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Optimization of Effective Parameters on Biohydrogen Production Using Bioaugmentation

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ABSTRACT

Biohydrogen production is a green method that utilizes organic materials in activated sludge as a substrate. The process involves microorganisms contracting the volume of the sludge content to produce hydrogen, CO_2, CH_4, and etc. However, the efficiency of this process is low. In this study, bioaugmentation was carried out by inoculating a 10% Escherichia coli suspension adapted in whey and activated sludge medium. The effects of parameters such as pH, temperature, and stirring, the concentration of whey as carbon source and nitrate as nitrogen source on hydrogen production were screened using the Plackett-Burman method with Minitab 21 software. Among the selected parameters, pH, temperature, concentration of whey and nitrate were found to be the most effective parameters in hydrogen production and were further optimized using Response surface methodology. Stirring wasn't statistically significant. The optimum conditions for hydrogen production were pH=5.4, temperature=39 ˚C, whey concentration=30 g/L, and nitrate concentration=3.6 g/L. Under these conditions with a 10% inoculation, the total volume of gas production was extended to 1.61 L per liters of activated sludge with 0.046 mole H_2 per liters of activated sludge. Comparing the bioaugmentation method with other method showed that the total time of the process decreased by 8 hours. Additionally, hydrogen production started after 10 hours of incubation and reached its maximum value in 16 hours, resulting in a 59% increase in productivity in less than 16 hours.

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INTRODUCTION

The growing energy demand has led to an increased emphasis on identifying environmentally sustainable and renewable energy sources (Wang et al., 2022). Given the concerns over greenhouse gas emissions and global warming, clean and renewable energy sources are essential for ensuring a sustainable future. Hydrogen (H_2) is a promising alternative fuel that meets these requirements, as it is a clean fuel with high energy content (122 kJ/g) and renewable. The combustion of hydrogen results in only water as an emission, making it a promising green fuel (Khesareh & Ataei, 2023; Pugazhendhi et al., 2019). Biological hydrogen production represents an alternative method for producing fuel from low-cost, renewable, and environmentally friendly resources (Srivastava et al., 2019). This method is performed at ambient temperature and pressure and offers a sustainable route for producing hydrogen with minimal environmental impact (Silva et al., 2018), making it a viable option for businesses and academic institutions looking to reduce their carbon footprint and promote a sustainable future. Dark fermentation is a biological decomposition process that offers a promising approach for treating organic waste and producing sustainable bioenergy. According to a recent study by the World Bank in 2018, global waste production is expected to reach 3.4 billion tonnes by 2050, with more than 50% of the waste composition generated from agricultural sectors. Therefore, sustainable management of this waste is of utmost importance. Dark fermentation offers two simultaneous benefits of waste treatment and sustainable bioenergy generation, making it an attractive option for waste management (Wang & Yin, 2019). Methane is currently the most commonly produced bioenergy from organic waste. However, hydrogen production is gaining attention as part of the hydrogen economy to substitute the hydrogen produced from fossil fuels. Hydrogen has three times higher energy content than hydrocarbon fuels, and its combustion is clean and carbon-free, producing only water as a by-product (Zhang et al., 2020). Dark fermentation is an attractive biohydrogen production option due to its low demand for light, high biohydrogen production rate, versatile substrate utilization, and low energy intensity. Dark fermentation is more appealing than other biological processes due to its environmentally friendly nature, versatile substrate utilization, and less energy-intensive process (Ghimire et al., 2015; Mishra et al., 2019). Moreover, the use of organic waste as feedstocks in dark fermentative biohydrogen production is potentially cost-competitive since organic waste is relatively abundant, cheap, renewable, and highly biodegradable (Sharma et al., 2020). In dark fermentation, diverse microbial communities work synergistically to ensure a stable degradation of organic substrates (Abendroth et al., 2015; Stolze et al., 2016). The main biochemical pathways in dark fermentation overlap with those of anaerobic digestion and can be divided into four phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Hydrogen (H_2) is produced during acidogenesis and acetogenesis by hydrolytic and fermentative bacteria, while it is later consumed during methanogenesis when methanogenic archaea use H_2 and CO_2 to produce methane (CH_4) (Hassa et al., 2018). The reactions that consume acid and hydrogen (H_2) are either fast or potentially faster than the ones that produce acid and H_2. When H_2 partial pressure exceeds 0.18 pa, it can negatively affect the breakdown of propionate and butyrate intermediates, leading to their accumulation. Therefore, it is critical to maintain the balance of these processes to ensure optimal digestion (Feldewert et al., 2020; Venkiteshwaran et al., 2015). Bioaugmentation is a promising strategy for enhancing bioproduct production in various industries. Studies have shown its effectiveness in improving energy valorization in anaerobic digestion processes (Inamuddin, 2023), Addressing stress factors in the anaerobic digestion of biomass waste to promote sustainable biofuel production (Mazzurco Miritana et al., 2023), and enhancing the degradation of contaminants in the environment

