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Role of Temperature, pH and Substrate (Banana peel) Concentration for Maximizing Biohydrogen Production

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Abstract

Dark fermentation is a potentially renewable energy source that can be used to produce biohydrogen, which might lead to sustainable fuel production. The goal of this study is to maximize Biohydrogen production using banana peel as the substrate and *Enterobacter* species as the inoculum. The research discovers the impact of three critical parameters (temperature, pH, and substrate concentration) on biohydrogen production, employing Response Surface Methodology (RSM) to determine the ideal conditions for maximum yield. According to experimental results, the maximum amount of biohydrogen (2.6 L H_2/L) was produced at 35°C, neutral pH 7.0, and substrate concentrations ranging from 10% to 25%. By maximizing microbial activity without creating substrate overload, which can result in metabolic inhibition and decreased hydrogen generation, moderate substrate concentrations improve biohydrogen yield, according to data analysis. Furthermore, it was shown that high substrate concentrations and temperatures beyond the optimal range inhibit the synthesis of biohydrogen because they cause microbial stress and the buildup of inhibitory byproducts. By establishing certain ideal condition for the generation of biohydrogen utilizing banana peels, an abundant agricultural waste, this work fills a gap in the existing literature. The findings highlight the necessity of exact control over fermentation settings in order to optimize biohydrogen production and microbial efficiency. These discoveries have potential for expanding the process in industrial settings in addition to aiding in our knowledge of biohydrogen generation. This study opens the door to new environmentally friendly and sustainable energy options by demonstrating the possibility of employing agricultural waste in the production of bioenergy.

Introduction

The urgent problems of fossil fuel depletion of resources, greenhouse gas emissions, and climate change are causing a revolutionary transformation in world's energy prospect toward sustainable and renewable energy sources Deshmukh et al., 2023 and Singh, S. (2021). Because of this, there has been an over consumption of these non-renewable and unsustainable resources, which has raised the concentration of overall carbon dioxide in the atmosphere. Increasing energy demand of today necessitates the development of sustainable and cost-effectively as an alternative energy source useful (Mumtha and Mahalingam, 2023). Biohydrogen production is one of the greatest promising energy carriers among the options. Making use of particular microbes to break down organic materials, biohydrogen is generated organically and provides a clean, efficient energy source for fuel cells, combustion engines, and other industrial operations. Compared to hydrocarbon fuels, the hydrogen has a three times greater energy content (120 MJ/kg), burns cleanly, releases no carbon dioxide during combustion, and produces only water as a byproduct (Mumtha and Mahalingam, 2023) and (Zhang et al., 2020)

The most fascinating feature of AD is that, it allows to introduced additional additives that improve the process and permits for the simultaneous digestion of different substrates (Mumtha and Mahalingam, 2023. Three primary processes which involved in the synthesis of biohydrogen: the dark fermentation, photofermentation, and electrohydrogenesis (Rajesh et al., 2020). Dark fermentation stands out among them all for its effectiveness in producing hydrogen gas from a range of organic substrates, frequently in anaerobic condition (Ndayisenga et al., 2021 and Xue et al., 2021). According to a 2018 World Bank report, organic wastes produced by the agriculture sector would make up for over fifty percent (50%) of the whole waste composition, with global trash output expected to grasp 3.4 billion tons by 2050 (Dzulkarnain et al., 2022). This huge number of wastes has to remain sustainably managed. For this reason, dark fermentation offers two simultaneous benefits for waste treatment and sustainable biogas generation (Patel et al., 2021) and (Soares et al., 2020). Various feedstocks have been investigated to maximize hydrogen production, including the organic waste, agricultural wastes, and specialized energy crops. This approach has greater significance due to its combined advantages of waste management and renewable energy; these benefits are in linked with the fundamental principles of a circular economy.

