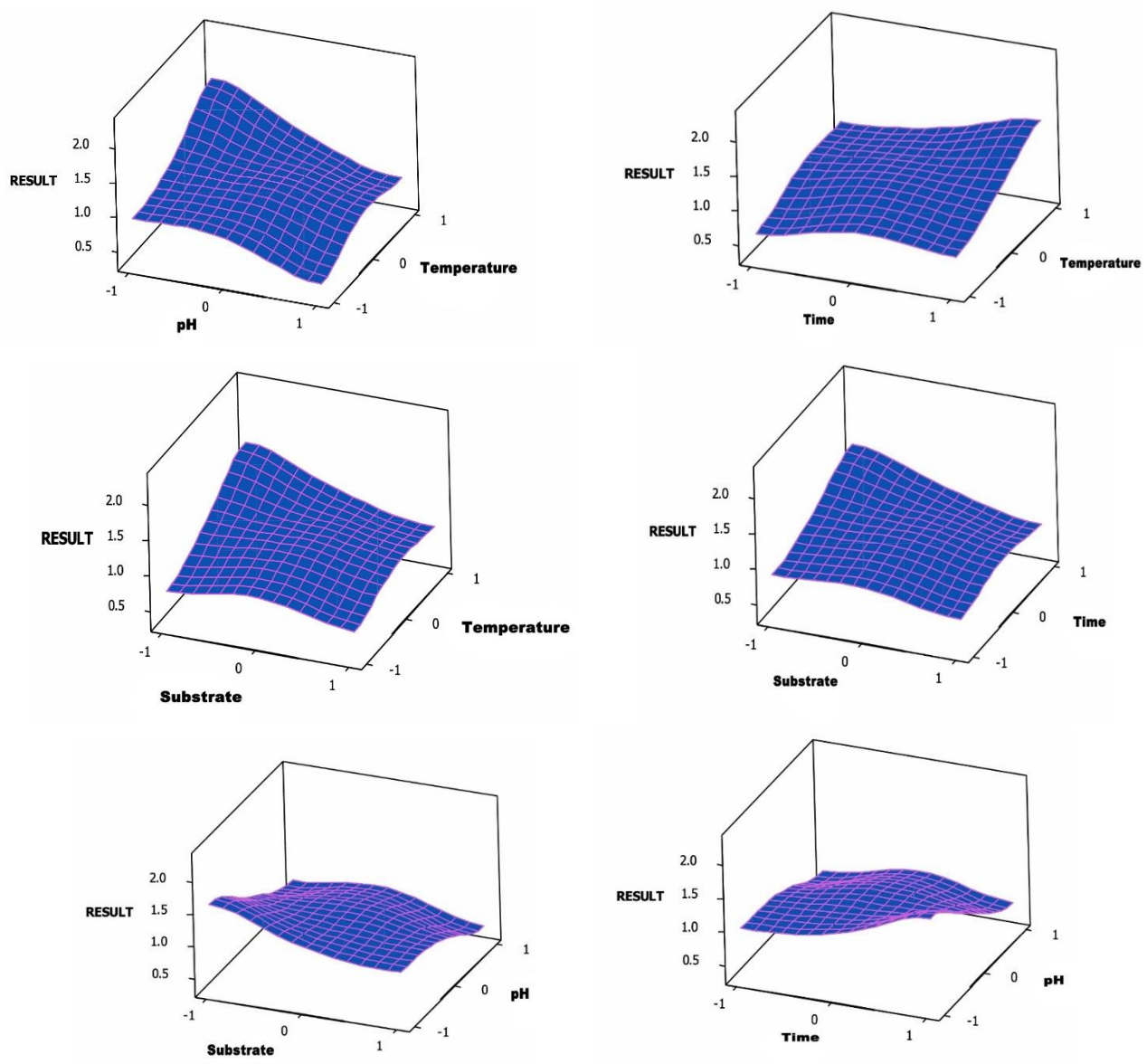


***Fig. 4: Effects of temperature (A), pH (B), time (C) and substrate (D) parameters on bio-hydrogen production (mol$H_2$/mol glucose).***

probably due to differences in studies. The slight differences can be because of systematic studies, experiment design, and the process of optimizations. Also, the contour diagram showed the behavior of pH (A), temperature (B), substrate (C), and time (D) on hydrogen production (Fig. 6). According to the results, these behaviors are agreed to the RSM optimization with a desirability of 1.000. As an important result, RSMoptimizations and contour diagrams provide the possibility of products with high performance.

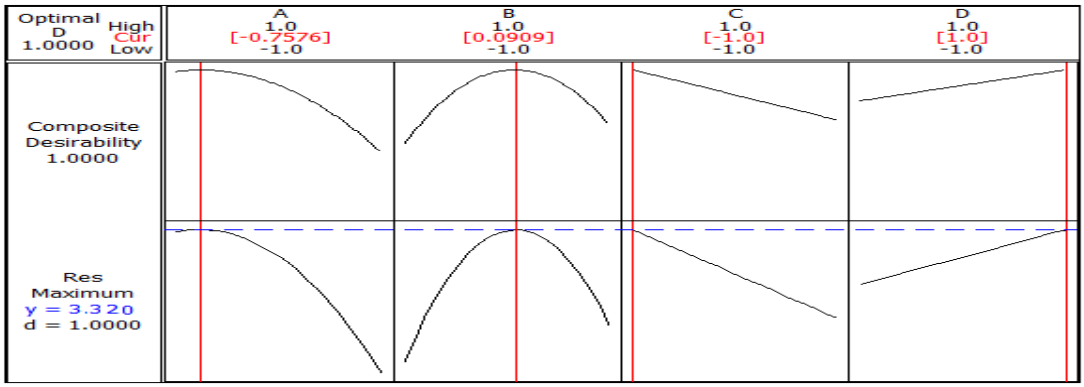


***Fig. 5: Parameter optimization of pH (A), temperature (B), the substrate (C), and time (D) affecting hydrogen production;***

