

DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**BUTYRIC ACID PRODUCTION FROM WASTE
SUBSTRATES**

by

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June, 2024

İZMİR

BUTYRIC ACID PRODUCTION FROM WASTE SUBSTRATES

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Program**

by

Gizem KARAKAYA

June, 2024

İZMİR

M. Sc THESIS EXAMINATION RESULT FORM

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BUTYRIC ACID PRODUCTION FROM WASTE SUBSTRATES

ABSTRACT

Butyric acid, produced via dark fermentation giving a valuable renewable energy of hydrogen gas as an end product, is a green chemical. In this thesis, different parameters were investigated to enhance butyric acid and hydrogen gas production.

At the beginning of the experiments, lactose and anaerobic sludge were used to investigate the effects of sodium chloride (0-6 g/L), and lactose (5-100 g/L) for butyrate and hydrogen production. Then, the impacts of sodium chloride, sodium thiosulfate, and mineral concentrations on butyric acid and hydrogen gas production was examined using the Box-Behnken Design with Cheese Whey Powder (CWP) via *Clostridium acetobutylicum*. Additionally, calcium carbonate concentrations (0-4 g/L) were optimized to maximize the butyrate and hydrogen production with *C. acetobutylicum*. Furthermore, the organism enhancing butyrate via hydrogen production pathway with CWP was selected. Anaerobic sludge, *C. acetobutylicum*, *C. beijerinckii*, and *C. pasteurianum* was used. Lastly, the effect of organism concentration (0.5-3 g/L) and substrate concentration (40-200 g/L) with *C. pasteurianum* was evaluated.

Finally, the evaluation of all experimental designs showed optimum conditions as follows. The highest butyric acid production by *C. pasteurianum* was observed as 6.8 g/L at 20 g/L CWP. The maximum butyric acid production yield and butyric acid production rate (BAPR) was 197.9 g BA/g TS and 1.6 g BA/g TS h at 5 g/L sodium chloride and 0.4 mM mineral concentrations, respectively. The highest butyric acid to acetic acid ratio was 7.68 g BA/g AA with 0.1 g/L calcium carbonate, and 15 g/L CWP.

Keywords: Butyric acid fermentation, Hydrogen Gas Production, Lactose, Cheese Whey Powder, *Clostridium pasteurianum*.

ATIK SUBSTRATLARDAN BÜTİRİK ASİT ÜRETİMİ

ÖZ

Bütirik asit karanlık fermantasyon yoluyla üretildiğinde ortaya çıkan son ürün olan değerli hidrojen gazı sayesinde yenilenebilir, temiz ve yeşil bir kimyasaldır. Bu tezde, bütirik asit ve hidrojen gazı üretimini arttırmak için farklı parametreler araştırılmıştır.

Öncelikle, substrat olarak laktoz, mikroorganizma olarak anaerobik çamur kullanılıp sodyum klorür (0-6 g/L) ile performans denemeleri yapılmış, daha sonra laktoz konsantrasyonu değiştirilerek (5-100 g/L) bütirik asit üretimi verimi incelenmiştir. Ardından NaCl, Na₂S₂O₃ ve mineral konsantrasyonlarının (Ni, Fe, Cu, Zn) bütirik asit ve hidrojen gazı üretimi üzerindeki etkisi, laktoz ve *C. acetobutylicum* ile Box-Behnken Tasarımı (BBD) kullanılarak araştırılmıştır. Ayrıca eser mineral optimizasyonuna ek olarak CaCO₃ konsantrasyonları (0-4 g/L) *C. acetobutylicum* ve Peynir Altı Suyu Tozu (PAT) ile bütirik asit ve hidrojen gazı üretimini maksimuma çıkaracak şekilde incelenmiştir. Daha sonrasında, peynir altı tozu ile bütirik asit üretim verimi için organizmanın seçimi değerlendirilmiştir. Bu amaçla anaerobik çamur, *C. acetobutylicum*, *C. beijerinckii* ve *C. pasteurianum* kullanıldı. Son olarak, *C. pasteurianum* ile organizma konsantrasyonunun (0,5-3 g/L) etkisi ve substrat konsantrasyon (40-200 g/L) değişiminin değerlendirilmesi ayrı ayrı yapılmıştır.

Sonuç olarak tüm deneysel tasarımların değerlendirilmesi aşağıdaki optimum koşulları gösterdi. En yüksek bütirik asit üretimi *C. pasteurianum* ile 20 g/L PAT'da 6,8 g/L olarak bulundu. Maksimum bütirik asit verimi ve hacimsel bütirik asit üretim hızına 5 g/L NaCl ve 0,4 mM mineral konsantrasyonlarında sırasıyla 197,9 g BA/g TS ve 1,6 g BA/g TS/saat olarak hesaplandı. Bütirik Asit/Asetik Asit oranı açısından en yüksek değer 0,1 g/L CaCO₃ ve 15 g/L peynir altı tozu ile 7,68 g BA/g AA olarak bulundu.

Anahtar kelimeler: Bütirik asit fermantasyonu, Hidrojen gazı üretimi, Peynir Altı Tozu, *Clostridium pasteurianum*.

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CHAPTER ONE

INTRODUCTION

1.1 Problem Statement

Nowadays, as we are familiar with the harmful effects of traditional fuel methods and synthetic chemicals on the environment, especially on global warming, humanity is turning towards green chemistry technologies (Sauer & Marx, 2023). The basic structure of green chemistry is to reduce the use of hazardous substances and earth-damaging methods in the production of chemical products and to achieve these products in more environmentally friendly ways (Clark & Deswarte, 2008). Green chemistry technologies aim to be sustainable and at the same time encourage economical means. In 2018, the European Union adopted a bio-economy strategy to achieve the goals of sustainable growth, environmental protection, and global warming set out in the 2030 agenda with the Paris Agreement commitments (Dessi et al., 2020). This context encourages production systems to implement the biorefinery concept, where waste is treated as an opportunity to diversify the product range, reducing the costs of biomass output, and waste treatment, and thus meeting increasingly stringent legislation on emissions (Dessi et al., 2020). Microbial transformation pathways are among the most sustainable and economical of all these processes. Unlike many chemical processors, it can be achieved without the need for excessive pressure, low temperature, and excess water. These microbial pathways can achieve complex, multi-step reactions catalysed by enzyme cascades (Sauer & Marx, 2023). In addition, microorganisms can reproduce themselves. No toxic waste streams are generated after microbial processes. With this microbial process, it has become possible to make the production of organic acids such as green hydrogen, butyric acid, and solvents sustainable.

The need for renewable energy has increased even more today. These valuable products were realized by forming acetone-butanol-ethanol (ABE) with fermentation processes. Scientists usually produce ABE with *Clostridium* species, which are also favoured in organic acid production. By *Clostridium* species via ABE fermentation process produced n-butanol with a ratio of 3:6:1, respectively (Bankar et al., 2013). The fermentation occurs in two stages: the acidogenic phase during exponential

growth and the solventogenic phase at the end of exponential growth. The acidogenic phase includes volatile fatty acids (VFA), hydrogen, CO₂, and other compounds. During the first phase, the pH decreases from 7 to 4.5-5. Then, the solventogenic phase follows, during which solvents such as butanol, acetone, and ethanol are produced. In the final phase, organic acids are reassimilated to produce solvents and other gases, such as CH₄ and CO₂.

Butanol is considered to be superior to other fermentation-derived fuels (Ezeji et al., 2007). Butanol has a lower vapour pressure than ethanol, making it more reliable for transport and is also known to be less flammable (Narueworanon et al., 2020). As a result of this study, it was reported that *C. beijerinckii* TISTR 1461 was the most suitable microorganism for butanol production among four *clostridia* species that are *C. beijerinckii* JCM 1390, *C. acetobutylicum* JCM 7829, *C. beijerinckii* TISTR 1461 and *C. beijerinckii* TISTR 1462 (Wechgama et al., 2017).