(Gao et al., 2023). Bioaugmentation involves introducing specific microbial strains to optimize fermentation, hydrolysis, and methane production, leading to increased biogas yields (Zhang et al., 2022). It addresses challenges such as ammonia inhibition, inefficient biomass degradation, Volatile fatty acid (VFA) accumulation, and the presence of recalcitrant pollutants in bioreactors (Gokulapriya et al., 2022). Through the utilization of bioaugmentation techniques, industries can achieve heightened efficiency in biogas production, improved waste management, and increased production of renewable biofuels. Recently the discussion focused on bioaugmentation strategies for mitigating various stress factors that affect bioenergy production. The study faced challenges related to the lack of efficient bioaugmentation strategies impacting biohydrogen production efficiency and technical bottlenecks associated with ineffective bioaugmentation, particularly about substrate challenges. To address these issues, the approach involved the utilization of repeated inoculation methods and pretreatment aids (Zhang et al., 2022). A comparison was conducted between an unaugmented culture and a bioaugmented one, and another study examined the timing of bioaugmentation. The results revealed that in the absence of augmentation, H2 production was significantly reduced during downward temperature fluctuation, with no production occurring during upward fluctuation. Furthermore, it was observed that applying bioaugmentation to the culture after the temperature fluctuation resulted in better performance compared to applying it during the fluctuation (Okonkwo et al., 2020). In a separate study, the challenges related to the longevity of bioaugmented microorganisms and the issues associated with scaling up in pollutant removal tasks were examined. When evaluating and optimizing bioaugmentation processes, it is essential to consistently consider sustainability concepts at every stage of these activities. Additionally, the application of bioaugmentation techniques is increasingly gaining traction in other industries, such as biogas production (Rashama et al., 2022). This study's findings suggest that utilizing agricultural solid waste as a substrate for microorganisms with a complete cellulase system can enhance thermophilic hydrogen production through bioaugmentation. The research demonstrated that the thermo-anaerobic bacteria R. thermocellum M3 played a significant role in augmenting the hydrogen production of the consolidated bioprocessing (CBP) of raw lignocellulosic agricultural wastes. This presents a promising solution for the industrial application of lignocellulose bioconversion using the CBP strategy (Sheng et al., 2021). The objective of this study was to identify the key factors affecting biohydrogen production and to determine the fermentation conditions responsible for this process. According to the literature review, the partial pressure of hydrogen in the fermenter tank emerged as a critical factor in biohydrogen production. Therefore, it is crucial to accurately determine the timing for removing the produced gas from the fermenter tank, not only to prevent methanogenesis initiation but also to maximize the yield of biohydrogen production. Establishing an effective mechanism to mitigate the impact of hydrogen partial pressure is essential. Additionally, gaining an understanding of the behaviour of Escherichia coli within a consortium of microbes is vital for future research. Achieving the optimal conditions for biohydrogen production is imperative to maximize the yield of hydrogen.

MATERIALS AND METHODS

Microorganism

The required Escherichia coli for the experiments was procured from the Persian Type Culture Collection (PTCC 1222) in lyophilized form. The Escherichia coli species were cultured in a pre-culture medium containing Beef extract (1g), peptone (5g), yeast extract (2g), sodium chloride (5g) per liter. Following aerobic incubation at 37°C for 24 hours, the resulting culture was employed as the inoculum.