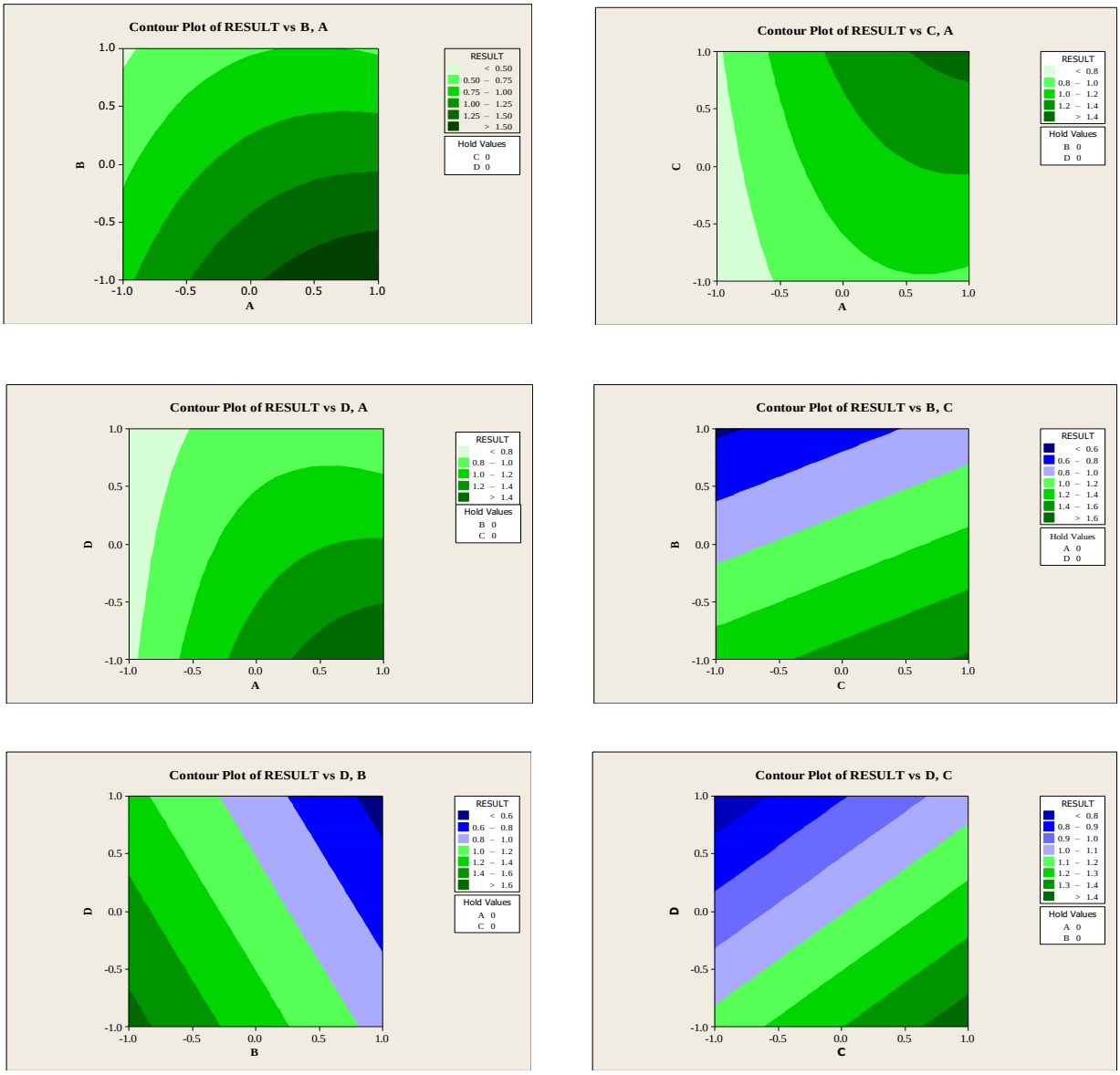


***Fig. 6: contour diagrams for pH (A), temperature (B), the substrate (C), and time (D) affecting hydrogen production.***

## CONCLUSIONS

In this essay, the effectiveness of different parameters such as pH, sugar substrate concentration, temperature, and fermentation time on bio-hydrogen productivity using active sludge and RBW as the substrate has been studied. Results from the $2^{k-1}$ factorial design, variance analysis, and regression equation have been shown that these four parameters have a great impact on the productivity rate. According to RMS, in optimal conditions 3.30 mol $H_2$/mol glucose can be produced when parameters of pH, temperature, sugar substrate concentration, and fermentation duration are equal to 6.240, 35.090 (˚C), 5.400 (g/L), and 36 respectively. Furthermore, the proposed theoretical mod, and the acquired experimental data match each other. Therefore, due to the capability of $2^{k-1}$ factorial design, test design and RMS this method can be used to investigate the effect of other effective parameters such as nutrient concentration and metal ions, on producing biohydrogen by inexpensive substrates.

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