The dairy industry processes 170 billion L of milk per year in Europe, creating on average of 2.5 L of wastewater per L of milk processed and 9-10 L of whey (CW) per kg of cheese produced (Dessi et al., 2020). Like other food production activities, the dairy industry is known to generate large quantities of liquid waste characterized by high organic load. Specifically, cheese whey production is estimated at 0.8-0.9 L per liter of processed milk or 9 kg per kilogram of cheese produced (Siso et al., 1996). If whey is discharged without proper treatment, it can cause major environmental problems. For example, when discharged into water, it can increase eutrophication, reduce oxygen in the water, threaten the life of aquatic life, and cause the formation of mucilage that pollutes the water (Carvalho et al., 2013). When discharged into the soil, due to the intense and high amount of minerals, mostly salt, it disrupts the characterization of the soil and reduces its fertility, affecting fertile soils and negatively affecting crop production in agriculture (Barba et al., 2021). As for CW waste management, the main uses vary depending on the size of the industries, but mainly include whey protein recovery and conversion to animal feed (Montecchio et al., 2018a). However, recent studies have shown that it is often used as a substrate due to its high organic matter content (50-100 g COD/L, 90% of which is in the form of lactose)(Davila-Vazquez et al., 2008). Studies targeting physicochemical and

biological production have used CW to convert it into many useful products such as hydrogen, solvents, volatile fatty acids (VFA) (Akhlaghi et al., 2017). All these valuable products can be applied in the bioplastics, electrical energy, or pharmaceutical industries (Polettini et al., 2022). CW is a substrate of particular interest for dark fermentation as sugars are the preferred substrate for fermentative microorganisms. CW fermentation results in H_2 yields typically ranging between 1-4 mol H_2 /mol lactose (or 0.5-2 mol H_2 /mol glucose equivalent), depending on operating conditions such as pH, temperature, and organic loading rate (Dwidar et al., 2012). Besides H_2 , up to 20-30 g/L of VFA, mainly acetic, propionic, and butyric acid, are produced via CW fermentation at different mass ratios depending on the operating parameters, especially pH (Dessi et al., 2020).

In recent decades, there has been a significant shift in focus towards biochemicals, rather than synthetic chemicals produced from fossil fuels. Butyric acid is one such biochemical with extensive applications in the industry (Yang et al., 2003). Typically, operating conditions that promote H_2 production in CW fermentation also favour the production of butyric acid among soluble organic fermentation products (Ribeiro et al., 2022). In response to the approval of butyric acid as a food flavouring agent by the US Food and Drug Administration (FDA), butyric acid finds numerous applications in the chemical, pharmaceutical, perfume and animal feed sectors with a market size of approximately 125 MV, which is expected to grow further at an annual rate of 15.1% (Dessi et al., 2020). As butyric acid is a precursor to the production of polyhydroxyalkanoates (PHAs), this already favourable context could be even more beneficial with the development of the bioplastics sector over the next decade. Therefore, the development of a process for the combined production of H_2 and butyric acid contributes significantly to a modern and environmentally sustainable CW management (Dessi et al., 2020).

1.2 Purpose & Scope

This thesis aims at the production of a commercial green chemicals, butyric acid along with production of hydrogen gas. The optimum operating conditions that provide the highest butyric acid and hydrogen gas production potential were determined. Anaerobic sludge, *Clostridium acetobutylicum*, *Clostridium beijerinckii* and *Clostridium pasteurianum* were used as different microorganisms in different trials.

Objectives of the Study:

- 1- Utilization of cheese whey powder, as the waste of dairy industry to transform it into a useful product.
- 2- Comparison of different conditions for increasing the production of butyric acid and reaching the highest butyric acid production potential.
- 3- Evaluation of salt concentration on biohydrogen and butyric acid production with anaerobic sludge.
- 4- Estimation of lactose concentration on biohydrogen and butyric acid production with anaerobic sludge.
- 5- Optimization of mineral addition to the fermentation media for enhancement of biohydrogen and butyric acid production with *C. acetobutylicum*.
- 6- Effects of CaCO_3 on biohydrogen and butyric acid production with *Clostridium acetobutylicum*.
- 7- Selection of organisms for enhancing butyric acid and hydrogen gas potential.
- 8- Effect of organism (biomass) concentration (0.5-3 g/L) with *Clostridium pasteurianum* for butyric acid and hydrogen gas potential.
- 9- Impact of substrate (CWP) concentration ranging from 40 to 200 g/L on butyric acid and hydrogen gas potential.

CHAPTER TWO

LITERATURE SURVEY

2.1 Butyric acid

Butyric acid (C_3H_7COOH) is a short-chain fatty acid with 4 carbons (Câmara-Salim et al., 2021). Concentrated butyric acid has an unpleasant odour. Butyric acid esters occur naturally in animals and some plants. (Fuess et al., 2020). The substance is commonly used in the food and pharmaceutical industries. It is typically synthesized chemically from petroleum-based raw materials (Huang et al., 2018). In the food industry, it is frequently employed as a pure acid to intensify butter-like flavours, enhance fruit flavours, and as an aromatic compound in perfume production (Luo et al., 2018).

As a result of scientific studies, food waste can be converted into various products such as chemicals, biogas, biofertilizer, and biofuel, which are commercially useful products (Atasoy & Cetecioglu, 2020). In many recent studies to produce useful products that can be produced in the fermentation process, scientists have sought different types of renewable raw materials (Fu et al., 2019). In this context, butyric acid is a promising biochemical alternative.

2.2 Butyric acid production methods, and pathways

In fermentation, the process typically proceeds in two stages: Acidogenesis and Solventogenesis, during which the cells are in exponential and constant growth phases, respectively (Gedam et al., 2019). During acidogenesis, cells grow actively, and their main products are acetic acid, butyric acid, CO_2 and H_2 . The pH drops as these acids accumulate. In the solventogenesis phase, the cells enter the stationary phase. These organic acids are used to produce acetone, butanol, and ethanol. The fermentative production of butyric acid and butanol is reported to be influenced by several factors, including the levels of nitrogen sources, cations, and buffering substrates, as well as the size of the inoculum. Figure 1 shows the production of butyric acid and the pathways by which it is converted into butanol (Kushwaha et al., 2019).