Approximately 1.3 billion tons, or one-third, of all food that produced are wasted each year, making food waste a serious worldwide problem (Pandey, 2021). Geographically, the issue takes diverse forms. While advanced countries tend to waste more food at the consumer level because of behavioral issues including overbuying and misinterpreting expiry dates, developing nations frequently lose food during manufacturing and delivery due to inadequate infrastructure (Bjornlund et al., 2022). Food waste has extensive negative influence on environment since it's not only using up resources like land and water but also produces greenhouse gasses, especially methane, when it breaks down in landfills. Food waste has a huge financial cost roughly 1 trillion dollar a year and worsens food insecurity by taking resources away from those who could be feed on (Barrera, 2021) and Sethi et al., (2020). To address this issue, a number of strategies are being used, such as waste reduction legislation being implemented by the government, educating consumers about food management, and utilizing creative solutions like apps which connect extra food with people in need (Al-Obadi et al., 2022). Effective strategies for reducing food waste are shown in case studies from nations like Sweden and France, emphasizing the need of cross-sector collaboration in tackling this complex issue (Matzembacher et al., 2021).

Food waste and its ability to produce biohydrogen point to a viable path for managing food waste and producing renewable energy (Habashy et al., 2021 and Srivastava et al., 2021). A large amount of municipal garbage is made up of food waste, which is rich in organic compounds that may be transformed into biohydrogen via a variety of biochemical processes. According to research, food waste may produce significant amounts of hydrogen through anaerobic digestion and fermentation because some bacteria can decompose organic materials in the absence of oxygen (Sharma et al., 2024). Research has indicated that the efficiency of hydrogen generation may be increased by regulating variables including pH, temperature, and microbial consortiums (Camargo et al., 2021) and (Martinez-Burgos et al., 2020). In addition, a sustainable option that enables the concurrent reduction of food waste and the creation of clean energy is incorporating the process of biohydrogen production into currently operating waste management systems (Kazmi, et al., 2024). This methodology not only alleviates the ecological consequences linked to the disposal of waste, but also advances energy stability and the shift towards a circular economy.

For this reason of its high consumption, fruit waste like peels and seed are generated. Proper handling of all these wastes was emerged as a global concern (Mumtha and Mahalingam, 2023). The most promising solution to this energy source and waste issues is to use the agricultural residues from banana waste as a profitable substrate for energy generation (Kholik, 2017). As one (1) of the most extensively grown fruit crops worldwide, bananas produce large amounts of fruit each year. The harvesting of the fruit produces a lot of debris, such as peels, leaves, and pseudo stems, which when improperly disposed it could lead to environmental problems (Gupta et al., 2023). In addition, to address waste management concerns, using banana waste as a substrate for biohydrogen production takes use of its high organic content. Because of their high carbohydrate content, banana substrates are ideal for microbial fermentation processes (da Silva et al., 2021). Banana waste is an appealing and underused resource for the generation of bioenergy because some bacterial strains can effectively convert the sugars in it into biohydrogen.

In the recent years, there was an increase in research on the production of biohydrogen from banana substrates. Several research have investigated the fermentation of various banana waste by optimizing parameters which including temperature, pH, substrate concentration and inoculum types (da Silva et al., 2021) and (Ulma et al., 2024). Advanced strategies are also being investigated, such as the use of genetically modified microbes, pre-treatment procedures to improve substrate digestibility, and co-fermentation with other organic materials (Zhang et al., 2022). Despite the promising results, issues with scalability, process effectiveness, and economic feasibility still exist, necessitating further research and innovation (Kuspanov et al., 2023).

This article prepares a phase for the detailed examination of the methods for optimizing the synthesis of biohydrogen from banana substrate. By studying the complexities of microbial fermentation, improving operating conditions, and investigating cooperative strategies might help us exploit banana waste's full potential as a sustainable feedstock for biohydrogen. As the research landscape evolves, the insights gained will contribute significantly to the development of efficient and eco-friendly bioenergy systems.