Microorganism adaptation

To expedite bacterial growth, the bacteria must be acclimated with a seed culture. The adaptation process of activated sludge involves an initial centrifugation step at a speed of 6000 rpm for 5 minutes to effectively separate solid and floating particles from the wastewater. Following this, five different solutions were prepared in five separate Erlenmeyers, as detailed in Table 1. 50 ml of each solution prepared.

Table 1. Required solution for bacterial adaptation

Solution	Whey/wastewater + whey (g/l)
A	200
B	100
C	50
D	20
E	0

Gradual adaptation is key in preventing bacterial shock, which can increase the lag phase and delay hydrogen production, ultimately proving disadvantageous to the industry. Additionally, as methanogenesis begins, the amount of hydrogen production decreases, as 4 moles of hydrogen and 1 mole of CO_2 are consumed in the process of methane production. As indicated in Figure 1, the adaptation process has been occurred. The present discourse pertains to the detection of biomass concentration through employment of a spectrophotometer (optizen 3220 uv) at a 600 nm wavelength. It is worth noting that a blank sample was procured for each detection, and in the case of biomass concentration in Figure 1, line (a), the blank sample was Nutrient broth in which Escherichia coli was cultured.

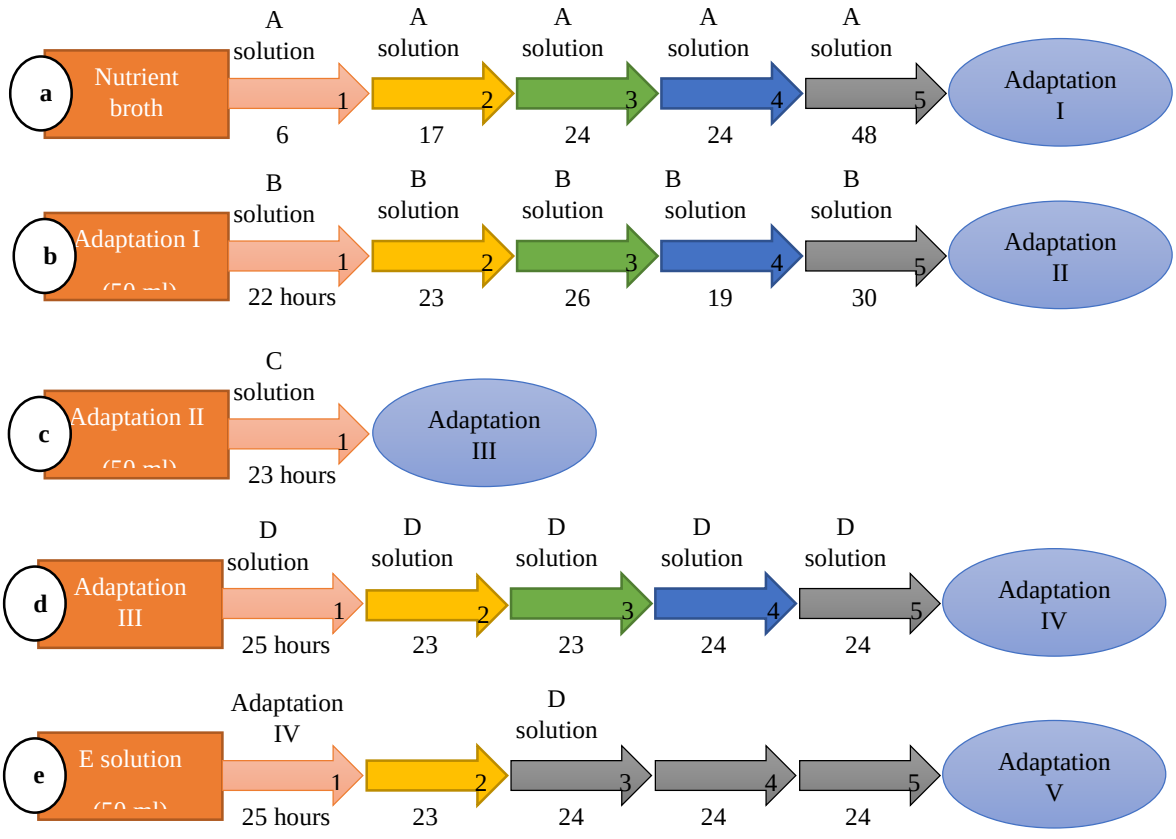

Figure 1. Microorganism adaptation process

Effective factors evaluating

In this study, twelve experiments were conducted using plackett-burman methodology of Minitab 21 software to evaluate the volume of gas production and the amount of hydrogen produced during batch dark-fermentation. The experiments were carried out using twelve 250ml vacuum Erlenmeyer flasks and 50ml of medium with a two times dilution activated sludge. Table 2 shows design of experiments.