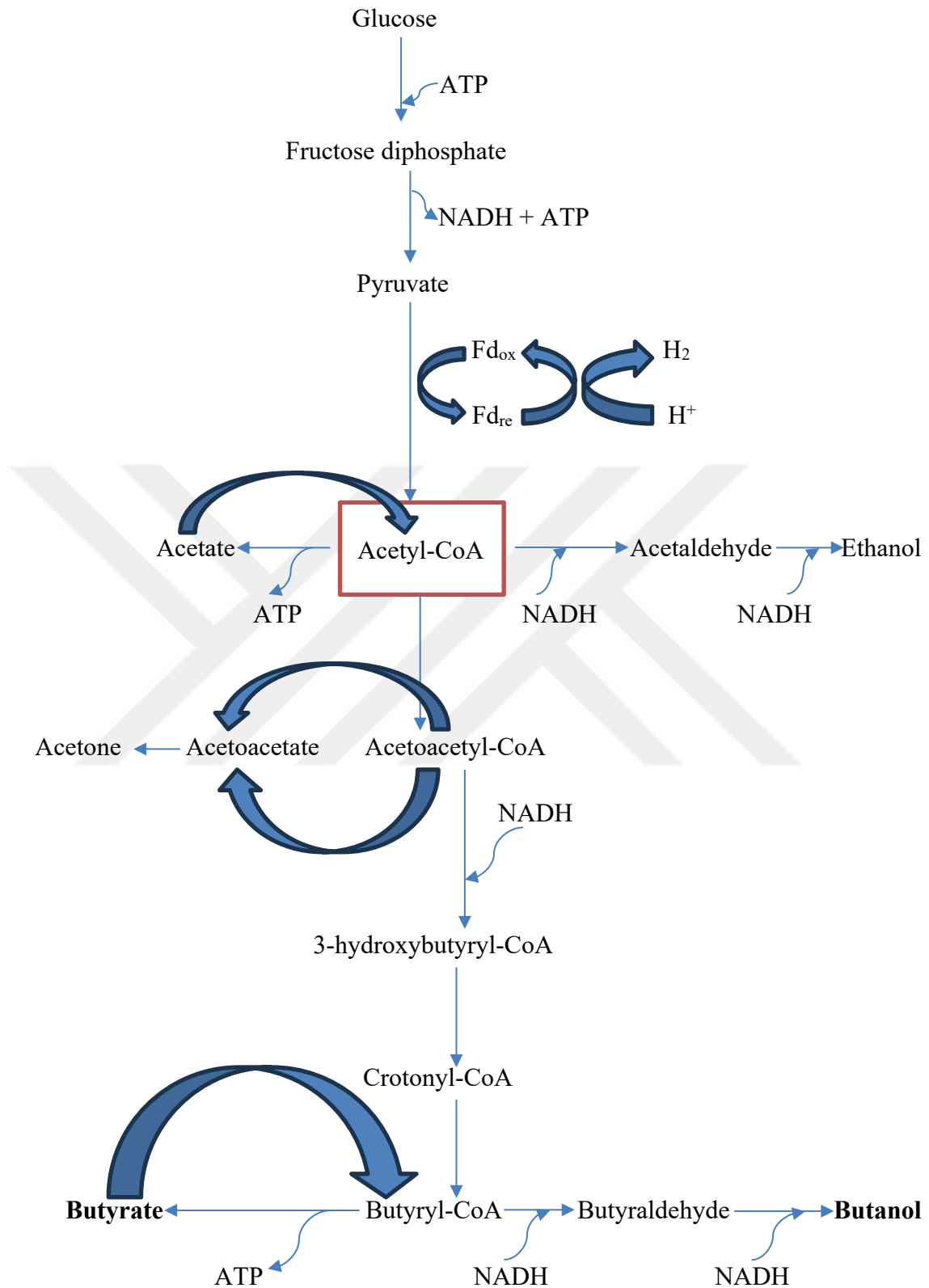


Figure 1 Butyrate and Butanol pathway (Kushwaha et al., 2019)

The production of hydrogen through dark fermentation is understood to be constrained in terms of yield. The theoretical maximum of 4 mol H₂ mol⁻¹ glucose is theoretically achieved with strict anaerobic pure cultures in which hydrogen production occurs via the Pyruvate Ferredoxin Oxidoreductase (PFOR) pathway, with acetate representing the primary metabolite. Maximum yields of 2 mol H₂ mol⁻¹ glucose are associated with butyrate production by strict anaerobes or with formate production by facultative anaerobes through the Pyruvate Formate Lyase (PFL) pathway (Yousuf & Tomás-Pejó, 2023).

Production of butyric acid from microbial fermentation has attracted great interest in recent years, due to its limited petroleum resources and some specific medical and food-related applications (H. Fu et al., 2017). Butyric acid is one of the organic acids produced by bacterial fermentation of various substances (S. Liu et al., 2018). Most butyric acid fermentation studies are carried out using *Clostridium* species under strict anaerobic fermentation conditions (Jang et al., 2012).

For increasing butyric acid production, *Clostridium butyricum* organism species was selected by Atasoy et al., (2020) in mixed culture fermentation, it was stated that it is one of the most important butyric acid producers thanks to its high productivity and relatively higher stability. Atasoy et al., (2020) increased the production of butyric acid from 260 ± 36(mg COD/L) to 2889 ± 180, and total volatile fatty acids 1434 ± 217(mg COD/L) by using *C. butyricum* DSMZ 10702. Since the *Clostridium* producing butyric acid generally produces acetic acid as well as a by-product where the production efficiency of butyric acid decreases. Liu et al., (2021) and Zhang et al., (2017) aimed to reduce acetic acid production from *C. tyrobutyricum* by cleaving the genes *ack* (acetate kinase gene) and *PTA* (phosphotransacetylase gene) which are related to acetic acid formation; however, some (4-8 g/L) of acetic acid was still produced. Jang et al., (2014) cleaved the *CTF*, *PTA*, *buk*, and *adhE1* genes, providing low acetic acid production (1.04 g/L) and high butyric acid production (32.5 g/L).

Since butyric acid induces morphological and biochemical differentiation in various cells, it is also used in hemoglobinopathies, cancer and gastrointestinal diseases, and vasoconstrictor drugs due to its anti-cancer effects (Atasoy et al., 2020). In the food, beverage, and cosmetic industries, butyric acid esters are used as a flavoring in food and beverages, and as fragrance and aroma in cosmetic products (Noh et al., 2019). Although fermentation is the most sustainable and environmentally friendly production method for butyric acid production, it is not economically preferred due to its low production efficiency and high substrate costs (Cetecioglu et al., 2020). Furthermore, fermentation can lead to several process-related issues, including the self-inhibiting effect of end products and limited productivity at lower concentrations. Hence, more comprehensive studies are needed to reduce overall costs and increase production efficiency. In the literature, some trace element additions have been made to increase the production efficiency of butyric acid.

As a result of recent studies, it has been observed that the dissolution of transition metals such as iron or nickel and its effect on microbial activities can increase butyrate production together with hydrogen production. Taherdanak et al., (2015) observed that when 37.5 mg/L iron and 37.5 mg/L nickel were added to the fermentation reactor, ten and twelve times more butyrate and hydrogen production were realized, respectively.

For this reason, it is assumed that adding some trace elements to the fermentation system can increase the production of hydrogen and butyrate. However, Suanon et al., (2016) observed that iron, nickel, and other trace elements do not always support butyrate production. In addition, although Taherdanak et al., (2015) observed in several experiments that metal addition improves butyrate production from glucose and starch, it is still unknown whether and exactly how this method can be applied to butyrate production in other food waste fermentation systems.

2.3 Hydrogen Production Processes

Hydrogen has recently gained attention as a potential energy carrier for the future. Hydrogen has a specific energy content of 142 kJ/g, according to Badea et al., (2021). Its high efficiency, ability to be converted into electricity, and ease of combustion make it a promising energy source for the future (Hosseini et al., 2015) .

2.4 Biohydrogen Production Processes

Hydrogen is produced worldwide largely by steam reforming of hydrocarbons (Fowles et al., 2021). Although this process is widely used, it is not considered environmentally friendly due to its reliance on fossil fuels. As a result, scientists have turned their attention to biohydrogen production as a more sustainable alternative. This method involves using waste food and wastewater as a substrate for hydrogen production through fermentation by microorganisms. By producing biohydrogen through fermentation, we can close the worldwide energy gap using green chemistry. Biohydrogen can be produced by methods such as bio-photolysis, dark fermentation, photo fermentation, microbial water-gas shift reaction of carbon monoxide (Guo et al., 2010; Mishra et al., 2019; Montecchio et al., 2018) . It can also be produced from biogas and by gasification of biomass, like bio-SNG production (Skorek et al., 2022). Different groups of microorganisms, including strict anaerobes (*Clostridium*), aerobes (*Bacillus*), and facultative (*Enterobacter*), can produce biohydrogen in environments ranging from mesophilic to hyper thermophilic (Gabra et al., 2019; Moura et al., 2020; Torres de Souza et al., 2019). Studies have shown that it is best carried out, in the absence of oxygen, in anaerobic conditions and by dark fermentation. In most cases, dark fermentation produces hydrogen in addition to other products. Examples of other products are alcohols and organic acids. Although it seems advantageous to produce useful by-products and at the same time produce biohydrogen, due to the low yields in hydrogen production, it is an important question how to increase this yield and at the same time make the waste usable as substrate.

Since each bioreactor has its own unique characteristics, a reactor should be selected for specific uses. These reactors can be used in either batch or continuous mode. One of the purposes of building these reactors is to minimize the effects of toxic inhibition. The other is to increase hydrogen production rates or the efficiency of production of desired solvents and organic acids (Anzola et al., 2016; Banu et al., 2021; Ghimire et al., 2015).