Methodology

Substrate collection and preparation

Banana peels were collected from the local market at Sharda University as organic substances for the production of biohydrogen. The sample (banana peels) were reduced to a smaller size. Two pretreatments were carried out. firstly, the physical pretreatment using hot air oven at a temperature usually around 120°C, which broke down lignocellulosic materials. After oven dry, a grinder was used (hammer mill) and pulverized the dried substrate into a fine powder. Secondly, about 10 to 30g of Banana peel with 2M H_2SO_4 (sulfuric acid) were mixed in a conical flask and covered, then autoclaved at 121°C for 15 min as per the method presented Jayachandran and Basak (2023). Substrate was allowed to cool down at room temperature. The banana peel was filtered by the use of Whatman filter paper (high quality filter) under a vacuum. After successful acid pretreatment of the substrate collected, the pH was adjusted to 7.0

using 1N of NaOH and the sample remain was used for further process.

Enrichment of hydrogen-producing bacteria

The Enrichment of biohydrogen ($BioH_2$) producing bacteria (*Enterobacter species*) was carried out in 500 mL conical flask with 300mL working volume at 37°C, following a technique previously reported [31]. The sterile pre-reduced Maltose-yeast extract -Dextrose (MYD) medium contains maltose 10 g/L, yeast extract 4 g/L and Dextrose 10 g/L. Prior to autoclaving, the pH was adjusted to 7.0 by the use of either 1N HCl (Hydrochloric acid) or NaOH (Sodium hydroxide). To get the maximum growth, three consecutive transfers in MYD medium were carried out. Freshly grown (24hr) of the enriched cultures was used as the inoculum for the fermentation processes.

Batch Fermentation/ Experimental Setup and Procedures

To evaluate each inoculum's ability to produce hydrogen, a number of batch hydrogen fermentation tests were conducted. Batch tests were conducted under strict anaerobic conditions in a 2.7L bioreactor with a 2L working volume. As the inoculum, 0.15L of enriched culture was introduced to the fermentation medium using the procedure outlined by (Amekan et al., 2018). The batch fermentation of Banana peel which carried out in individual basal medium containing substrate and some important nutrient with inoculum which incubated at 37°C under magnetic stirrer at 270rpm. All batch bioreactors were incubated on a magnetic stirrer with temperature controlled at 37°C. Prior to the autoclave, the initial culture pH was adjusted to 7.0 by the use of either 1 N HCl or NaOH. This process of fermentation continued till the gas generation stopped.

Analysis of Biohydrogen Produced

Using a Gas Chromatography (SHIMADZU GC-2014, Japan) with a thermal conductivity detector that had an 80/100 mesh stainless-steel column packed with Porapak Q and nitrogen (N_2) as a carrier gas at a flow speed of 20 mL min^{-1}, and the hydrogen gas generated during the bacterial monocultures in the lab-scale batch biohydrogen reactors which was measured. In gas chromatography, the injector (syringe), column (which sample passing through), and detector were maintained at 80°C, 100°C, and 150°C, respectively. TCD was used to assess the amount of biohydrogen produced after 0.5 ml of the sample was placed in a 1 ml Hamilton glass syringe and subjected to gas chromatography (Mumtha and Mahalingam, 2024). Hydrogen produced was measured at regular intervals of 48hrs using gas collection systems and analyze gas samples with chromatography and record the hydrogen yield over time to maintain the process safety and reproducibility while assessing the effectiveness of biohydrogen generation using data gathered.

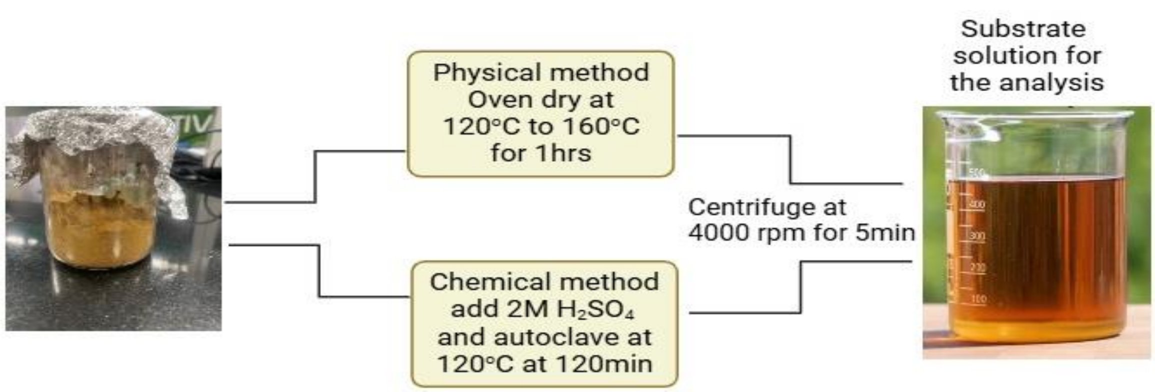

Figure 1: Pretreatment processes (Physical and Chemical) for the preparation of analysis