Table 2. Design of experiment for effective factors determination

Test no	pH	Temp ˚C	Blocks	RPM	KNO_3 g/L	Whey g/L
1	8	30	2	150	0	0
2	8	45	1	300	0	0
3	6	45	2	150	10	0
4	8	30	2	300	0	10
5	8	45	1	300	10	0
6	8	45	2	150	10	10
7	6	45	2	300	0	10
8	6	30	2	300	10	0
9	6	30	1	300	10	10
10	8	30	1	150	10	10
11	6	45	1	150	0	10
12	6	30	1	150	0	0

The evaluation of the volume of gas produced has been carried out through two distinct experimental conditions that have been applied and illustrated as blocks in the design of the experiment. The first condition involves measuring gas production under anaerobic conditions at the outset of the process, which is referred to as Block 1. The second condition entails initiating the process in an aerobic environment for 24 hours, followed by continuing the process in anaerobic conditions for the subsequent 24 hours, which is referred to as Block 2.

Optimization of effective factors

To optimize the yield of conversion, it is critical to optimize the effective factors. In this regard, response surface methodology of Minitab 21 software has been utilized to achieve the desired results. 31 experiments have been defined to assess the optimal conditions for achieving the maximum volume of hydrogen production. The optimization design of experiment has been presented in Table 3.

Experimental set up for measuring the volume of produced gas

The measurement of gas production, water displacement method, was done using a glassy tube filled with water and two smaller hoses. One of the hoses was used to transfer the produced gas into the tube, while the other hose brought out extra water. Two ways of a Y-shaped joint was used to connect the vacuum Erlenmeyer to the glassy tube with a longer hose. Third way of Y-shaped joint was used to sample from the produced gas. A 1 cm stirrer magnet was placed in each vacuum Erlenmeyer and made anaerobic with N_2 gas and then located within the incubator and the stirring process was initiated. Figure 2 depicts a hydrogen production setup.