Bio-reactor types:

- Anaerobic downflow structured-bed reactors (ADSBR)
- Anaerobic fluidized-bed reactors (AFBR)
- Anaerobic membrane bioreactors (AnMBR)
- Anaerobic packed-bed reactors (APBR)
- Anaerobic sequencing batch reactor (ASBR)
- Continuous multiple tube reactor (CMTR)
- Continuous stirred-tank reactor (CSTR)
- Expanded granular sludge bed (EGSB)

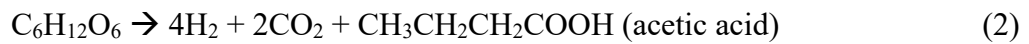
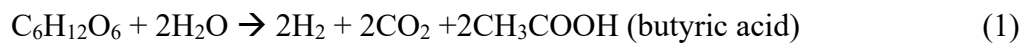
2.4.1 Biohydrogen Production by Dark Fermentation

Fermentation is one of the first steps of degradation by microorganisms and forming new products. It can be obtained by the fermentation process, including other by-products such as short and medium-chain organic acids, alcohols, and solvents that can also be used in industry (Kargi et al., 2012; Ozmihci et al., 2006). The performance of fermentative systems is determined by different factors such as inoculation, substrate type, pH, temperature, F/M ratio which are interrelated (Y. Chen et al., 2021; Montecchio et al., 2018; Srivastava et al., 2017). pH is a key factor in fermentation systems. It has been reported in the literature that the products produced in the fermentation medium differ when the pH is increases or decreases. The pH also determines the hydrogenase activity and how the metabolic pathways will be shaped. Based on the literature, it has been observed that extremely high pH values negatively affect the activity of hydrogen-producing microorganisms (Ates et al., 2022; X. M. Guo et al., 2010; Kapdan et al., 2006; Xia et al., 2015). On the other hand, extremely low pH values may cause inhibition of hydrogenase activity by diverting the pathways corresponding to the production of alcohols (Rosales et al., 2010; J. Wang & Yin, 2018). Similarly, the concentration of active biomass (inoculum) and consumable substrate in the system is expressed as the food-to-microorganism (F/M) ratio. This ratio can go from limited growth of the substrate to adequate growth. Furthermore, it can conclude with substrate saturation, destabilization of anabolic and catabolic reactions. As a result, the conversion efficiency of the substrate to by-products may be affected.

Dark fermentation is the anaerobic conversion of glucose to Volatile Fatty Acids (VFAs), hydrogen, and alcohols. Unless an electron transport chain is artificially introduced into the anaerobic environment, the oxidation of glucose and VFA proceeds via dehydrogenation, resulting in large amounts of hydrogen (Ghimire et al., 2016; Srivastava et al., 2017). There are three forms of fermentation: butyric acid fermentation, propionic acid fermentation, and ethanol fermentation (Qu et al., 2022). Several factors influence the fermentation process, including pH, hydrogen partial pressure, and the concentration of fermentation metabolites such as lactic, propionic, butyric, and acetic acids, as well as ethanol; all these compounds are produced in varying ratios to maintain an appropriate NADH/NAD⁺ ratio, which determines the type of fermentation definition (Qu et al., 2022).

2.5 Microorganisms Producing Bio-Hydrogen Gas, Butyric Acid, and Butanol

The identified microbes that produce bio-hydrogen through fermentation mainly belong to the genera *Clostridium*, *Enterobacter*, and *Escherichia*. This suggests that *Clostridium* species are the most likely to achieve near-theoretical hydrogen production yield values (Calli et al., 2008). Hydrogen is a high-energy power source obtained mostly during primary fermentation through the production of acetate, and butyrate. Butyric acid produced by dark fermentation from glucose or lactose produces 2 moles of H₂ while acetic acid pathway results with 4 moles of H₂ (Polettini et al., 2022).



Theoretically, up to 12 mol of hydrogen can be obtained from 1 mol of glucose (Eq. (3)). Hydrogen production by *Clostridium* fermentation mainly depends on the two metabolic pathways of butyrate and acetate fermentation (Eqs. (1) and (2)). According to the equation, it can be calculated that the theoretical maximum hydrogen production can reach 4 mol H₂ per mol glucose (Eqs. (1) and (2)) (Hallenbeck & Ghosh, 2009). However, considering the influence of various parameters of the operation process,

such as pH, temperature, substrate, etc., the actual hydrogen production is much lower than 4 mol H₂/mol glucose (Hallenbeck & Ghosh, 2009).

Clostridium pasteurianum is a gram-positive, strict anaerobe that forms endospores (Ahn et al., 2011). These bacteria are known for their high substrate concentration tolerance, inhibitor resistance, and sustainability against adverse conditions during hydrogen, butyric acid, and butanol production (Ahn et al., 2011; L. Chen et al., 2021; Krasňan et al., 2018). *C. pasteurianum* is a strictly anaerobic bacterium, digesting glycerol to form butanol as the primary product and 1,3-propanediol, ethanol, butyrate, acetate, lactate, CO₂ and H₂ as secondary products (Andrés & Marusich, 2013). Sarma et al., (2017) achieved 1.19 mmol/L h specific butyric acid production rate (SPBR), 3.85 mmol/L h specific hydrogen production rate (SHBR), and 0.95 mol/mol glycerol hydrogen production yield by fermentation from glycerol with *C. pasteurianum* microorganism. (Gallardo et al., 2014) used batch fermentation of 35 g/L crude glycerol with *C. pasteurianum* DSM 525 at 37 °C without pH control to obtain a yield of 0.2 g/g butanol, 6.9 g/L butanol and 0.43±0.08 g/L.

Sirisantimethakom et al., (2018) performed validation experiments in a 2-L fermenter under optimal conditions and obtained results for butanol concentration (16.91 g/L) and productivity (0.47 g/L h). Statistical analyses showed that the optimum concentrations for maximum butanol production were 7.14 g/L CaCO₃, 1.27 g/L (NH₄)₂SO₄ and 5.18 g/L butyric acid. In this study, sweet sorghum stem juice (60 g/L total sugar) was used as a substrate by *Clostridium beijerinckii* TISTR 1461. Wu et al., (2017) studied pH and ferrous iron on hydrogen and butanol production by *Clostridium beijerinckii* IB4 and obtained the following results: butanol 12.06 g/L, butanol productivity 0.31 g/L h, butanol yield 0.234 g/g, H₂ productivity 148 mL/L h, H₂ yield 112 mL/g and finally butyric acid 2.25 g/L.

Arachchige et al. (2020) studied the effects of Fe⁰ and Ni⁰ nanoparticles against Fe²⁺ and Ni²⁺ ions on the efficiency of mesophilic dark hydrogen fermentation of glucose using heat-shock pre-treated anaerobic sludge to produce 247 mL/g VS H₂ yield and 1.7 mg/L maximum butyric acid. Dessì et al., (2020) studied with anaerobic sludge the simultaneous production and extraction of VFA and hydrogen production from whey in UASB reactors at two temperatures (20°C and 35°C) and pH values (5.0

and 4.5). At 35°C and pH 5.0, batch experiments showed higher yields of 0.9 mol H₂/mol glucose and 25 g/L butyric acid production. In this study, Jang et al., (2013)metabolically engineered *Clostridium acetobutylicum* for highly selective butyric acid production. Batch fermentation of the metabolically engineered *C. acetobutylicum* strain HCBEKW at pH 6.0 resulted in production of 32.5 g/L butyric acid. The butyric acid to acetic acid ratio (BA/AA ratio) was 31.3 g/g, the butyric acid yield was 0.39 g/g, the butyric acid productivity was 0.89 g/L h, and 0.4 g/L butanol from 83.3 g/L glucose was produced in this study. Tsai et al., (2020) used *Clostridium acetobutylicum* ATCC 824 as the organism. In the absence of rice straw hydrolysate and yeast extract as feedstock, the titer of biobutanol was 13.80 g/L,the yield, and productivity was 0.90 g/L h and 0.58 mol biobutanol/mol glucose (0.23 g biobutanol/g glucose), the cumulative H₂ production was 9000 mL/L and 3.6 g/L butyric acid were obtained.

