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Biohydrogen Production From Lignocellulosic Biomass Using Escherichia coli

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Abstract

A rising emphasis is being placed on switching to renewable energy options with less impact on the environment, given the growing environmental issues linked to nonrenewable energy sources. The importance of biohydrogen as a sustainable, clean energy source made from organic materials is highlighted by this change. This study investigates a novel method for producing biohydrogen through dark fermentation using commonly available and sustainable feedstock: paper and cardboard waste from our surroundings. To break down the complex structure and make the material easier for microbes to digest, lignocellulose-rich materials (paper and cardboard) were first treated to improve bioconversion efficiency by fungal and acid hydrolysis methods (using *Trametes versicolor* and H_2SO_4, respectively). In the next stage, *Escherichia coli* was used to produce hydrogen by taking advantage of its metabolic formate pathway. The degradation of waste materials for biohydrogen production not only provides a sustainable energy source but also helps reduce waste and promotes environmental sustainability. In this study, we aimed to discover which is more suitable, paper or cardboard, and which treatment is better, acid or fungal, and this paper describes a clear-cut answer to our question. Results show that homogeneous cardboard, acid-treated paper, and fungal-treated paper and cardboard are the best substrates to work with, and fungal treatment is much more efficient than acid treatment.

Categories: Bioprocess Engineering, Biotechnology and Engineering, Environmental and Sustainable Engineering
Keywords: renewable energy, dark fermentation, acid hydrolysis, lignocellulose, bio-hydrogen.

Introduction

In the fast-growing economy, the need for vehicles and automobiles for travel is increasing simultaneously, and the changes in the environment can be seen in cities like Delhi, Mumbai, and others, so as an alternative, we have to rely on more sustainable solutions that can down-regulate the risk caused by conventional fuels. Biohydrogen emerges as a plausible solution for these problems, presenting us with an opportunity to grow as a sustainable world and encourage emerging economies of developing countries like India. As we look around us, the amount of waste, especially from the primary sector, which is abundant in lignocellulose as well as cellulosic biomass, is also a big challenge before us. Combining both of these problems, we can open a golden gate of opportunity. Waste accumulation has also been a major problem that the world faces. Landfills and pollution continue to rise due to urbanization. Making use of waste and recycling has been a mission toward sustainability and healthy living. If these two problems can be solved by producing hydrogen biologically, it would hold huge potential as a powerful renewable energy source. The benefits of biohydrogen can be narrowed down to the following:

1. It has nearly zero greenhouse emissions.

2. It is a clean and homogeneous fuel.

3. It can increase flame speed and combustion stability.

4. No noise or visual pollution.

5. Low maintenance cost.

6. Long usage time.

The ever-booming population of developing countries leads us to the craving for a clean fuel, and the current emerging and best answer is biohydrogen, a renewable fuel that can be developed from different sources, the current trend being lignocellulose biorefineries; it can be harvested from agricultural waste, which counts in trillions in countries like India, transforming our weakness into powerful resources. The clutch that we have on fossil fuels can be minimized along with tackling waste management. Still in the development stages, more focus on fuels like biohydrogen can help our future generations.

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Microorganisms in biohydrogen production

Escherichia coli (*E. coli*), a commonly found bacterium in food, water, and intestines of warm-blooded animals, can be developed into a powerful tool for developing biohydrogen, the reasons being:

• Natural Hydrogen Production: *Escherichia coli* has the inherent ability to produce hydrogen gas during a process called fermentation, particularly under oxygen-depleted conditions with the help of an enzyme known as the hydrogenase enzyme.

• Genetic Engineering: With the help of genetic engineering, using tools like CRISPR-Cas9 and bioinformatics tools, we can help develop strains that can be utilized to improve their efficiency.

• Fast Growth: *Escherichia coli* reproduces rapidly, allowing for large-scale hydrogen production in a relatively short time frame.

However, there are challenges to consider:

• Low Hydrogen Yield: Currently, natural hydrogen production by *E. coli* is inefficient. Need genetic engineering as wild type isn't build for growing under anaerobic conditions.

• Competition with Other Fermentation Products: During fermentation, *E. coli* produces various byproducts besides hydrogen. Optimizing the process to favor hydrogen production is crucial.

• Oxygen Sensitivity: *Escherichia coli*'s hydrogen production ceases in the presence of oxygen. Maintaining oxygen-free conditions in large-scale production systems presents an engineering challenge.

Despite these hurdles, *E. coli* remains a promising candidate for biohydrogen production due to its well-understood biology and ease of manipulation. Continued research on metabolic engineering and fermentation optimization can unlock *E. coli*'s full potential as a clean and renewable hydrogen source.

Native microorganisms in paper and cardboard waste

Paper and cardboard waste harbors a diverse community of native organisms, primarily consisting of various microorganisms. Here is a breakdown of some common types:

• Bacteria: Species like *Bacillus*, *Pseudomonas*, and *Cellulomonas* are naturally present. These bacteria play a crucial role in decomposing cellulose and lignin, the main components of paper and cardboard. They break down these complex molecules into simpler sugars, which they can then utilize for energy.

• Fungi: Fungal species like *Aspergillus* and *Penicillium* are also commonly found. Fungi contribute to the degradation process by secreting enzymes that further break down cellulose and lignin. Some fungi can also degrade other components, like starches and adhesives present in certain paper products.

• Mould: Moulds, which are a type of fungus, can also be present. They thrive in moist environments and contribute to the decomposition process. However, excessive mould growth can be detrimental, as it can weaken the paper and create unpleasant odors.

• Yeast: While less common, some yeast species may be present in paper and cardboard waste. These yeasts can ferment sugars released during the degradation process, producing alcohols and carbon dioxide as byproducts.

It is important to remember that the specific composition of this microbial community will vary depending on factors like the type of paper or cardboard, moisture content, and storage conditions. However, these native organisms play a vital role in the natural biodegradation process, breaking down paper and cardboard waste into simpler components that can be reabsorbed into the environment.

Acetate Production and Biohydrogen Production

Acetate production and biohydrogen production can be interconnected but have opposing goals. Here we can have a breakdown of how the process happens:

• Microbial Fermentation: The breaking down of organic compounds like cellulose and lignocellulose by microorganisms in the absence of oxygen, producing products like lactate, acetate, formate, ethanol, hydrogen sulphide, and methane.

• Competing Metabolic Pathway: The metabolic pathway that *E. coli* selects depends on factors such as whether there is any presence of oxygen; then it would carry out normal respiration and hydrolyze

hydrogenase enzymes and prevent the hydrogen pathway.

• Electrochemical Inhibition: In some biohydrogen production methods using microbial electrolysis cells (MECs), acetate can accumulate and interfere with the electrical processes needed for hydrogen production.

Strategies to Minimize Acetate Production

• Optimizing Culture Conditions: Scientists can manipulate factors like pH, temperature, and substrate composition in the fermentation process to favor hydrogen production pathways over acetate production pathways.

• Selecting Hydrogen-Producing Bacteria: Certain bacterial strains are naturally more efficient hydrogen producers with lower acetate production. Research focuses on identifying and utilizing these strains.

• Two-Stage Processes: Some approaches involve separating the fermentation process into two stages. In the first stage, hydrogen is produced. Then, the remaining organic matter is processed in a separate stage to convert it into valuable products like acetate.