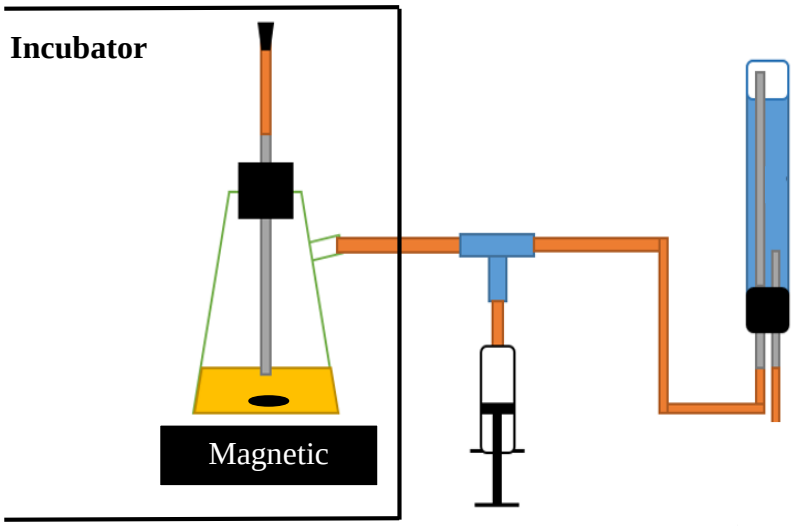

Figure 2. Gas production set up

Table 3. Design of experiment for effective factors optimization

Test no	Whey (g/l)	KNO_3 (g/l/)	Temp ˚C	pH
1	0	0	30.0	5.00
2	20	0	30.0	5.00
3	0	10	30.0	5.00
4	20	10	30.0	5.00
5	0	0	45.0	5.00
6	20	0	45.0	5.00
7	0	10	45.0	5.00
8	20	10	45.0	5.00
9	0	0	30.0	7.50
10	20	0	30.0	7.50
11	0	10	30.0	7.50
12	20	10	30.0	7.50
13	0	0	45.0	7.50
14	20	0	45.0	7.50
15	0	10	45.0	7.50
16	20	10	45.0	7.50
17	0	5	37.5	6.25
18	30	5	37.5	6.25
19	10	0	37.5	6.25
20	10	15	37.5	6.25
21	10	5	22.5	6.25
22	10	5	52.5	6.25
23	10	5	37.5	3.75
24	10	5	37.5	8.75
25	10	5	37.5	6.25
26	10	5	37.5	6.25
27	10	5	37.5	6.25
28	10	5	37.5	6.25
29	10	5	37.5	6.25
30	10	5	37.5	6.25
31	10	5	37.5	6.25

Analytical methods

The amount of gas produced is quantified by both a ruler and equations, which are outlined below. Initially, the total empty volume is measured by taking into account the absolute pressure. At the end of the process, the volume of gas produced is calculated using the equations provided in 1 and 2.

$$P_{Erlenmeyer} = P_{atm} - \gamma h \quad (1)$$

$$P_{Erlenmeyer} V = nRT \quad (2)$$

The following variables have been defined: $P_{Erlenmeyer}$, γ, h, V, n, R, and T, which refers to the absolute pressure of gases, specific weight, the height of water in a glassy tube, the volume of produced gas, total mole of gas , the gas constant, and the temperature of the system respectively. In order to determine the percentage of presented hydrogen (n_{H_2}), a gas chromatograph (Varian CM 3500) was employed. The chromatograph was fitted with a thermal conductivity detector (TCD) and a stainless steel column (2 m × 3 mm) packed with molecular sieve 5 A. N_2 was selected as carrier gas. The column, injector, and detector were maintained at, 80, and 90 °C, respectively. One of the most important factors of bio-product is the amount of product per unit of feed and time that called productivity. For each test 50 ml of medium was prepared and the maximum mole of produced hydrogen that calculated using equations 1 and 2 determined in its time. According to equation 3 below productivity is calculated. The yield of hydrogen production is equal to the amount of hydrogen production per unit of media shows in equation 4.

$$\text{Productivity} = \frac{\text{mole of produced hydrogen}}{\text{Volume of medium} \times \text{Time}} \quad (3)$$

$$\text{Yield} = \frac{\text{mole of produced hydrogen}}{\text{Volume of medium}} \quad (4)$$

RESULTS AND DISCUSSION

Microorganism adaptation

The reduction of the lag phase of bacterial growth is a critical aspect of industrial biological activity, as it leads to an increase in productivity and yield of the reaction and bio-conversion. Figure 3 displays the outcomes of adaptation, which are indicative of the aforementioned benefits. Therefore, it can be inferred that shortening the lag phase of bacterial growth is crucial for optimizing industrial biological activities (Karadag et al., 2014).

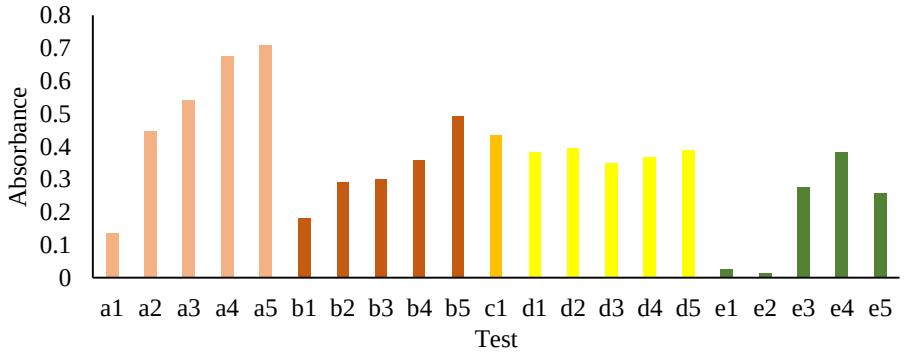

Figure 3. Optical density of microbial concentration in adaptation process using spectrophotometry in 600nm wavelength. Y-axis refers to figure 1