2.6 Raw Materials for Bio-hydrogen and Butyric Acid Production

Different groups of microorganisms, ranging from mesophilic to hyper thermophilic, including facultative (*Enterobacter*), strict anaerobes (*Clostridium*) and even aerobes (*Bacillus*), can produce hydrogen, VFAs and solvents from various substrates, including simple sugars and various agricultural, industrial, or domestic waste streams. Recent studies are therefore investigating the possibility of producing such products from carbohydrate-rich materials such as agricultural, agro-industrial and food wastes. Lignocellulosic wastes (e.g. maize stalks, barley, and wheat straw), which are very abundant worldwide, have the potential to be used for hydrogen production after certain pre-treatments, but these are still costly and need further development. Among these, the genus *Clostridium*, which are anaerobic, Gram-positive, spore-forming bacteria, can produce butyric acid with high titer, yield, and productivity from a variety of substrates, including glucose, xylose, lactose, and glycerol (Cheng et al., 2019; Vamsi Krishna et al., 2022).

Fuess et al. (2021) used sugarcane vinasse as substrate and an enriched mixed culture as microorganism and obtained 12.7 g/L butyric acid, 0.52 g-Hbu/g substrate butyric acid yield and about 0.44 to 0.52 Specific Butyric Acid Production Rate (SBAPR). Atasoy M. et al. (2020) produced 2580 mg/L butyric acid with the

microorganism *Clostridium butyricum* (No. 10702, DSMZ, Germany) in an anaerobic sequencing batch reactor using $20,000 \pm 60$ mg COD/L wastewater from a cheese-making industry as the initial substrate. Stein et al., (2017) obtained 13.00 g/L butyric acid from 100.84 g/L food waste in batch mode with mixed culture in a thermophilic environment at 55°C. Liu T. et al. (2021) using *Clostridium tyrobutyricum* CtΔack::cat1 from rice straw in batch mode achieved 26.25 g/L butyric acid and 0.41 g/L.h SBAPR in 24 h in mesophilic medium.

2.6.1 Cheese Whey Powder as Substrate Source

In recent years, different alternatives have been discovered to take advantage of the valuable components of cheese whey (CW). Barba et al. (2021), concluded that approximately 200 million tons of whey is produced worldwide in 2020. Simple options, such as past land application or direct use as animal feed, have been significantly restricted by specific regulations due to obvious concerns about associated environmental impacts and potential adverse effects on animal nutrition and health conditions. In some geographic areas, CW is used to produce ricotta/cottage cheese but creates a secondary waste of CW with different properties and therefore cannot be treated with an effective treatment method (Akhlaghi et al., 2017). More assertive and process-intensive alternatives aim to take advantage of CW's nutritional content by restoring proteins and lactose. Other options include CW refining, typically through biological anaerobic processes, to take advantage of its main components producing biofuels or chemical products (sugars, organic acids, biopolymers, etc.) for industrial applications(Akhlaghi et al., 2017). More specifically, due to the typically high carbohydrate content of CW, biohydrogen production by dark fermentation has recently received significant attention from the scientific community as a strategy to optimize the degradation process and allow for enhanced substrate stabilization and conversion to biofuels.

The chemical composition and characteristics of CW depend upon the type of milk as well as cheese production techniques used. On average, CW accounts for ~80% of the original fermentation medium and retains ~55% of the milk nutrients(Venetsaneas et al., 2009). The chemical composition and properties of whey depend on the type of milk used and cheese production techniques, but on average, CW contains lactose (45–

50 g/L), proteins (6–8 g/L), lipids (4–5 g/L), and mineral salts (8–10% dried extract). Also, NaCl and KCl (> 50%), calcium salts and others are consisted in CWs characteristic (Venetsaneas et al., 2009). Other components of CW include lactic and citric acids, urea and uric acid, and B-group vitamins. The dairy industry has investigated various alternatives for utilising the valuable components of cheese whey wastewater and cheese whey powder (CWP).

CWP is used in the food industry (for example, bread, biscuits, pastry), and in areas such as supplementary food for athletes. Generally, CW has high BOD (50 g/L) and COD (80 g/L) content (Gannoun et al., 2008; J. Xia et al., 2021). Biological treatment of CW with the traditional activated sludge process is very costly (J. Xia et al., 2021). In addition, the rich nutrient content in its content and the cost of its treatment are the most sustainable form of converting waste whey into useful products through fermentation.

Cheese whey can be utilized as a substrate for dark fermentation, yielding hydrogen production yields ranging from 1-4 mol H₂/mol lactose depending on operation variables such as temperature, pH, and organic loading rate (Davila-Vazquez et al., 2009; Gannoun et al., 2008; J. Xia et al., 2021). In addition to H₂, CW fermentation can produce VFAs up to 20-30 g/L in various mass ratios depending on the working parameters, particularly pH, primarily acetic, propionic, and butyric acid (Ferraz et al., 2021; Ferreira Rosa et al., 2014; X. Guo et al., 2017). The studies using CW as substrate can be carried out in various types of reactors such as UASB reactor, anaerobic fixed bed reactor, CSTR reactor, anaerobic digester, and batch feed reactors (Azbar et al., 2009; Carrillo-Reyes et al., 2012; Davila-Vazquez et al., 2009; Perna et al., 2013). Atasoy et al. (2020) used an anaerobic sequential batch reactor and chose the wastewater of the Swedish whey industry as a substrate at a pH below 10 and a temperature of 35°C. The bio-growth performance of the organism (*Clostridium butyricum*) was injected targeting 10% of the active reactor volume daily for 7 days before and during application. As a result of this study conducted in 2020, butyric acid production increased from 260 ± 36 to 2889 ± 180 mg COD/L.

In this view, whey could represent an important renewable resource to exploit, given that the global production of liquid whey from cheese and casein is over 180 million tonnes and accounts for more than 80% of the volume of processed milk (Pires et al., 2021). Indeed, CW production contains 8.6 million tons of lactose, a very important source of carbohydrates. Lactose is a disaccharide composed of one molecule of glucose and one molecule of galactose. Lactose is the main component of whey and makes up more than 70% of the total dry weight: other whey components are mineral salts (12-15%) and proteins (usually below 10%) (Akhlaghi et al., 2017; Montecchio et al., 2018). In this ultrafiltered whey, the protein concentration corresponded to less than 2% of the total COD. In an anaerobic environment, lactose is rapidly hydrolysed to glucose and galactose, the fermentation products are the same as those obtained from glucose; In general, after hydrolysis is complete, lactose follows the same fermentation pathways as glucose.

2.7 Energy and Economics of Hydrogen

Hydrogen is an adaptable energy source that has a wide range of possible uses, such as power generation, energy storage, mobility, and warmth (Badea et al., 2021). Hydrogen fuel is expanding into large fleets and heavy-duty applications for road transport, transportation, shipping, and aviation in some countries, starting with the expansion of infrastructure (Aarhaug et al., 2024). By the end of 2021, there were 729 hydrogen refuelling stations (HRS) in operation worldwide (Samsun et al., 2022). South Korea has installed 162 HRSs in less than 3 years (Samsun et al., 2022). The development of hydrogen technologies as a potential sustainable energy source has sparked interest in many nations. Among these, South Korea and Germany are the two global leaders. With this commitment, they have become global leaders with massive investments and research (Ashari et al., 2024).

2.8 Energy and Economics of Butyrate and Butanol Production

2.8.1 Energy and Economics of Butyrate

Current industrial production of butyric acid is exclusively via chemical synthesis and has a global market of approximately 80,000 tonnes per year, sold at a price of ~\$1.8/kg (Jiang et al., 2018a). However, with increasing demand from food and

pharmaceutical manufacturers, who generally prefer biologically produced products, bioproduction of butyric acid from renewable biomass is becoming an inevitable trend. In the chemical industry, butyric acid is mainly used as a precursor to produce thermoplastic cellulose acetate butyrate (CAB), which has excellent performance in terms of solubility in organic solvents due to improved hydrophobicity, light and cold resistance and flexibility (Dwidar et al., 2012). Butyric acid can also be used as a direct additive to plastics and textile fibres to improve heat and sunlight resistance.

It has been reported that blending CAB with another polymer such as poly(3-hydroxybutyrate) (PHB), which can also be produced from butyric acid, can improve product properties, and reduce the cost of producing PHB (Kataoka et al., 2017).