Using Acetate for Biohydrogen Production (Indirectly): While directly minimizing acetate production is often desired for biohydrogen, there are alternative approaches:

• Photosynthetic Bacteria: Certain photosynthetic bacteria can utilize acetate as a fuel source to produce hydrogen gas through a light-dependent process. This offers a way to utilize the acetate produced during fermentation as a feedstock for further hydrogen generation.

Overall, acetate production and biohydrogen production are intertwined. While minimizing acetate is often preferred for efficient biohydrogen, there are also strategies to utilize acetate for further hydrogen generation in a two-step process or through specific types of bacteria.

Materials And Methods

Lignocellulosic biomass

Lignocellulose, the most abundant raw material for biofuels, consists of three challenging components: lignin, a tough and hydrophobic polymer primarily valued for its fuel potential; hemicellulose, a polysaccharide closely linked to lignin, complicating sugar extraction; and cellulose, a glucose-based polymer that requires enzymatic breakdown for biofuel conversion. The percentage composition of paper and cardboard can vary widely depending on factors such as the type of material, manufacturing process, and intended use. However, in general, it consists of cellulose fibers ranging from around 60% to 95%, depending on factors such as the quality of the pulp used and the desired properties of the final product. Lignin ranges from 2% to 30%. Higher-quality papers, such as those used for printing or packaging, tend to have lower lignin content, while recycled papers may contain higher levels. Hemicellulose comprises around 5% to 20% of the composition. Additives, such as sizing agents, fillers, and coatings, can make up a small percentage of the composition, usually less than 5% [1].

Significance of lignocellulosic biomass

Lignocellulosic biomass (LCB), such as crop residues and wood chips, is crucial for sustainable energy [2]. It offers a renewable feedstock for biohydrogen production. Its abundance supports waste reduction, resource efficiency, and economic opportunities for farmers. Despite these benefits, challenges such as improving technology and ensuring sustainable sourcing need attention for widespread use, highlighting the crucial role of LCB in promoting a greener future. Moreover, harnessing lignocellulose for biohydrogen production provides a renewable energy source and addresses waste utilization, contributing to a more sustainable and environmentally friendly energy source.

Hydrogen production using lignocellulosic substrates like paper and cardboard waste

Hydrogen production from materials like paper and cardboard waste offers several significant benefits. These materials, derived from plants, are renewable and abundant, providing a sustainable energy source [3]. By converting these waste products into hydrogen, we effectively manage and repurpose them, contributing to waste reduction and a circular economy. The resulting hydrogen is considered environmentally friendly, as its production and use are roughly carbon-neutral. Diversifying feedstocks, including paper and cardboard, enhances the flexibility of hydrogen production.

Additionally, research in biomass conversion technology promotes advancements, improving efficiency and cost-effectiveness. Localized production using abundant waste materials reduces the need for long-

distance transportation. Hydrogen, derived from these substrates, is a versatile energy carrier applicable across various sectors. Integrating hydrogen production with biorefineries adds value by simultaneously generating other useful products, supporting economic viability. In summary, hydrogen production from lignocellulosic substrates contributes to sustainability, waste reduction, and the development of cleaner energy, aligning with global efforts to move away from fossil fuels and address environmental challenges.

Microorganisms in biohydrogen production

Escherichia coli

Escherichia coli is a rod-shaped bacterium often found in the intestines of warm-blooded organisms. It thrives at 37°C and can live on different substances. When oxygen is scarce, it undergoes mixed-acid fermentation, producing various compounds such as lactate, succinate, ethanol, acetate, and carbon dioxide. *Escherichia coli* can undergo various fermentation pathways, such as mixed-acid and butyric acid fermentation, in anaerobic conditions, leading to the production of hydrogen gas. When conditions are favorable, *E. coli* can reproduce every 20 minutes and needs a specific medium containing carbon and energy. It is heavily researched and commonly used in biotech and microbiology, especially for studying DNA recombinants.

Escherichia coli, a common bacterium, holds promise as a biological tool for hydrogen production. Here is why:

• Natural Hydrogen Production: *Escherichia coli* can produce hydrogen gas during fermentation, particularly under oxygen-depleted conditions [4].

• Metabolic Engineering: Scientists can genetically modify *E. coli* to enhance its hydrogen production capabilities. This involves introducing genes for enzymes that increase hydrogen yield or optimize metabolic pathways toward hydrogen generation.

• Fast Growth: *Escherichia coli* reproduces rapidly, allowing for large-scale hydrogen production in a relatively short time frame.

However, there are challenges to consider:

• Low Hydrogen Yield: Currently, natural hydrogen production by *E. coli* is inefficient. Research is ongoing to improve this.

• Competition with Other Fermentation Products: During fermentation, *E. coli* produces various byproducts besides hydrogen. Optimizing the process to favor hydrogen production is crucial.

• Oxygen Sensitivity: *Escherichia coli*'s hydrogen production ceases in the presence of oxygen. Maintaining oxygen-free conditions in large-scale production systems presents an engineering challenge.

Despite these hurdles, *E. coli* remains a promising candidate for biohydrogen production due to its well-understood biology and ease of manipulation. Continued research on metabolic engineering and fermentation optimization can unlock *E. coli*'s full potential as a clean and renewable hydrogen source.

Hydrogen Production using Escherichia coli

Escherichia coli is one of the common organisms used as a model for biohydrogen production. This is because *E. coli* has a specialized enzyme known as hydrogenase in it, notably [Ni-Fe] and [Fe-Fe] hydrogenases, which are crucial for hydrogen production. In the wild strain or native conditions, the yield of hydrogen can be minimal, but it can be solved by turning to genetic engineering to produce *E. coli* strains that can produce high amounts of hydrogen. Another benefit of utilizing *E. coli* as our chief microorganism is that it can utilize substrates such as glucose or glycerol, and the choice of substrate can be factored into hydrogen yield. The challenges in front of us due to using *E. coli* include pathway competition and toxicity due to hydrogen. To overcome such challenges co-culturing method can be adapted; despite its ability to thrive in both aerobic and anaerobic conditions, practical applications can be limited by substrate costs and process scalability.
Under anaerobic conditions, *E. coli* carries out the formate pathway, a central role in hydrogen production. In the formate pathway, phosphoenolpyruvate is converted to oxaloacetate via phosphoenolpyruvate carboxylase, leading to succinate formation through malate and fumarate intermediates. Also, phosphoenolpyruvate is directly converted to pyruvate, which splits into formate and acetyl-CoA by pyruvate formate lyase under anaerobic conditions. Formate is then converted to hydrogen and carbon dioxide by the formate hydrogenase complex, while acetyl-CoA is converted into acetate [5].

Dark Fermentation

Escherichia coli can produce biohydrogen through dark fermentation in the absence of oxygen. It uses different substrates, like glucose or formate, to initiate the process. *Escherichia coli* has special pathways in its metabolism that help it produce hydrogen gas as a byproduct. It consists of enzymes called hydrogenases that help in converting protons and electrons into hydrogen gas. Along with hydrogen, *E. coli* also produces other products like acetate, formate, ethanol, and lactate. The efficiency of making biohydrogen depends on parameters like pH, temperature, and genetic modifications that enhance the production of more hydrogen. This way, using *E. coli* for dark fermentation to make biohydrogen can be a good way to create renewable energy when oxygen is not around.