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Figure 2: Biohydrogen production set-up using water displacement procedure and Inoculum Enrichment

RSM Design Setup

A Response Surface Methodology (RSM) technique, more especially a Central Composite Design (CCD), is utilized to investigate the combined impacts of pH, temperature, and substrate concentration on biohydrogen generation. An effective and methodological evaluation of every variable at several levels and their interactions is made possible by this architecture. Compared to more straightforward experimental designs, a CCD provides a more complete model by using a factorial design with extra "star points" to represent curvature. This usually consists of 15–20 experimental runs for three components.

$$Y = \beta_0 + \sum_{\{i=1\}}^{\{3\}\beta}{}_{iX_i} + \sum_{\{i=1\}}^{\{3\}\beta}{}_{\{ii\}X_i^2} + \sum_{\{i<j\}}^{\{3\}\beta}{}_{\{ij\}X_{iX_j}}$$

Where: Y represents Biohydrogen yield while X_1, X_2 and X_3 represent temperature, pH and Substrate concentration, respectively.

Preliminary studies and the body of research on *Enterobacter spp*.-mediated biohydrogen generation serve as a reference for the selection of values for each variable. For example, temperature levels are chosen within a range that promotes microbial activity without denaturing important enzymes, and pH may be adjusted at low, medium, and high levels based on previous research. Levels of substrate concentration are also selected to strike a balance between microbial tolerance and available carbon sources.

A polynomial model that explains the link between the factors and biohydrogen production may be created by utilizing CCD inside RSM. The outcomes of these studies will pinpoint ideal circumstances and offer a forecasting model for the generation of biohydrogen in a range of scenarios.

Result

Relationship Between Temperature & pH for Biohydrogen Production

The result obtained output data represented in RSM for dark fermentation of biohydrogen production showed that, both the surface and contour plot of biohydrogen production in relation to temperature & pH is increased with temperature range from 32° C to 38°C and pH range from 6.8 - 7.4 respectively, with constant substrate concentration as 20% (figure 3).

Relationship of pH & Substrate Concertation for Biohydrogen Production

The result of the two relationships of pH and substrate concentration showed a maximum hydrogen production at constant temperature

35°C with the pH range of 6.8 – 7.2 and substrate concentration of 10 - 25% respectively (figure 4).

Relationship Between Temperature & Substrate for Biohydrogen Production

Various factor like temperature and substrate concentration in this relationship increase the biohydrogen production, the result of this relationship showed a maximum biohydrogen production at a constant pH = 7. The maximum produced biohydrogen production was achieved at the temperature 32 - 38°C and substrate conc. of 17% to 25% respectively (figure 5).

Discussion

The experiment of this study uses response surface methodology (RSM) with data processing using minitabs 21, three factors (Temperature, pH and Substrate) were use with 14 experimental runs, the response data were modulated as the function of the input variable to optimize the hydrogen production.