Butyric acid can also be used to improve butanol production as a biofuel. Naturally, butyric acid is the precursor of butanol biosynthesis in acetone-butanol-ethanol (ABE) fermentation with solventogenic clostridia. Some butyric acid-producing bacteria are also engineered to produce butanol as a major product (Jang et al., 2012; Raganati et al., 2015). Several biological effects of butyrate have been reported. For example, butyrate induces morphological and biochemical differentiation in various cells, while suppressing neoplastic features. Furusawa et al., (2013) showed that butyrate can induce colonic regulatory T (Treg) cells, which play a central role in suppressing inflammatory and allergic responses. Butyrate also positively modulates mitochondrial function, including increasing oxidative phosphorylation and beta-oxidation, and has been proposed as a neuroprotectant. Various butyric acid derivatives have been developed as antithyroid drugs, vasoconstrictors and used as anaesthetics. A family of acyloxy alkyl butyrate prodrugs is currently in clinical development. Because of its health benefits, particularly for young animals, butyrate is increasingly being used as a substitute for antibiotics in animal feed (Furusawa et al., 2013).

Current industrial production of butyric acid is almost exclusively by chemical synthesis using petrochemical feedstocks. It is dominated by the "oxo" process (Jiang et al., 2018). Due to its availability, low cost and historical links with the petrochemical industry, chemical synthesis from propylene feedstock is used (Dwidar et al., 2012; Jiang et al., 2018). Several methods can be used for the extraction of VFAs, including electrochemical (electrodialysis), physical (membrane contactors, distillation, vapour

permeation, liquid-liquid extraction, gas stripping and nanofiltration), and chemical (solvent extraction, adsorption) (Dessi et al., 2020). The comparison of current mainstream technologies for the production of butyric acid is shown in Table 1.

Table 1 Comparison of current mainstream technologies to produce butyric acid (Jiang et al., 2018)

<u>Method</u>	<u>Reaction</u>	<u>Advantages</u>
Hydroformylation of propylene	$\text{CH}_2\text{CHCH}_3 + \text{CO} + \text{H}_2 + 0.5 \text{O}_2 = \text{CH}_3(\text{CH}_2)_2\text{COOH}$	Propylene is available and cost-effective for use in the petrochemical industry.
Oxidation of butyraldehyde	$\text{CH}_3(\text{CH}_2)_2\text{CHO} + 0.5 \text{O}_2 = \text{CH}_3(\text{CH}_2)_2\text{COOH}$	The production control process is convenient and straightforward, resulting in a high product yield of over 99.5% and selectivity of over 95%.
Fermentation	$\begin{aligned} &\text{Glucose} = 0.8 \\ &\text{CH}_3(\text{CH}_2)_2\text{COOH} + 0.4 \\ &\text{CH}_3\text{COOH} + 2 \text{CO}_2 + 2.4 \\ &\text{H}_2 \end{aligned}$	Renewable biomass and ambient conditions are utilized in the process, making it sustainable and environmentally friendly.

2.8.2 Energy and Economics of Butanol Production

The production of butanol from butyric acid and its use as a green and environmentally friendly energy source is vital for the future. Butanol has been shown to have several advantages over other biofuels such as ethanol and methanol (Sang et al., 2011). It has a lower vapour pressure and a higher energy content (Krasňan et al., 2018; Venkataramanan et al., 2014). As a result, it is a safer option for use in petrol blends and has better fuel economy than ethanol. In addition, butanol has a higher tolerance to water contamination in petrol blends, which makes butanol-petrol blends less susceptible to segregation and facilitates their use in the existing petrol supply and distribution channels (Raganati et al., 2015; Schwarz et al., 2017). In particular, butanol can be transported through existing pipelines and is much less corrosive than

ethanol. Furthermore, branched chain 4-carbon alcohols such as iso-butanol, 2-methyl-1-butanol and 3-methyl-1-butanol have higher octane ratings than n-butanol (Sirisantimethakom et al., 2018). Lastly, butanol is similar enough to petrol to be used directly or mixed with petrol in any petrol engine without alteration, even in higher concentrations than ethanol (Costa-Silva et al., 2021).



CHAPTER THREE

METHODOLOGY & DATA ANALYSIS

3.1 Experimental setup and procedure

Lactose and CWP were used as substrates in the experiments. Lactose was used first to observe which minerals have positive effects on butyric acid that CWP may contain. After the effects of minerals added into lactose-based media were observed, CWP was used as substrate in different concentrations and its effects were examined. The experiments were carried out in 250 mL serum flasks (Isolab-Germany Boro 3.3) with a working volume of 100 mL for the first and 150 mL for the second experiments. Dark fermentation was carried out under mesophilic (37°C) conditions for 10 days with a 10% inoculum.

Three different experiments were carried out with media containing lactose as carbon source. In the first two experiments, anaerobic sludge was used as the organism consortium. In the first experiment a lactose concentration of 20 g/L and different NaCl concentrations (0-6 g/L) were used. In the second experiment, different concentrations of lactose (5-100 g/L) were tested with the amount of 5 g/L NaCl concentration that gave the highest butyric acid production based on the results from the first experiment. In the third experiment, 10 g/L lactose was subjected to *Clostridium acetobutylicum*-NRLL-B 527 (CAB). This experiment was carried out 150 mL with a working volume. The Box-Behnken experimental design (BBD) experiment with sodium chloride (NaCl: 0.1-2.505-5 g/L), sodium thiosulfate (Na₂S₂O₃: 0-100-200 mg/L) and trace metals (Ni, Fe, Zn, Cu) independent variables were tested for maximizing butyric acid production. FeSO₄ (0-0.1-0.2 mM), ZnSO₄ (0-0.1-0.2 mM), CuCl₂ (0-0.1-0.2 mM), NiCl₂ (0-0.1-0.2 mM) were added as trace minerals to the medium.

The fourth experiment, *Clostridium acetobutylicum*-NRLL-B 527 (CAB) was used as microorganism, 17.5 g/L CWP was used as substrate, and 150 mL was used as a working volume. For protein removal, CWP was autoclaved at 90°C for 30 minutes. The total sugar amount was analysed as 15 g/L after protein removal.

For the fourth experiment, it was aimed to observe whether different CaCO₃ concentrations would have an impact on butyric acid production. Therefore, different

CaCO₃ concentrations (0-0.7-3.3-6.7-13.3-20.0-26.7 g/L) were examined in 7 different experimental vessels. According to the previous experiments NaCl (2.505 g/L,) Na₂S₂O₃ (100 mg/L,) NiCl₂ (0.1 mM), FeSO₄ (0.1 mM), ZnSO₄ (0.1mM), CuCl₂ (0.1mM), CaCO₃ (0.7 g/L) was added into the fermentation media where CWP was used as the carbon source in further experiments.

In the fifth experiment, 20 g/L CWP was used as substrate and 150 mL was used as a working volume. The aim of this experiment was to investigate the effects of four different microorganisms: anaerobic sludge, *Clostridium acetobutylicum*-NRLL-B 527 (CAB), *Clostridium beijerinckii* DSM 791, *Clostridium pasteurianum* DSM 525.

In the sixth experiment, different CWP concentrations (40-80-100-150-200 g/L) were tested with *Clostridium pasteurianum* DSM 525, and the effects of substrate concentrations was observed. A working volume of 200 mL was used in, Isolab-Germany Boro 3.3, experimental vessels.

Finally, in the seventh experiment, 45 g/L CWP was used as substrate, *Clostridium pasteurianum* DSM 525 as microorganism in a 150 mL working volume. In the experiment, the effect of different microorganism concentrations (0.5-1-1.5-1.5-2-2.5-3 g/L) were observed.

Before fermentation, the pH of the medium for all experiments was adjusted to 7.2 using 10 M NaOH and the experimental vessels were autoclaved at 121°C for 15 minutes. Nitrogen gas was passed through the top of the experimental vessels to ensure anaerobic conditions, the experimental vessels were sealed with stoppers and caps and finally sealed with silicone to verify the gas tightness. The fermentation medium was consisted of 2.8 g/L K₂HPO₄, 3.9 g/L KH₂PO₄, 0.25 g/L MgSO₄, 0.1 g/L L-Cysteine, 0.6 g/L yeast extract, 1.0 g/L peptone with addition of different elements mentioned above.