Culturing Techniques

Culturing is a process by which microorganisms are allowed to grow and multiply in number in a particular medium under laboratory conditions. It involves providing suitable conditions and nutrients for bacterial growth and reproduction. Here are some common culturing techniques used for bacteria:

• Agar Plate Culture: Agar plate culture is a widely used method for bacterial culturing. It involves preparing a solid growth medium by adding agar to a nutrient broth. The medium is poured into Petri dishes, allowed to solidify, and then inoculated with a small amount of bacterial sample. The bacteria grow as visible colonies on the agar surface, allowing for the isolation and identification of different bacterial strains.

• Liquid Broth Culture: Liquid broth culture involves growing bacteria in liquid media. Nutrient-rich broth, such as Biebl-Pfenning, is to be used. Bacterial samples are added to the broth and incubated with gentle shaking to promote growth. Liquid broth cultures are often used for large-scale bacterial growth and protein expression or to obtain a high bacterial cell count for downstream applications.

Acetate Production

Acetate production and biohydrogen production can be interconnected but have opposing goals. Here is a breakdown of the relationship:

Acetate Production: Acetate (acetic acid) is a common byproduct of various biological processes, including:

• Microbial Fermentation: When bacteria break down organic matter (like sugars or starches) in an oxygen-limited environment, they can produce acetate alongside other products like hydrogen, ethanol, or carbon dioxide.

Biohydrogen Production: Biohydrogen production aims to harness certain bacteria to generate hydrogen gas as the main product. Acetate production during biohydrogen fermentation is often undesirable because:

• Competing Pathway: Microbial pathways that lead to acetate production compete with pathways for hydrogen generation. This reduces the overall yield of hydrogen.

• Electrochemical Inhibition: In some biohydrogen production methods using MECs, acetate can accumulate and interfere with the electrical processes needed for hydrogen production.

Strategies to Minimize Acetate Production:

• Optimizing Culture Conditions: Scientists can manipulate factors like pH, temperature, and substrate composition in the fermentation process to favor hydrogen production pathways over acetate production pathways.

• Selecting Hydrogen-Producing Bacteria: Certain bacterial strains are naturally more efficient hydrogen producers with lower acetate production. Research focuses on identifying and utilizing these strains.

• Two-Stage Processes: Some approaches involve separating the fermentation process into two stages. In the first stage, hydrogen is produced. Then, the remaining organic matter is processed in a separate stage to convert it into valuable products like acetate.

Methodology

Collection and Processing of Raw Materials

Cardboard and paper were gathered, dried, and blended into a slurry. A vacuum filter was used to filter the ground substrate, and the resulting filtrate was gathered for additional processing. Additionally, pulp was created by combining sterilized water with the ground substrate.

Culture Collection and Storage

Escherichia coli was ordered in active form from the University of Kerala. It was, therefore, stored and subcultured. Through MTCC, a culture of *Trametes versicolor* was ordered from the CSIR-Institute of Microbial Technology in Chandigarh. Additionally, it was subcultured.

Pretreatment Methods

Pretreatment is done to overcome the recalcitrant nature of LCB. Pretreatment can disintegrate the lignin and hemicellulose present in the complex structure. In general, LCB consists of cellulose (35%-50%), hemicellulose (20%-35%), and lignin (5%-30%) as its three major components. These fibers can be broken down into oligosaccharides that are easily utilized by microbes to produce hydrogen. Chemical pretreatment was carried out by using 0.75% sulfuric acid and autoclaving for one hour. Biological pretreatment was performed by treating the slurry with *Trametes versicolor* to enhance hydrogen production through the laccase activity of fungus for 7 days.

Pretreatment using Trametes versicolor

Trametes versicolor, commonly known as turkey tail mushroom, is a species of fungus belonging to the genus *Trametes*. It is characterized by its fan-shaped or semicircular fruiting bodies, which typically grow in overlapping clusters on dead or decaying wood. The upper surface of the fruiting body displays concentric bands of various colors, resembling the tail feathers of a turkey, and hence its common name. This fungus is widely distributed and can be found in forests, woodlands, and other natural habitats throughout the world. It is a saprotrophic organism, meaning it obtains nutrients by decomposing dead organic matter, particularly wood. *Trametes versicolor* plays an important ecological role as a decomposer, contributing to the breakdown of lignocellulosic materials such as fallen branches and tree trunks. *Trametes versicolor* is known for its production of laccase enzymes, which are members of the multicopper oxidase enzyme family. Laccases play a crucial role in lignin degradation, essential for the breakdown of LCB. They catalyze the oxidation of phenolic compounds in lignin, leading to its depolymerization and eventual breakdown into smaller, more accessible molecules [6]. Overall, *Trametes versicolor* is a significant organism in ecosystems, contributing to nutrient cycling and possessing biotechnological potential through its enzymatic activities.

Acid Hydrolysis

Acid hydrolysis is a crucial process in converting LCB into sugars, which can then be utilized for various purposes, notably biofuel production. Initially, the LCB, which could be sourced from materials like wood chips or agricultural residues, undergoes pretreatment to enhance its accessibility for hydrolysis. Acid hydrolysis using H_2SO_4 facilitates the breakdown of LCB to release fermentable sugars, which are used in biohydrogen production through microbial fermentation [7]. Biomass is subjected to acid hydrolysis, commonly utilizing dilute acids such as sulfuric acid or hydrochloric acid. During this step, the acid acts to break down the cellulose and hemicellulose polymers into their constituent sugars, including glucose, xylose, and other monosaccharides. The hydrolysis reaction is carefully controlled in terms of temperature, pressure, and acid concentration to maximize sugar yields while minimizing undesired side reactions. Figure *1* shows the substrate after acid treatment. Following hydrolysis, the mixture is neutralized to halt the acid reaction and then filtered to separate the liquid containing the sugars from the solid residue. This sugar-containing liquid, known as hydrolysate, can then be used for biohydrogen production.

FIGURE 1: Substrate sample following acid pretreatment

Fermentation

For four days, anaerobic fermentation was conducted with *E. coli* utilizing treated and pretreated substrates. By using quantitative analysis, the amount of hydrogen generated in both substrates was compared. Figure *2* depicit the anaerobic provision done to carry out dark fermentation.