According to Figure 3, the presence of whey in the medium facilitated bacterial consumption, resulting in optimal bacterial growth between steps a1 and a5. In the subsequent steps, b1 to b5, the whey concentration decreased, and the composition of the master medium, nutrient broth, was modified. Notwithstanding these changes, a substantial biomass concentration was maintained. Steps c and d exhibited biomass concentrations that were similar, potentially due to the same underlying cause as before. When the whey concentration reached zero in step e, the biomass concentration declined. However, after the addition of solution D, the biomass

concentration increased. It appears that Escherichia coli is incapable of breaking down complex carbon sources and necessitates simpler carbon sources to enable growth.

Effective factors evaluating

Table 4 displays the results of the designed experiments regarding both the volume of produced gas and the amount of mole hydrogen.

Table 4. The volume of produced gas and mole of hydrogen produced values

Test no	Yield of produced hydrogen (mole $H_2/L_{Activated\ sludge}$)	$V_{Total\ produced\ gas}/V_{Activated\ sludge}$
1	0.0035	0.55
2	0.0167	0.66
3	0.015	0.58
4	0.022	0.64
5	0.0046	0.51
6	0.019	0.68
7	0.033	1.32
8	0.012	0.7
9	0.016	0.76
10	0.008	0.49
11	0.025	1.05
12	0.016	0.59

According to the findings presented in Table 5, it appears that the amount of whey used as a carbon source, the amount of KNO_3 used as a nitrogen source, pH, and temperature are all recognized as effective factors. This recognition is attributed to their P-Value being less than 0.05.

Table 5. Effective factors determination using plackett-burman methodology

Source	P-Value
Model	0.024
Blocks	0.365
pH	0.015
TEMP	0.045
RPM	0.170
N	0.043
C	0.020
Error	-
Total	-

The coded coefficient of factors displayed in Table 6 provides insight into the efficiency of each factor. Within the design of the experiment, two key levels have been defined for each factor, namely the upper level and the lower level. Notably, the upper level is denoted by a "+" sign, while the lower level is denoted by a "-" sign in Table 5.

Table 6. Coded coefficient of the mentioned factors

Term	Coef	P-Value
Constant	0.7131	0.000
Blocks		
1	-0.0333	0.365
pH	-0.1216	0.015
TEMP	0.0887	0.045
RPM	0.0536	0.170
N	-0.0898	0.043
C	0.1127	0.020

The results indicate that the carbon source, RPM, and temperature factors all display a positive coefficient, suggesting their upper levels are more efficient in gas production compared to lower levels. The positive sign for temperature and RPM factors is attributed to their effect on enzyme folding and function. Similarly, the high RPM level can reduce mass transfer prevention and facilitate the exit of produced gas from the solution. Moreover, a higher level of carbon

source is preferred as the bacteria can consume it more easily due to gas production. Conversely, the pH factor displays a negative coefficient, indicating that the lower level is more optimal for enzyme folding. Additionally, the lower level of the nitrogen source is significant because it can alter the total charge and pH of the inner cell. Based on the results, Block 2 seems to be a better option since the biomass growth is rapid in the first 24 hours under aerobic conditions, making it more suitable for gas production. However, statistically, the block is not significant, and batch culture conditions can also be considered as one of the best options (Bao et al., 2013; Choi et al., 2020).

Optimization of effective factors

As shown in Table 7 the values of the volume of produced gas and mole of hydrogen are presented below.

To optimize the fermentation process, it is essential to have a comprehensive understanding of the factors that influence it. In this regard, response surface methodology utilized through Minitab 21 software to design and conduct 31 experiments. The outcomes were analyzed to determine the approximated values of the influential factors, which are displayed in Figure 4.