3.2 Substrate Characterization

The characterization of the whey powder used in the experiments are given in Table 2 and Table 3.

Table 2 The main characterization of the whey powder (10 g/L) used in the experiment

pH	6.8
TP	260 mg/L
TN	280 mg/L
NH₄⁺	3.51 mg/L
NO₃⁻	100 mg/L
SS	778 mg/L
TSS	9980 mg/L

Table 3 The trace mineral characteristic of whey powder used in the experiment (mg/kg)

⁷ Li	1.95	⁶³ Cu [He]	2.00
⁹ Be	0	⁶⁶ Zn [He]	3.97
¹¹ B [He]	33.38	⁷⁵ As [He]	0.87
²³ Na [He]	132342.94	⁷⁸ Se [He]	0.22
²⁴ Mg [He]	17940.54	⁸⁸ Sr [He]	123.36
²⁷ Al [He]	<0.000	⁹⁵ Mo	9.48
³⁹ K [He]	152848.84	¹⁰⁷ Ag	0.03
⁴³ Ca [He]	173537.15	¹¹¹ Cd	0.10
⁵¹ V [He]	76.40	¹¹⁸ Sn	0.23
⁵² Cr [He]	1.78	¹²¹ Sb	0.13
⁵⁵ Mn [He]	10.63	¹³⁷ Ba	13.02

Table 3 The trace mineral characteristic of whey powder used in the experiment (continued)

⁵⁶ Fe [He]	41.46	²⁰⁵ Tl	0.08
⁵⁹ Co [He]	0.24	²⁰⁸ Pb	2.15
⁶⁰ Ni [He]	13.12		

3.3 Microorganisms

Anaerobic sludge was obtained from the anaerobic wastewater treatment plant of PAK-MAYA Baker's Yeast Co, Izmir, Turkiye, operating at 35 °C. Anaerobic sludge, was thermally pretreated by adjusting the pH to 5 for 1.5 hours. The pure anaerobic culture of *C.acetobutylicum*-NRLL-B 527 (CAB) was obtained from the USDA National Center for Agricultural Utilization Research, Peoria, IL, USA. *C. pasteurianum* DSM 525 and *C. beijerinckii* -DSMZ-791 were achieved from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in lyophilized form. All microorganisms were inoculated into a medium containing 10 g/L lactose, 2.8 g/L K₂HPO₄, 3.9 g/L KH₂PO₄, 0.25 g/L MgSO₄, 10 g/L peptone, 0.6 g/L yeast extract and 0.1 g/L L-cysteine [37]. Anaerobic conditions were provided by passing nitrogen gas through the top of the experimental vessel, and the organisms were incubated for 2 days at 37°C, at an initial pH of 7.2. The organisms were inoculated into the fermentation media at the end of the 2nd day.

3.3.1 Optimization of mineral addition to the fermentation media with Box–Behnken design

The third experiment used a three-level (-1, 0, +1) Box-Behnken Design (BBD) to optimise sodium chloride (A), sodium thiosulphate (B) and metal complex (C) concentrations. Sodium thiosulphate and sodium chloride concentrations varied between 0 to 200 mg/L and 0.1 to 5 g/L, respectively. The metal complex included four different elements that were nickel (Ni⁺² in the form of NiCl₂), zinc (Zn⁺² in the form of ZnSO₄), copper (Cu⁺² in the form of CuCl₂), and iron (Fe⁺² in form of FeSO₄).

All the metals were added to the fermentation media as a trace mineral complex at a level of 0-0.1-0.2 mM. The total concentration of trace elements in the fermentation media varied between 0-0.4-0.8 mM. The values and range of the variables are given in Table 4. The BBD was used for the quadratic response and generated a third-degree polynomial model to predict the optimum conditions. Statistical designs were determined using Design Expert 13.0.5.0 software. The calculation of the response (Y) is shown in equation (4).

$$Y = B_0 + \sum_{i=1}^3 B_i X_i + \sum_{i=1}^3 B_{ii} X_i^2 + \sum_{i=1}^3 \sum_{j=1}^3 B_{ij} X_i X_j \quad (4)$$

The estimated highest butyric acid was represented by Y as the response related to the X_i (A, B, C) variables, B_0 as the offset term, B_i as the i^{th} linear coefficient, B_{ii} as the quadratic coefficient and B_{ij} as the ij^{th} interaction coefficient. A total of 17 runs with dissimilar combinations of three variables were generated by Box Behnken Design. The experiments were run in duplicate, and the average of the duplicates was taken as the response.

Table 4. Variables and experimental Box-Behnken design levels

Variables		Experimental range			
Factors	Parameters	Units	Low (-1)	Middle (0)	High (+1)
A	Sodium Chloride	g/L	0.1	2.505	5
B	Sodium Thiosulfate	mg/L	0	100	200
C	Minerals	mM	0	0.4	0.8

3.4 Analytical techniques

The liquid portion of the fermentation media was analysed by taking 5 mL of samples daily from the experimental vessels and centrifuged at 8000 rpm for 15 minutes. Total sugar concentration, pH, and total volatile fatty acid (TVFA) concentration were analysed in the clear supernatant obtained. Total sugar concentration was determined using acid-phenol spectrometry with Dubois method (Dubois et al., 1956). The analysis of simple sugars and TVFAs concentration was

conducted using High-Performance Liquid Chromatography (HPLC, Agilent 1100; Aminex HPX-84 H column). The mobile phase consisted of 5 mM H₂SO₄ at a flow rate of 0.6 mL/min.

Gas-phase analysis was performed by taking daily samples from the fermentation vessels with a gas-tight glass syringe. The hydrogen gas concentration was measured using gas chromatography (Agilent 6890 N-GC) with a GC column of Alltech, Hayesep D 80/100 (6 inch x 1/8 inch x 0.85 inch). Nitrogen was used as the carrier gas at a flow rate of 30 mL/min. The furnace, injection, detector, and filament temperatures were 35°C, 120°C, 120°C, and 140°C, respectively. The total gas produced was calculated using the water displacement method with 2% H₂SO₄ and 10% NaCl (Kim et al., 2016). The equations used to determine the potential for hydrogen production were sourced from Kargi et al., (2012).

3.5 Theoretical Background

- Hydrogen Gas Volume

$$V_{H_2} = C_{H_2} * V_{Gas} \quad (5)$$

C_{H_2} = Hydrogen Gas Concentration (%H₂)

V_{Gas} = Total Produced Gas (mL)

- mmol Hydrogen Gas (37°C)

$$P * V = n * R * T \quad (6)$$

P = Pressure (1 atm)

V_{H_2} = Total Produced Gas (mL)

n = mmol H₂ gas

R = 0.082 (L*atm*mol⁻¹*°K)

T = Temperature (°K)

- Hydrogen, Butyric Acid, Production Yield (Y)

$$Y = \frac{\Delta P}{\Delta S} \quad (7)$$

Y = Hydrogen, butyric, butanol / initial substrate (mL H₂/g TS) (mmol H₂/mmol glucose) (g/L)

ΔP = Hydrogen, butyric, butanol production (mL H₂ or mol H₂) (g/L)

ΔS = Consumed substrate (total sugar) (g)

- Cumulative Hydrogen Gas Volume (CHF)

$$V_{cumulative, i} = V_{cumulative, i-1} + V_{TG}C_{H_2, i} + V_{HS, i} C_{H_2, i} - V_{HS, i-1} C_{H_2, i-1} \quad (8)$$

$V_{\text{cumulative}, i}$ = The amount of H_2 produced at time (i) (mL)
 $V_{\text{cumulative}, i-1}$ = The amount of H_2 produced at time (i-1) (mL)
 V_{TG} = The volume of total gas (mL)
 $C_{H_2, i}$ = The concentration of H_2 at time (i) (%)
 $V_{\text{HS}, i}$ = Headspace volume at time (i) (mL)
 $C_{H_2, i-1}$ = The concentration of H_2 at time (i-1) (%)
 $V_{\text{HS}, i-1}$ = Headspace volume at time (i-1) (mL)