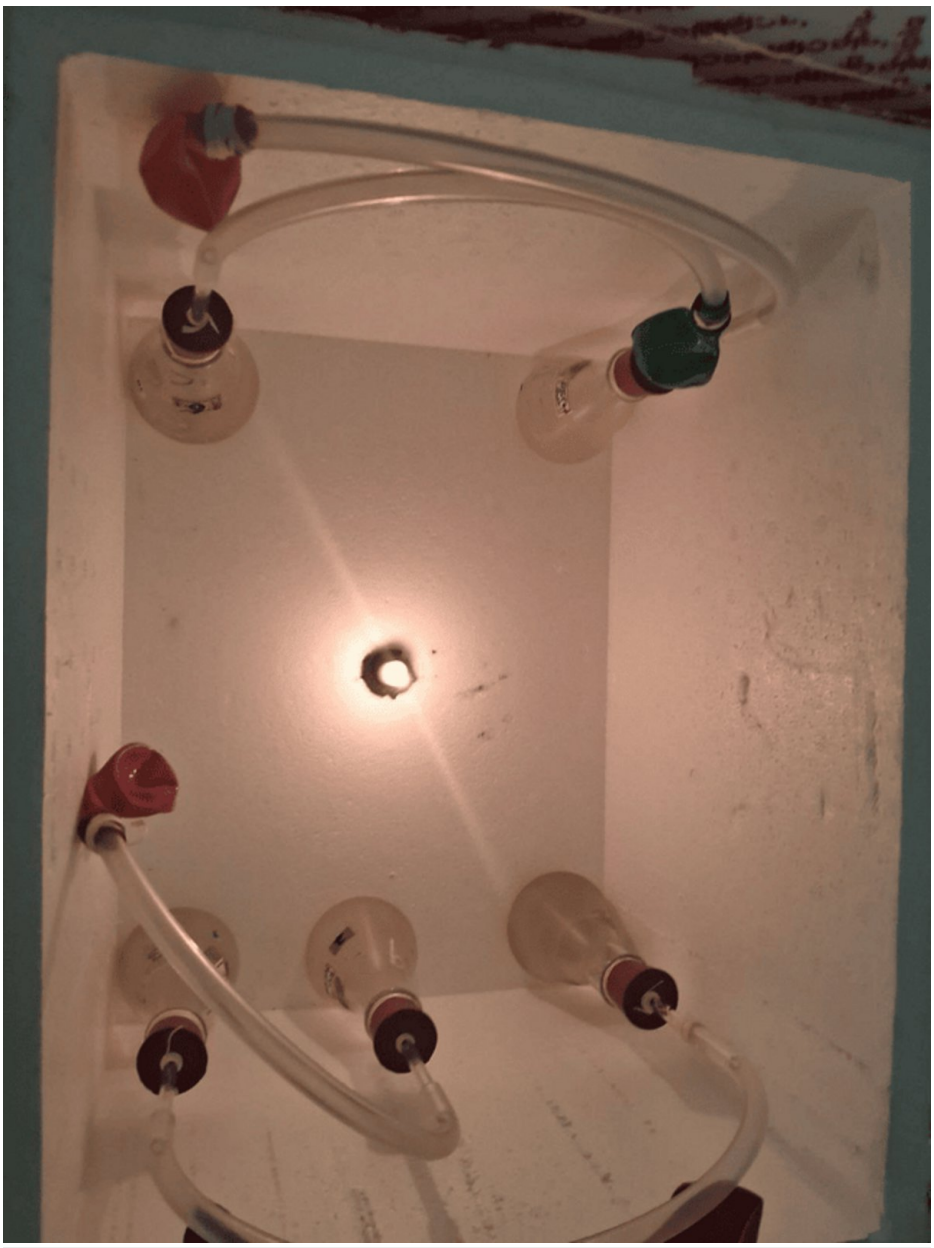

FIGURE 2: Provision of anaerobic environment for substrate fermentation

Biohydrogen Measurement

Multiple gas analyzer from Council of Scientific & Industrial Research - National Institute For Interdisciplinary Science and Technology, Thiruvananthapuram, were used to perform a quantitative analysis of the gas generated. The low concentration of hydrogen gas produced may be due to the production of acetate in the substrate. So we went for the estimation of acetate formed using the titration method.

Titration Method

Using phenolphthalein as an indicator, titration was performed against a known concentration of NaOH solution, and the amount of acetate produced was recorded.

Conductivity Measurement

To measure the conductivity of a solution containing acetate using a TDS meter, first calibrate the meter with a standard solution to ensure accuracy. Then, prepare the acetate solution by dissolving an appropriate amount of acetate compound, such as sodium acetate, in distilled water. Rinse the TDS meter

probe with distilled water to avoid contamination, then immerse it in the acetate solution. The TDS meter will measure the total dissolved solids, providing an indirect indication of the solution's conductivity.

Confirmation test for acetate

Ferric Chloride Test

Neutral $FeCl_3$ is prepared by adding dilute NaOH to ferric chloride until a permanent reddish-brown precipitate forms, then adding ferric chloride until the precipitate just dissolves, and the resulting solution is used to detect acetate ions by producing a red coloration when water is added and heated. The chemistry behind the $FeCl_3$ test involves the reaction between ferric chloride ($FeCl_3$) and acetate ions (CH_3COO-).

When ferric chloride is added to a solution containing acetate ions, the acetate ions react with the ferric chloride to form an iron(III) acetate complex, according to the equation:

$$FeCl_3 + 3CH_3COOH \rightarrow (CH_3COO)_3Fe + 3HCl$$

In this reaction, the acetate ions from acetic acid (CH_3COOH) replace the chloride ions in $FeCl_3$, resulting in the formation of iron(III) acetate [$(CH_3COO)Fe$] and the release of hydrochloric acid (HCl) as a byproduct. The iron(III) acetate complex formed in the reaction typically exhibits a red coloration, attributed to the interaction between the iron(III) ion and the acetate ligands in the complex. Consequently, the $FeCl_3$ test relies on the appearance of this red-colored iron(III) acetate complex when ferric chloride is introduced to a solution containing acetate ions, serving as a visual indicator of the presence of acetate ions in the solution.

Results

pH analysis of the substrate

pH is defined as the negative logarithm of H^+ ion concentration. Hence, the meaning of the name pH is justified as the power of hydrogen: $pH = -\log[H^+]$. As the anaerobic fermentation process progresses, *E. coli* utilizes its glucose substrate to produce formate and follows the formate cycle, during which hydrogen is produced. The hydrogenase enzyme plays an integral and vital role in hydrogen evolution. If the enzyme is inhibited instead of undergoing a formate cycle, *E. coli* will produce acetate. In our procedure, we divided our samples as controls, from which samples are taken periodically every two hours (from April 4, 2024, to April 8, 2024) for analysis. Another set of samples was analyzed before it was kept for anaerobic fermentation (on April 5, 2024) and after the fermentation process was completed (on April 11, 2024). The pH was analyzed with the help of a pH meter. When the pH of the control sample becomes stabilized or constant, the fermentation is said to be complete. Figure *3* and Figure *4* show the gradual change in pH of the substrate during the fermentation period. As we can see from Table *1*, the homogenous paper mixture's pH is the highest among the untreated substrates. This can be due to two reasons:

• The production of acetate, a conjugate base of acetic acid, which is a strong base, increases the pH of the sample.

• Also, there can be changes due to dyes, such as cadmium yellow and phenolic compounds in newspapers, which can also contribute to this increase in pH.

Substrate	pH before fermentation	pH after fermentation
Homogeneous paper	6.6	7.93
Homogeneous cardboard	6.56	7.29
Heterogeneous	6.7	7.98
Acid-treated homogeneous paper	7.54	8.08
Acid-treated homogeneous cardboard	5.85	6.96
Acid-treated heterogeneous	5.99	6.74
Fungal-treated homogeneous paper	6.57	8.05
Fungal-treated homogeneous cardboard	6.67	6.55
Fungal-treated heterogeneous	6.89	7.24

TABLE 1: Initial and final pH values of substrate in biohydrogen fermentation trials