Table 7. Optimization values of the volume of produced gas and mole hydrogen

Test no	V_{gas}/V_{as}	$molH_2/L_{as}$	Test no	V_{gas}/V_{as}	$molH_2/L_{as}$
1	0.8	0.021	17	1.02	0.021
2	1.3	0.039	18	1.68	0.046
3	0.75	0.022	19	1.115	0.032
4	1.21	0.023	20	1.02	0.022
5	0.88	0.025	21	0.94	0.025
6	1.35	0.039	22	0.88	0.019
7	0.8	0.021	23	0.89	0.0085
8	1.3	0.032	24	0.94	0.009
9	0.58	0.015	25	1.22	0.031
10	1.02	0.015	26	1.32	0.03
11	0.45	0.0071	27	1.27	0.032
12	0.69	0.019	28	1.29	0.031
13	0.5	0.015	29	1.24	0.032
14	0.99	0.029	30	1.3	0.033
15	0.47	0.008	31	1.27	0.032
16	0.97	0.02	-	-	-

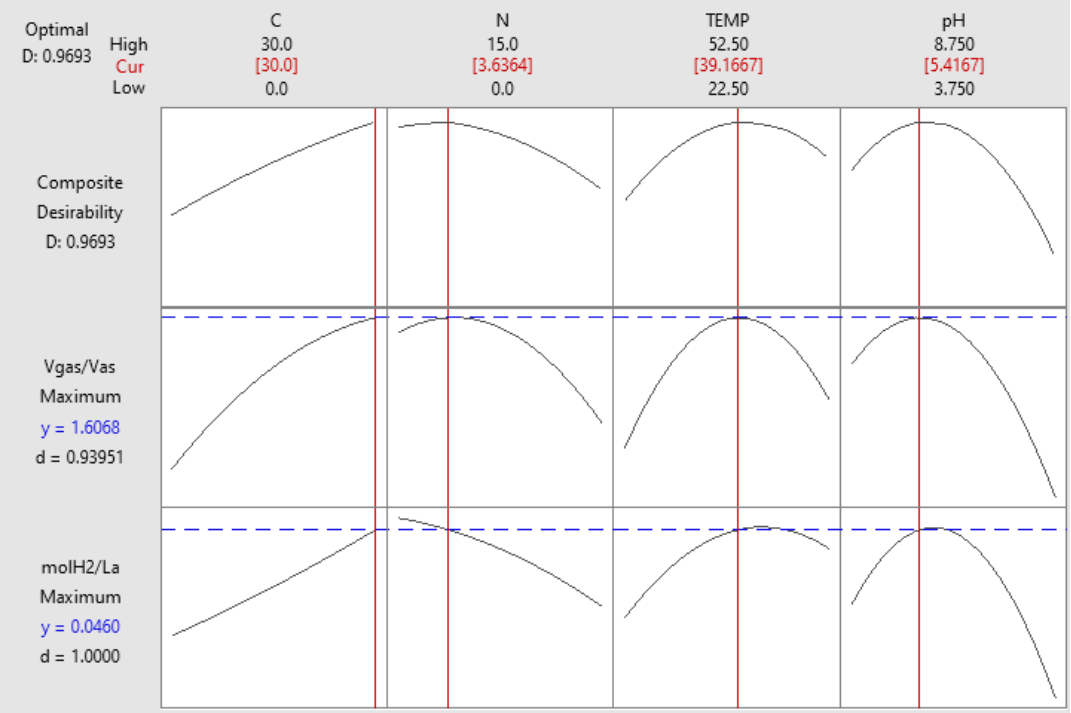

Figure 4. The value of the effective factors optimization

According to the statistical analysis, The results indicate that the optimal values of carbon source amount, nitrogen source amount, temperature, and initial pH are 30 grams per liter activated sludge, 3.6 grams per liter activated sludge, 39 degrees Celsius, and 5.4, respectively. It is noteworthy that under these conditions, the maximum volume of the produced gas is 1.6 per unit volume of activated sludge, and the maximum produced hydrogen is 0.04 mole H_2 per liter of activated sludge (as) (Argun & Kargi, 2011; Lee et al., 2015; Mu et al., 2006; Shaterzadeh & Ataei, 2017).

Based on the observations made, it can be concluded that the utilization of bio-augmentation results in a higher yield of hydrogen within a shorter duration as compared to the process without bio-augmentation. Table 8 presents a comprehensive overview of the benefits that bio-augmentation offers for the biohydrogen production process.