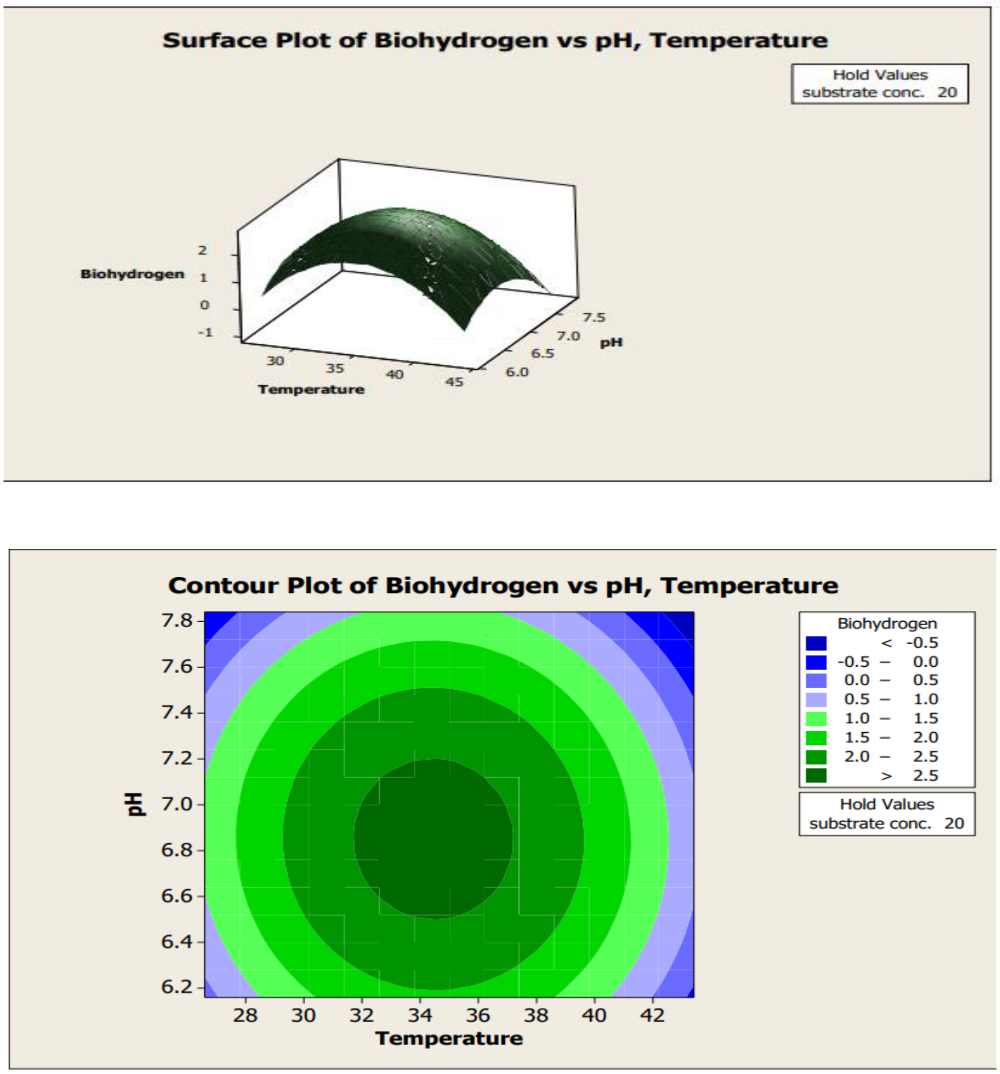


Figure 3: Response surface (3D) and corresponding contour (2D) curve shown interactive effect of Temperature and pH for Biohydrogen production