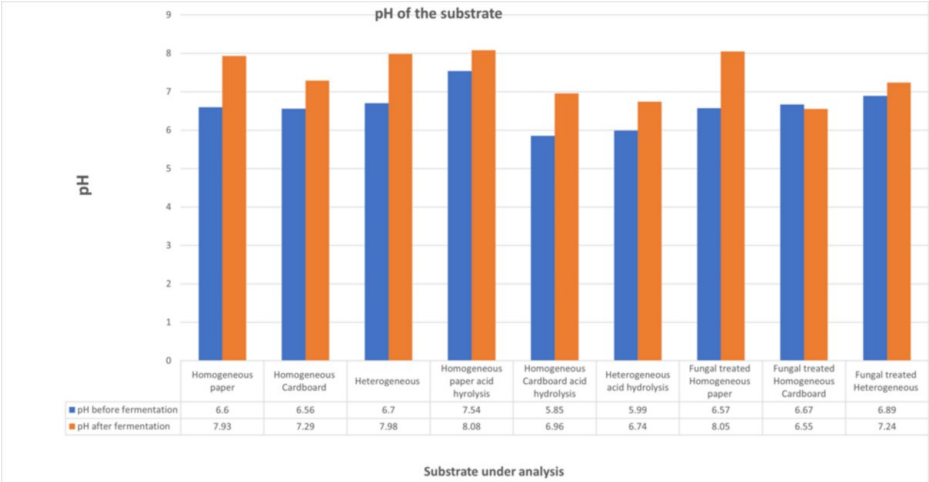

FIGURE 3: Variation in pH of the substrates during the fermentation period

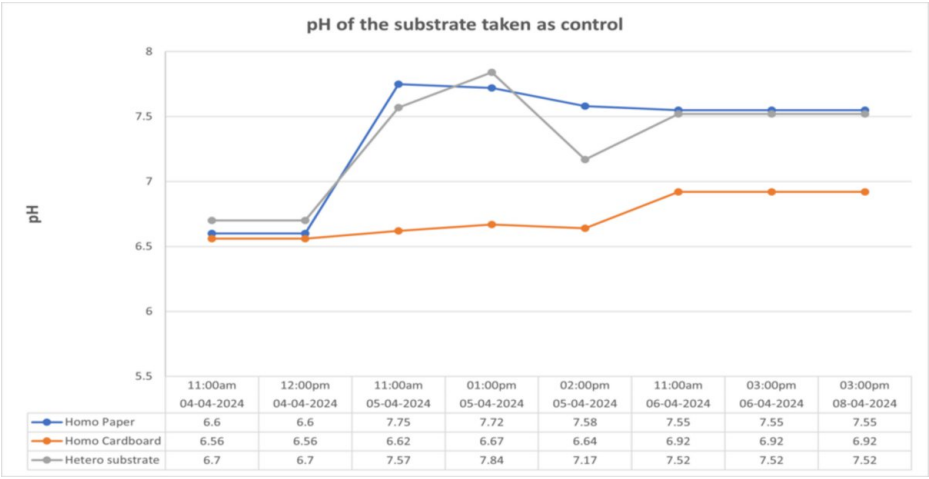

FIGURE 4: Measured pH of the sample during the experimental analysis

Absorbance of the substrate under analysis

In many scientific projects, a vital component is the absorbance, which can be determined with an optical density (OD) meter. This measure expresses a substance's concentration or purity by quantifying the amount of light it absorbs. We can learn more about the interactions of molecules by examining the absorbance spectrum at various wavelengths. The OD meter proved to be a dependable instrument for evaluating sample concentration, purity, and reaction kinetics in our project. The reliability and credibility of our findings were improved by the accurate experimental design and analysis made possible by this data. To precisely estimate cell growth from observed OD measurements, we used the spectrophotometer. For our required data, we measured the OD at 520 nm. For 1 week (05/04/24 to 11/04/24), we quantified the values of nine substrates, untreated, acid-treated, and fungal-treated (each of homogeneous paper, homogeneous cardboard, and heterogeneous) that had been inoculated with *E. coli*. From the results, it was observed that an equal trend was not followed, but noticeable changes in the initial and final values were observed. In the non-acid hydrolyzed substrate, OD increased for homogeneous paper and cardboard, showing that the growth of *E. coli* had occurred and favorable conditions were available for its sustenance. However, due to the presence of synthetic dyes in paper, the growth of *E. coli* was inhibited due to phenolic components, xylenes, and others. This was also a reason for the decrease in OD for the heterogeneous medium, which is the mixture of both paper and cardboard. Due to the absence of dyes in cardboard, the growth of *E. coli* was favorable, leading to an exceptional increase in the value. Figure *5* and Figure *6* denote how the OD of the solution changes as the microbial fermentation happens in the substrate. Regarding the acid-hydrolyzed substrate inoculated with *E. coli*, a noticeable trend of decreased OD values was observed. This was probably due to the extreme conditions offered by the acid H_2SO_4. Table *2* shows how the OD gradually changes over a period of time. Acid-pretreated media may not have provided a favorable environment for growth and development, resulting in this value. In conclusion, it can be assumed that homogeneous cardboard was expected to provide better conditions for the growth of *E. coli*, and it could be a sensible medium for giving maximum hydrogen production. In fungal-treated substrates, there was a noticeable decrease in the absorbance value, which may be due to inadequate elimination of fungus from the substrates before inoculating with *E coli*, which may have caused inhospitable conditions for the bacterium.

Substrate	OD before fermentation	OD after fermentation
Homogeneous paper	0.157	0.18
Homogeneous cardboard	0.204	0.245
Heterogeneous	0.214	0.182
Acid-treated homogeneous paper	0.222	0.187
Acid-treated homogeneous cardboard	0.342	0.152
Acid-treated heterogeneous	0.16	0.127
Fungal-treated homogeneous paper	0.208	0.066
Fungal-treated homogeneous cardboard	0.316	0.032
Fungal-treated heterogeneous	0.202	0.053

TABLE 2: Absorbance values of the substrate before and after fermentation

OD, optical density

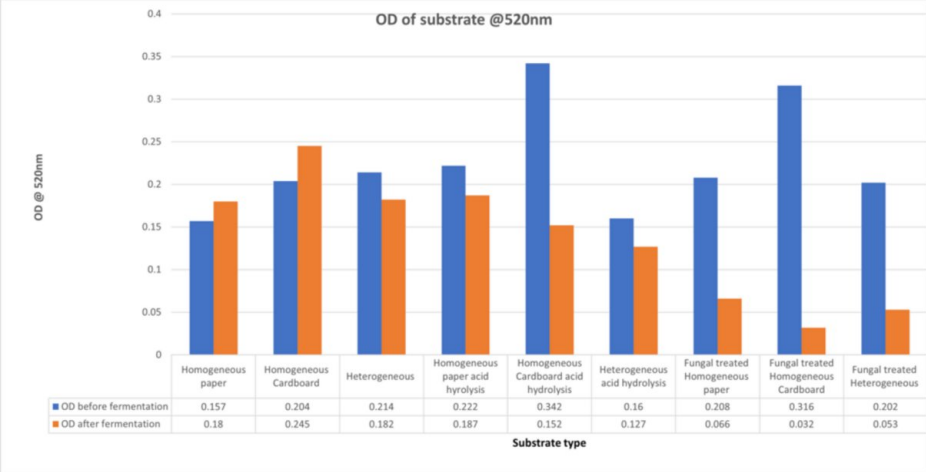

FIGURE 5: Variation in substrate absorbance (520 nm) throughout the fermentation process