Table 8. Advantages of bio-augmentation

Data	Without bio-augmentation	With bio-augmentation
mole H_2/L_{as}	0.98	1.61
Duration (hour)	24	16
Productivity (mole H_2/h.L_{as})	0.041	0.1

$$\text{Efficiency} = \frac{\text{Productivity}_{\text{with bio-augmentation}} - \text{Productivity}_{\text{without bio-augmentation}}}{\text{Productivity}_{\text{with bio-augmentation}}} = \frac{0.1 - 0.041}{0.1} = 0.59 \quad (5)$$

The experimental evidence demonstrates that integrating bio-augmentation in the biohydrogen production process leads to a higher productivity of hydrogen in a shorter time frame compared to the process without bio-augmentation. The study reveals that utilizing Escherichia coli as bio-augmentation can significantly enhance biohydrogen production and increase productivity of the reaction (Baskaran & Sathiavelu, 2022). Figure 5 indicates advantages of utilizing bio-augmentation to improve product output.

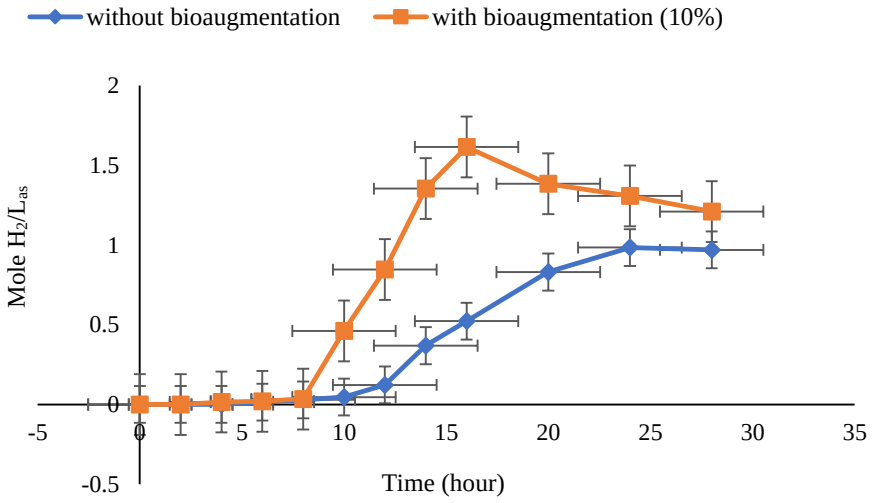

Figure 5. Comparison of biohydrogen production with bio-augmentation and without bio-augmentation

CONCLUSIONS

Eco-friendly fuels should be replaced with fossil fuels. One of the most favourable green fuels is hydrogen. The ways of hydrogen production are various. Hydrogen production by microorganisms is a considerable way that should be developed in efficiency. Bioproduct production rate is low and it is necessary to speed up. Using adapted Escherichia coli as bioaugmentation and utilizing urban wastewater as feed, illustrated that Escherichia coli can play a key positive role in biohydrogen production. The condition optimization was carried out for this study. Results show Factors such as temperature, pH, and carbon source amount (whey), and nitrogen source amount (nitrate) are all significant considerations in the process of biohydrogen production. Optimizing these factors can lead to a remarkable increase of up to 59% in hydrogen productivity as shown in equation 5. One of the effective methods for enhancing productivity is through bioaugmentation, which involves the deliberate increase of the microbial community responsible for hydrogen production. It is noteworthy that the ideal values for these factors may differ between biomass growth and hydrogen production. Nevertheless, an optimization process has revealed that the optimal values for temperature, pH, carbon source amount, and nitrogen source amount are 39 degrees Celsius, 5.4, 30 grams per liter of activated sludge, and 3.6 grams per liter of activated sludge, respectively. According to the statistical analysis, varying rotation speeds do not significantly impact the biohydrogen production process. However, it is objectively noted that the presence of the stirrer plays a crucial role in reducing mass transfer inhibitors and facilitating the removal of gas bubbles from the fermentation culture. This is particularly important as the commencement of stirring promptly eliminates air bubbles from the solution, thereby preventing the initiation of methanogenesis.

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