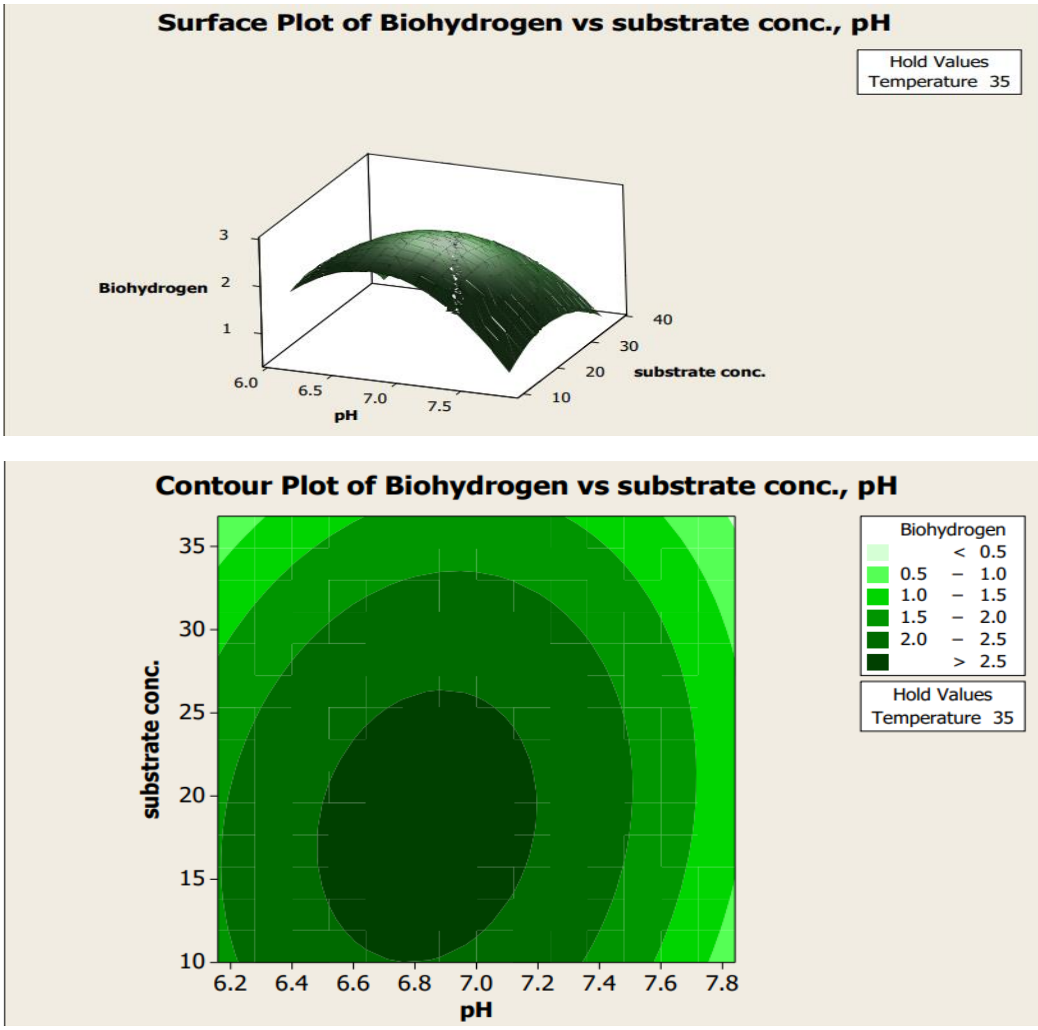
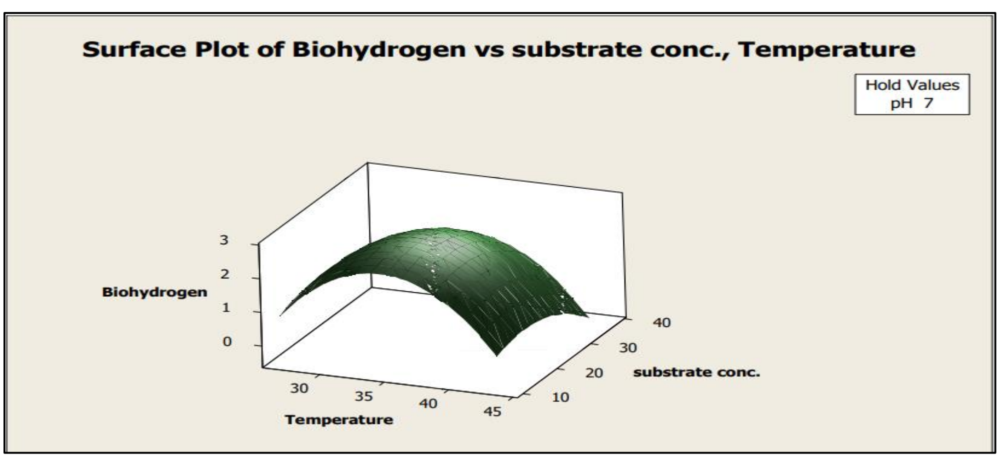


Figure 4: Response surface (3D) and corresponding contour (2D) curve shown interactive effect of Substrate and pH for Biohydrogen production