OD, optical density

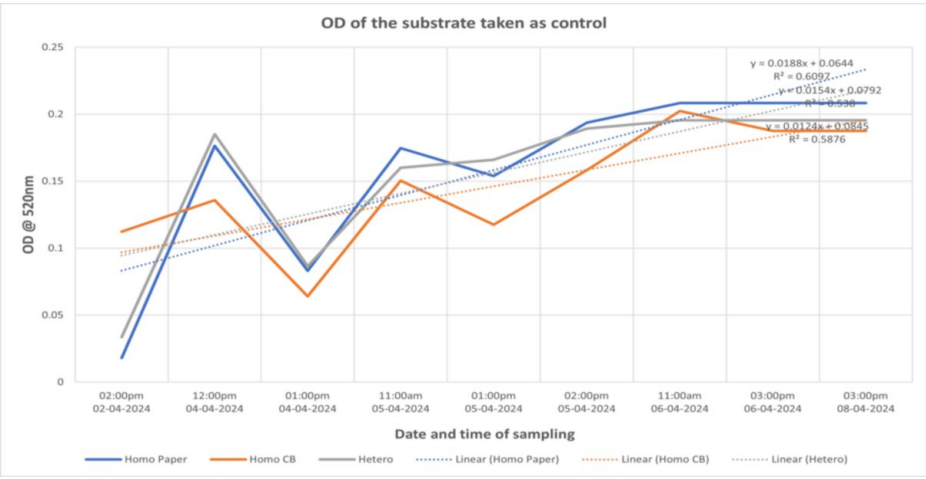

FIGURE 6: Control sample absorbance measured at 520 nm

OD, optical density

DNS estimation for reduced sugar

The dinitrosalicylic acid (DNSA) method is a non-specific method used to estimate reducing sugar concentration in a sample. It detects the presence of a free carbonyl group (C = O) of reducing sugars. This involves the oxidation of the aldehyde functional group (in glucose) and the ketone functional group (in fructose) [8]. During this reaction, DNSA is reduced to 3-amino-5-nitrosalicylic acid, which under alkaline conditions is converted to a reddish brown-colored complex with an absorbance maximum of 520 nm.

3, 5- Dinitrosalicylic acid + Reducing Sugar → 3-Amino-Nitrosalicylic acid + Oxidized sugar

Escherichia coli can use the reduced sugar in the substrate and use it for its metabolic pathway. As the time increases, there is the chance of using the reducing sugar in the sample by *E.coli*, which can be estimated using the DNS method. The values obtained with different substrates are given in Table 3 and Figure 7, showing the concentration of reducing sugar (mg/ml) for different substrates before and after fermentation using *E. coli*.

From Figure 7, we can see that there is a decrease in the concentration of reducing sugar due to the utilization of reducing sugars by *E.coli*. Also, it was observed that without treatment, for acid-treated substrate and for biologically treated substrate maximum utilization of reducing sugar was observed in

homogeneous cardboard. In biological treatment, we can see that there is less concentration of reducing sugar before fermentation. This may be due to the utilization of the reducing sugar by *Trametes versicolor* for its activity. Also, Homogeneous cardboard without any pretreatment was found to have more reduced sugar utilization than with pretreated Homogeneous cardboard substrate. It means that for untreated, acid-treated, and biologically treated substrates, homogeneous cardboard showed maximum utilization of reducing sugar by *E.coli*, with untreated homogeneous cardboard exhibiting higher levels of utilization compared to pretreated cardboard.

Substrate	Initial concentration (mg/ml)	Final concentration (mg/ml)
Homogeneous paper	0.0155	0.0077
Homogeneous cardboard	1.0065	0.0129
Heterogeneous	0.10444	0.102
Acid-treated homogeneous paper	0.1919	0.0147
Acid-treated homogeneous cardboard	0.2957	0.0103
Acid-treated heterogeneous	0.1383	0.0121
Fungal-treated homogeneous paper	0.025	0.0147
Fungal-treated homogeneous cardboard	0.1029	0.0129
Fungal-treated heterogeneous	0.0164	0.0008

TABLE 3: Quantification of reducing sugars by 3,5-dinitrosalicylic acid (DNS) method

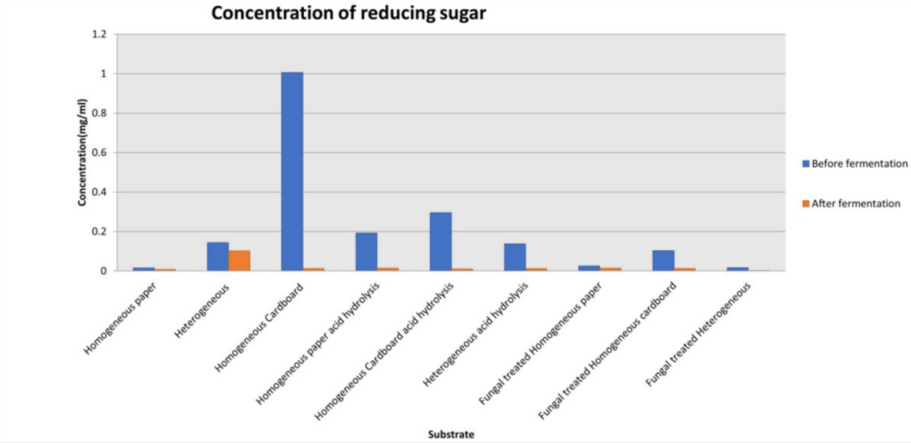

FIGURE 7: Reducing sugar levels estimated via DNS assay throughout the fermentation period

DNS, 3,5-dinitrosalicylic acid

Volume of gas evolved

The gas that evolved during the anaerobic fermentation period was collected in the balloon and the volume of gas was analyzed by noting down the circumference of the balloon and assuming the shape of the balloon to be a sphere. Figure *8* shows the increase in gas over the period of time. The volume of gas evolved was estimated. Table *4* shows the increase in circumference of the balloon corresponding to the evolution of gas.

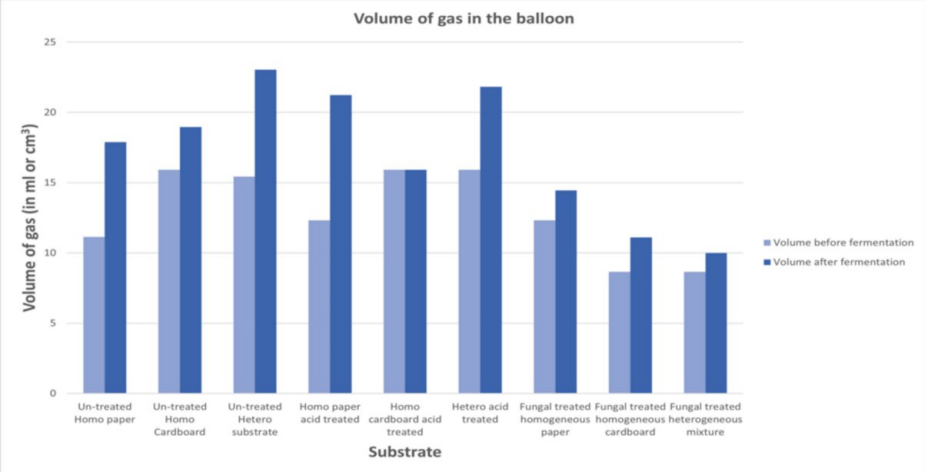

FIGURE 8: Comparison of gas volume evolved before and after fermentation

Substrate	Volume before fermentation (ml)	Volume after fermentation (ml)
Homogeneous paper	11.13	17.89
Homogeneous cardboard	15.91	18.95
Heterogeneous	15.43	23.05
Acid-treated homogeneous paper	12.32	21.23
Acid-treated homogeneous cardboard	15.91	15.91
Acid-treated heterogeneous	12.32	14.45
Fungal-treated homogeneous paper	12.32	14.45
Fungal-treated homogeneous cardboard	8.65	11.1
Fungal-treated heterogeneous	8.65	9.8

TABLE 4: Comparative analysis of gas evolution during fermentation

Gas composition analysis using multiple gas analyzer

The concentration of different gases evolved during the fermentation period was analyzed with the help of multiple gas analyzers. This process determined the absence of hydrogen, and the main component of the fermentation process was carbon dioxide. Table *5* shows the concentration of different gases such as carbon dioxide (ppm), hydrogen (ppm), methane (%LEL) and H2S (ppm) released during the fermentation process.