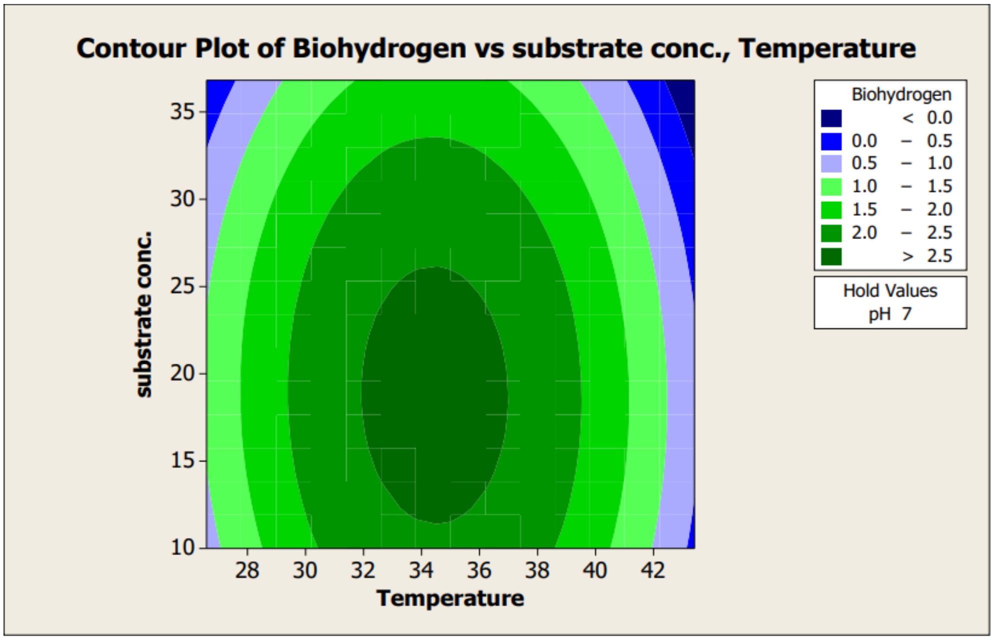


Figure 5: Response surface (3D) and corresponding contour (2D) curve shown interactive effect of Temperature and Substrate concentration for Biohydrogen production

The study shown the maximum and minimum hydrogen production which was observed to be 2.6L H_2/L media and 0.65L H_2/L of the media respectively with central composite design. The interaction between the temperature and pH was positively represented as three-Dimensional surface and two-Dimensional contour plot in figure 3**,** the dark circle is slightly stretched along the axis of pH indicated that, the pH has minor optimization range of pH range of 6.8-7.4 and temperature range of 32 to 38 when substrate concentration is kept constant 20%. This proposes that the optimal conditions for biohydrogen production require a moderately warm condition and a near-neutral pH (Chai et al., 2021). This bring into line with research findings indicating that *Enterobacter* species are highly active in moderate thermal and slightly acidic to neutral pH environment, which support the activity enzyme essential for hydrogen production (Jayachandran et al., 2023). Higher temperatures might influence the inhibition of microbial growth, while lower temperatures could slow metabolic rates, both leading to reduced biohydrogen yield.

Figure 4 data indicated that maximum production is achieved at constant temperature of 35°C, which was the optimum temperature for the maximum biohydrogen production at the pH range of 6.8–7.4 and substrate concentration of 10–25%. Further increase the substrate concentration beyond this range decreases the biohydrogen production in respect of the initial pH, which may be due to either acidic by-product or substrate overload, either could negatively affect microbial efficiency that may be caused as a result of inhibition of metabolic activities of microbes.

Figure 5 shows the relationship between the temperature and substrate concentration which presented in both 3D surface and 2D contour plot, this study shows that balanced combination of moderate temperature (32–38°C) and substrate concentration (10–25%) enhanced the biohydrogen production to the maximum level at an optimum pH of 7. Maintaining these factors likely prevent the buildup of the inhibitory by-product while utilizing the effective substrate concentration. Research support that excess substrate concentration or the temperature can lead rapid change in pH, metabolic inhibition or thermal pressure on microbes, thus reducing the efficacy of hydrogen production (Sangyoka et al., 2016).

Previous investigations on *Enterobacter* species-based biohydrogen generation has shown that certain circumstances have a major influence on hydrogen output (Yin et al., 2021). To enhance the process of producing of biohydrogen, scientists have adjusted important parameters including temperature, pH, and substrate concentration. For instance, Enterobacter enzymes that produce hydrogen are more active at neutral pH levels (about 7) and mild temperatures (around 30 to 37°C) (Gupta et al., 2023). This is in line with experimental result that reported the maximum hydrogen production was 1.580L H_2/L of media using potato waste as substrate (2.23g/) with combined Dark fermentation and the Photo fermentation using *Enterobacter aerogenes* and *Rhodobacter sphaeroides* respectively, which is lower than this study (Das and Basak, 2024). Another report shows high production using co-culture which result in maximum hydrogen production potential of 6.980L H_2/L with sugarcane bagasse which is higher than this present result (Sangyoka et al., 2016). Enterobacter species will probably produce much less biohydrogen if the substrate concentration is insufficient and falls below the necessary threshold. The microorganisms' inability to maintain their metabolic activity at their best due to a shortage of nutrition causes this decline. The major source of carbon and energy used in the biohydrogen production process is the substrate, such as organic material, which bacteria transform into hydrogen gas (Kalita and Sit, 2022). The rate at which the hydrogen is produced is directly impacted by low substrate levels, which result in restricted microbial growth and activity (Sangyoka et al., 2016). It might also experience a state of metabolic inactive or stress which decrease the efficiency in breaking down the substrate that utilize for the conversion to biohydrogen. Likewise, when the microbial pathways move toward less energy-intensive reactions, substrate shortages may result in incomplete fermentation processes, which might reduce hydrogen yields and perhaps increase the creation of alternate by-products (Aruwajoye et al., 2020). However, too much substrate might cause inhibition because of the accumulation of byproducts, which affects the effectiveness of the microbes.

Using response surface methodology (RSM) and the artificial neural networks to model and forecast optimal outcomes, *Enterobacter cloacae* proved to enhanced hydrogen yields under regulated substrate and ambient conditions in one research (Pradhan et al., 2024). These techniques shed light on how to control environmental factors and substrate concentration fluctuations to produce high yields in hydrogen generation systems.

Conclusion

The study of biohydrogen production using *Enterobacter* sp. and utilizing banana peel as a substrate highlighted several key parameters that influence the hydrogen yield, including temperature, pH, and substrate level. A range of temperatures between 32 and 38°C, the pH values between 6.8 and 7.4, and substrate concentrations between 10% and 25% were found to yield the best results of 2.6L H_2/L media. Achieving high biohydrogen results requires optimizing condition like temperature, pH, and substrate concentration using RSM according to current research. *Enterobacter* demonstrated effective metabolic activity within these limits, optimizing the highlighted condition for production of biohydrogen. Reduced yields were the result of deviations from these criteria, such as abnormally high or low substrate concentrations. High substrate concentrations ran the risk of accumulating byproducts, which might hinder microbial activity, whereas low concentrations restricted the availability of nutrients for microbial metabolism. Additionally, by addressing metabolic limitations and improving pathways that generate hydrogen more efficiently under different operating conditions, current research has concentrated on genetic alterations and the utilization of cocultures to further increase biohydrogen yields. According to these results, even though *Enterobacter species* are effective producers of biohydrogen, monitoring substrate overload and adjusting environmental condition are still crucial for achieving the best production rates. This field of study enhances the creation of more effective and sustainable methods for producing biohydrogen utilizing *Enterobacter* and other microbial systems. These results highlight the significance of regulated ambient conditions in maximizing biohydrogen generation and reducing by-product buildup, hence improving the effectiveness of dark fermentation processes.

Author Contribution

A.H Adam; Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **S. Pandit;** Validation, Supervision, Project administration, Methodology and Formal analysis. **R. Dhakar;** Editing and formal analysis. **A.U Maaji**; Writing – review & editing and investigation. **H.A Bawa**; Writing – review & editing, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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