Substrate	CO_2 (ppm)	H_2S (ppm)	CH_4 (%LEL)	H_2 (ppm)
Homogeneous paper	9	0	0	0
Homogeneous cardboard	8	0	0	0
Heterogeneous	24	0	0	0
Acid-treated homogeneous paper	8	0	0	0
Acid-treated homogeneous cardboard	50	0	0	0
Acid-treated heterogeneous	20	0	0	0

TABLE 5: Quantitative analysis of gaseous components using a multiple gas analyzer

Acetate confirmation test - neutral ferric chloride test

The sample after fermentation was confirmed to have acetate content by the ferric chloride test.

$FeCl_3 + 3\ CH_3COOH \rightarrow (CH_3COO)_3Fe + 3\ HCl$

Procedure: A few milliliters of the sample were taken in a test tube. Neutral ferric chloride was added to the solution. The solution was then filtered. The filtrate was divided into two parts: to one part, dilute HCl was added, which led to the discoloration of the solution. Water was added to the other part and boiled until a reddish precipitate was observed. Figure *9* shows the visual change in the sample after the test, showcasing the presence of acetate in the solution.

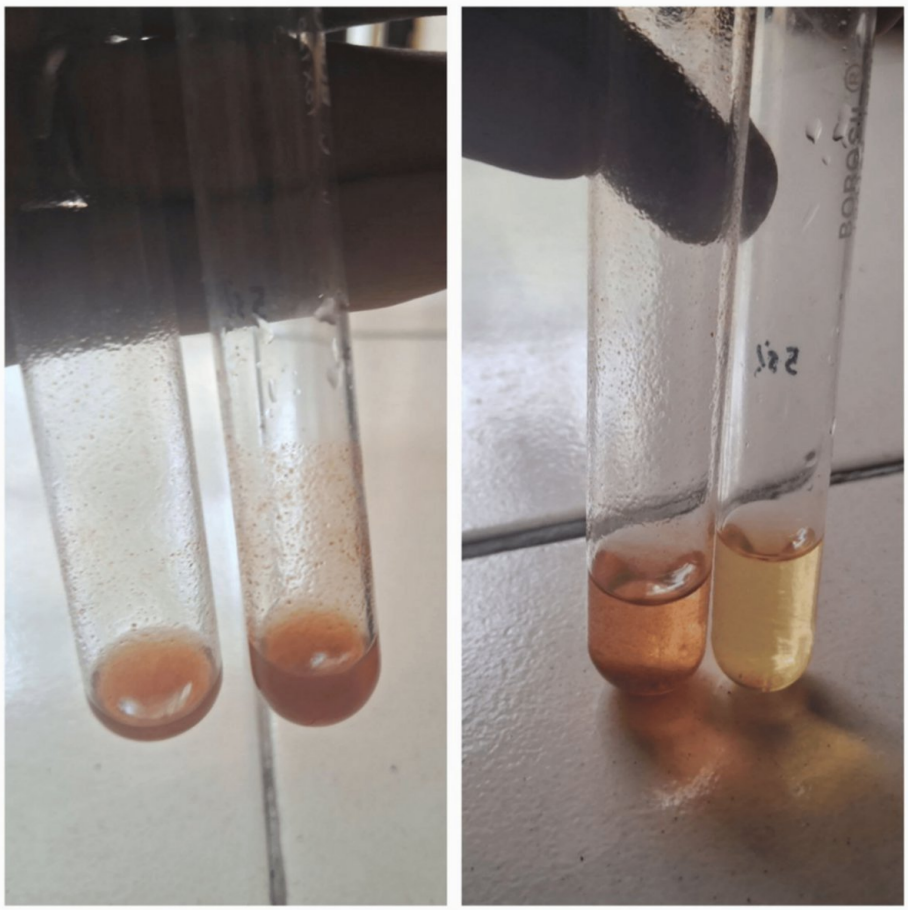

FIGURE 9: Visual changes in the sample before and after ferric chloride test

Quantification of acetate concentration by titration method

In the absence of hydrogen production during *E. coli* fermentation, more acetate may be produced as a byproduct. This is because hydrogen is typically consumed in the production of acetate through pathways like mixed-acid fermentation. So when hydrogen is not formed, more resources might be diverted toward acetate production. When *E. coli* undergoes fermentation, one of the pathways involved is mixed-acid fermentation, where various organic acids, including acetate, are produced as byproducts. In this process, hydrogen gas is consumed through reactions such as the reduction of NAD^+ to NADH. However, if hydrogen is not formed during fermentation, it implies that alternative pathways are being favored or that hydrogen-consuming reactions are not occurring as expected. In the absence of hydrogen production, the balance of redox reactions within the cell may shift, favoring pathways that produce more acetate. This could happen because the availability of hydrogen influences the activity of enzymes involved in different metabolic pathways. Without hydrogen being produced, these enzymes might favor reactions leading to the production of acetate, as opposed to pathways that would consume hydrogen. Additionally, the absence of hydrogen might indicate that the cells are experiencing conditions where the production of acetate is more advantageous for survival or growth. This could be due to factors such as the availability of nutrients, pH, or the presence of other fermentation byproducts. Therefore, the relationship between the absence of hydrogen production and increased acetate production during *E. coli* fermentation is complex and likely involves a combination of factors related to enzyme activity, redox balance, and cellular metabolic needs. The titration is based on a general acid-base reaction to form water and salt. Sodium hydroxide acts as a strong base and is titrated against weak acid (acetic acid). Phenolphthalein is used as an indicator, and colorless to pink is the endpoint. The ferric chloride test verified the presence of acetate in the provided sample. Rather than concentrating on the hydrogen generated in the test sample, the attention was on the acetate that was made in it. The concentration of the acetate is subsequently determined by titrating the test sample against a known concentration of NaOH. In both untreated and acid-treated substrates, homogeneous cardboard was shown to have a higher concentration than its counterparts because of the absence of dyes and other phenolic compounds that are present in the paper. Whereas in a fungal-treated substrate, both paper and cardboard substrates have the same concentration of acetate. It is due to the ability of *Trametes versicolor* to degrade the dyes and other resistant compounds

present in the paper. While comparing both untreated and treated substrates, pretreated substrates (acid- and fungal-treated) have shown a higher concentration of acetate due to the presence of the reducing sugar that were utilized in the *E.coli*'s metabolic pathway. Table 6 shows the concentration of acetate produced during the fermentation, and we can see that acid treated homogenous cardboard, and fungal treated homogenous paper and cardboard had the highest acetate concentration

Substrate	Volume of NaOH (ml)	Concentration of Acetate (N)	Molarity of Acetate (M)
Homogeneous paper	0.3	0.0249	0.0249
Homogeneous cardboard	0.5	0.0417	0.0417
Heterogeneous	0.2	0.0167	0.0167
Acid-treated homogeneous paper	0.8	0.0667	0.0667
Acid-treated homogeneous cardboard	0.6	0.0499	0.0499
Acid-treated heterogeneous	0.5	0.0417	0.0417
Fungal-treated homogeneous paper	0.8	0.0667	0.0667
Fungal-treated homogeneous cardboard	0.8	0.0667	0.0667
Fungal-treated heterogeneous	0.4	0.0333	0.0333

TABLE 6: Acetate concentration measured via titrimetric method

Quantification of acetate concentration by conductivity measurement

The concentration of acetate was quantified with the help of a digital conductivity measuring device (TDS meter). The TDS meter measures the conductivity of the solution, which can be converted to its respective concentration using the help of a standard curve constructed from known concentrations and their corresponding conductivities. Figure 10 shows a curve-fitted graph to analyze the conductivity of the substrate after fermentation. Table 7 shows the conductivity and its corresponding concentration for further analysis.

Substrate	Conductivity (micro Ohm)	Concentration (M)
Homogeneous paper	1520	0.0492
Heterogeneous	1516.67	0.05
Fungal-treated homogeneous paper	926	0.03
Fungal-treated homogeneous cardboard	1430	0.0463
Fungal-treated heterogeneous	1340	0.0434

TABLE 7: Quantification of acetate concentration using conductivity analysis

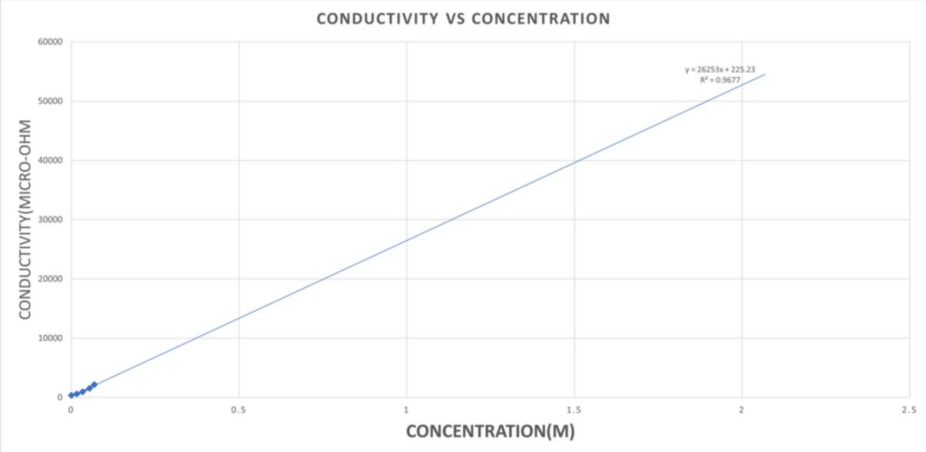

FIGURE 10: Calibration curve for acetate concentration determination

Discussion

Optimization for efficient biohydrogen production

For optimization purposes, different strains of bacteria must be searched to find the one with high hydrogen-producing capabilities. During the fermentation process, various inhibitory compounds could have accumulated, thus resulting in a negative impact. Optimization techniques involve identifying strategies to mitigate or manage these inhibitors, such as pH adjustment, feedstock supplementation, or using inhibitor-tolerant strains [9]. Controlling the pH and temperature in which it was carried on could also be adding factors to enhancing the efficiency of the process since it affects the metabolism and microbial growth. Sensors and feedback control algorithms can be used to monitor key process variables and adjust the conditions to optimize hydrogen production. Software such as MATLAB and COMSOL can be used to formulate and solve optimization problems such as parameters and conditions for enhancing the production of hydrogen.

Future studies

• We discovered that acetate, a crucial byproduct of our research, can be utilized to produce biohydrogen and other useful bio-products, highlighting the adaptability of our findings. Purple non-sulfur bacteria, such as *Rhodobacter*, can be employed in the dark fermentation process to create H_2 from acetate [10].

• Future directions for this research include genetically modifying *E. coli* to withstand inhibitors and exploring alternate bacterial strains. We can increase the efficiency of processing biomass by genetically modifying *E. coli* strains to be more resistant to furfurals, which are toxic byproducts of the acid hydrolysis process. By cultivating these altered strains on substrates such as cardboard and acid-hydrolyzed paper, the bioconversion process may be strengthened [11].

• To obtain even better findings, we plan to scale up our trials in the future. We can optimize the production process and yield of biohydrogen by adjusting critical variables such as the amount of substrate we utilize, the acidity of the substrate used, and the reactor design .

Conclusions

In the fast-moving economy, there is a need to find alternatives to fossil fuels and find sustainable solutions, and biohydrogen is one such solution. The production of hydrogen from the fermentation process is highly sustainable and underlooked and underappreciated, as researchers mostly focus on electrolysis methods. During this study, we utilized the formate metabolic cycle of *E. coli*, which produces formate from pyruvate and produces acetate as an intermediate during the production of hydrogen.

This study aimed to evaluate the potential of paper and cardboard waste as lignocellulosic feedstocks for biohydrogen production via dark fermentation using *Escherichia coli*. By utilizing pretreatment methods of acid and fungus, we tried to improve the substrate concentration by breaking down the hard lignin content and increasing hydrogen yield while continuously monitoring parameters such as pH, absorbance, reducing sugar concentration, and gas evolution.

Among the substrates we selected, homogenous cardboard claimed to be the best substrate, particularly in untreated and acid-treated parts. It showed significant microbial growth, indicated by an increase in OD

(from 0.204 to 0.245), and the highest reducing sugar utilization (from 1.0065 mg/mL to 0.0129 mg/mL in untreated and 0.2957 to 0.0103 mg/mL in acid-treated samples). The largest gas volume (23.05 mL) was observed in the untreated heterogeneous substrate, but homogeneous cardboard showed steady gas evolution (from 15.91 to 18.95 mL) across treatments.

Despite providing a setup for dark fermentation, hydrogen gas was not detected in the collected sample. It is due to either low-scale gas production, a problem in the anaerobic setup, or a wild strain of *E. coli*. Instead, acetate concentration was confirmed and quantified with the help of a neutral ferric chloride test and titration method, with maximum concentration observed in acid- and fungal-treated homogenous cardboard (0.0667 M). This suggests there was a shift in the *E. coli* metabolic pathway toward acetate production instead of hydrogen production, mainly due to the inhibition of hydrogenase because of the presence of oxygen in the fermentation setup.

From the results, we can conclude that homogenous cardboard, especially when acid- or fungally treated, is the most suitable candidate for hydrogen production through microbial fermentation. Even though hydrogen production was not achieved, the high acetate concentration indicates strong potential for the next stage in biohydrogen production, either by using a two-state hydrogen generation approach by utilizing *E. coli* for producing acetate and using purple non-sulfur bacteria for the production of hydrogen or by genetically engineering *E. coli* to ensure the production of hydrogen. The findings in the study show the feasibility of cardboard waste as a substrate in the lignocellulosic biorefinery system concerning the production of biohydrogen and give us a glimpse toward a sustainable future with renewable fuel and waste management.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: Arjun Babu SB, Gayathri Vijayakumar

Acquisition, analysis, or interpretation of data: Arjun Babu SB, Gayathri Vijayakumar

Drafting of the manuscript: Arjun Babu SB

Critical review of the manuscript for important intellectual content: Gayathri Vijayakumar

Supervision: Gayathri Vijayakumar

Disclosures

Human subjects: All authors have confirmed that this study did not involve human participants or tissue. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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