- Biomass concentration

$$X = \frac{Y_1 - Y_2}{V} * 1000 \quad (9)$$

X = Biomass calculation (g/L)
 Y_1 = Weight of filter paper with sample (g)
 Y_2 = Weight of blank filter paper (g)
 V = Volume of sample (mL)

- Specific Hydrogen Production Rate (SHPR, g H_2 /g biomass h)

$$\text{SHPR} = \frac{\Delta \text{CHF}}{\Delta X * \Delta t} \quad (10)$$

ΔCHF = CHF between initial to final ($\text{CHF} - \text{CHF}_0$) (mL)
 ΔX = Biomass Concentration between initial to final ($X - X_0$) (g/L)
 Δt = Fermentation time ($t - t_0$) (h)

- Specific Butyric Acid Production Rate (SBAPR, g BA/ g biomass h)

$$\text{SBAPR} = \frac{\Delta \text{BA}}{\Delta X * \Delta t} \quad (11)$$

ΔBA = BA between initial to final ($\text{BA} - \text{BA}_0$) (g/L)
 ΔX = Biomass Concentration between initial to final ($X - X_0$) (g/L)
 Δt = Fermentation time ($t - t_0$) (h)

- Hydrogen Production Rate (HPR)

$$\text{HPR} = \frac{Y_{H_2}}{\Delta t} \quad (12)$$

HPR = Hydrogen production rate (mL H_2 /g TS*h)
 Δt = Fermentation time ($t - t_0$) (h)

- Butyric Acid Production Rate (BAPR)

$$\text{BAPR} = \frac{Y_{\text{BA}}}{\Delta t} \quad (13)$$

BAPR = Butyric acid production rate (g BA/ g TS*h)
 Δt = Fermentation time ($t - t_0$) (h)

- BA/AA ratio

$$BA/AA = \frac{BA}{AA} \quad (14)$$

BA= Butyric acid amount for specific time

AA= Acetic acid amount for specific time



CHAPTER FOUR

RESULTS AND DISCUSSIONS

In this study butyric acid formation along with hydrogen fermentation potential was tried to be enhanced. For this purpose: variations of organism type, essential mineral requirements, substrate concentration and microorganism concentrations were tested for improvement of butyric acid and hydrogen gas production. Studies were separated for the use of substrate as lactose and Cheese Whey Powder (CWP). The experiments were performed in batch dark fermentation, at 37°C and at static conditions with a fermentation period of 10 days.

4.1 Experiments Performed with Lactose as Substrate

Lactose was preferred as a substrate in the experiments because Cheese Whey Powder (CWP) contains high amount of lactose (45-50 g/L) (Siso et al., 1996). In the initial stages of the experiments, only lactose was utilised as a carbon source in order to eliminate the impacts of the ingredients present in CWP, as well as to ascertain whether the mineral salts in CWP interfere with the fermentation of end products.

The experiments with lactose aimed:

- I. To evaluate the NaCl concentration on biohydrogen and butyric acid production with anaerobic sludge.
- II. To investigate the impact of lactose concentration on biohydrogen and butyric acid production with anaerobic sludge.
- III. Optimization of mineral (NaCl, Na₂S₂O₃, Ni, Fe, Cu, Zn) addition to the fermentation media for enhancement of biohydrogen and butyric acid production with *C. acetobutylicum*.

4.1.1 Evaluation of Salt Concentration on Biohydrogen and Butyric Acid Production with Anaerobic Sludge

The first experiment aimed to increase butyric acid production with varying amounts of NaCl (0-6 g/L). Anaerobic sludge was used as the culture under mesophilic conditions with an initial substrate concentration of 20 g/L lactose at dark fermentation for 10 days.

In the literature, sodium chloride was used to enhance butyric acid along with hydrogen (Kajal G Singh, 2016; Taheri et al., 2018; Y. Wang et al., 2013). Taheri et al. (2018) operated a sequencing batch reactor with an increasing concentration of 0.5-30 g/L NaCl to enhance biohydrogen production. Furthermore, they found that high levels of NaCl shifted the metabolic pathway from the acidogenic to the solventogenic phase (Taheri et.al, 2018). Similarly, increasing butyrate concentrations in the fermentation media triggers the pathway to produce butanol as the end product (Y. Wang et al., 2013). For this reason, studies focusing on the butanol pathway were also examined for trace element addition. It is important to note that when producing butyric acid during the acidogenic phase, the operating conditions, especially the pH, should be carefully managed to avoid a shift to the solventogenic phase.

Figure 2 indicates Cumulative Hydrogen Formation (CHF) with variation of sodium chloride concentrations. The highest CHF amount was 130.7 mL at 5 g/L NaCl. The lowest CHF was 53 mL at 0 g/L NaCl. According to this result, hydrogen gas production increased with NaCl concentrations (Kajal G Singh, 2016; Taheri et al., 2018; Y. Wang et al., 2013). Figure 3 shows the total sugar concentrations with time at different NaCl concentrations. Within the initial 24-hour period, 72% of the sugar in the experimental vessel without salt was consumed, making it the fastest sugar to be consumed compared to the other concentrations. Within 48 hours, lactose was almost spent at all concentrations except for 5 and 6 g/L NaCl.

In the tested fermentation mediums with 5 and 6 g/L NaCl, almost no consumption was observed in the first 48 hours, and it took 240 hours for almost all sugar to be consumed. These results revealed that high concentrations of NaCl delayed sugar consumption and slowed down the metabolic activity of organism.

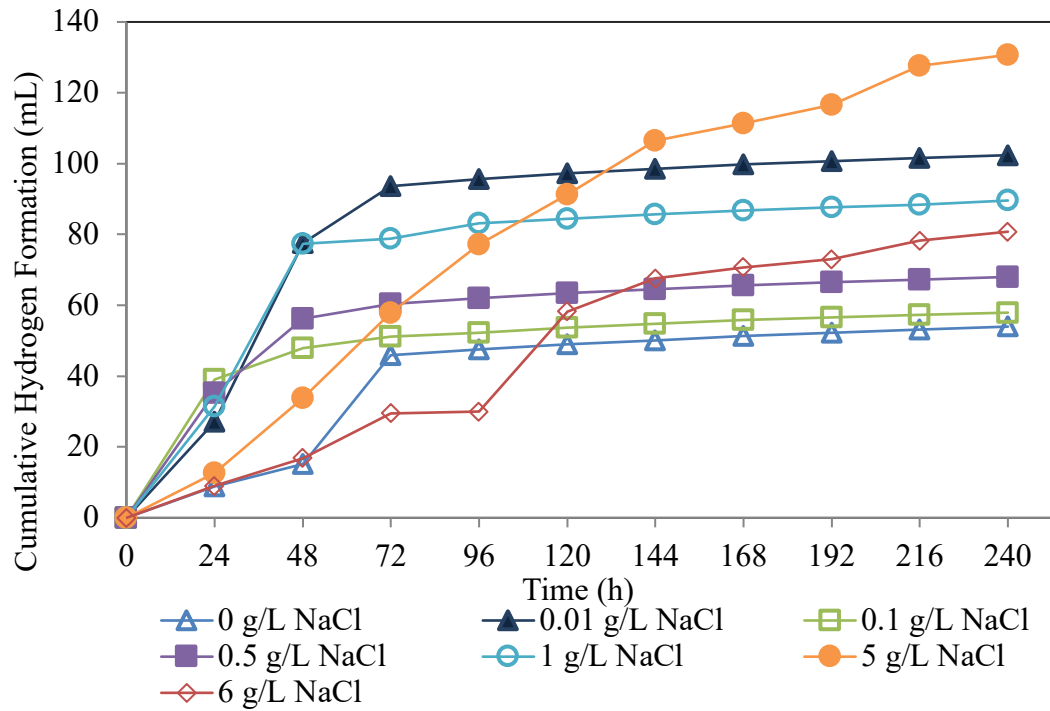


Figure 2 Cumulative Hydrogen Formation (mL) with time at different NaCl concentrations with anaerobic sludge

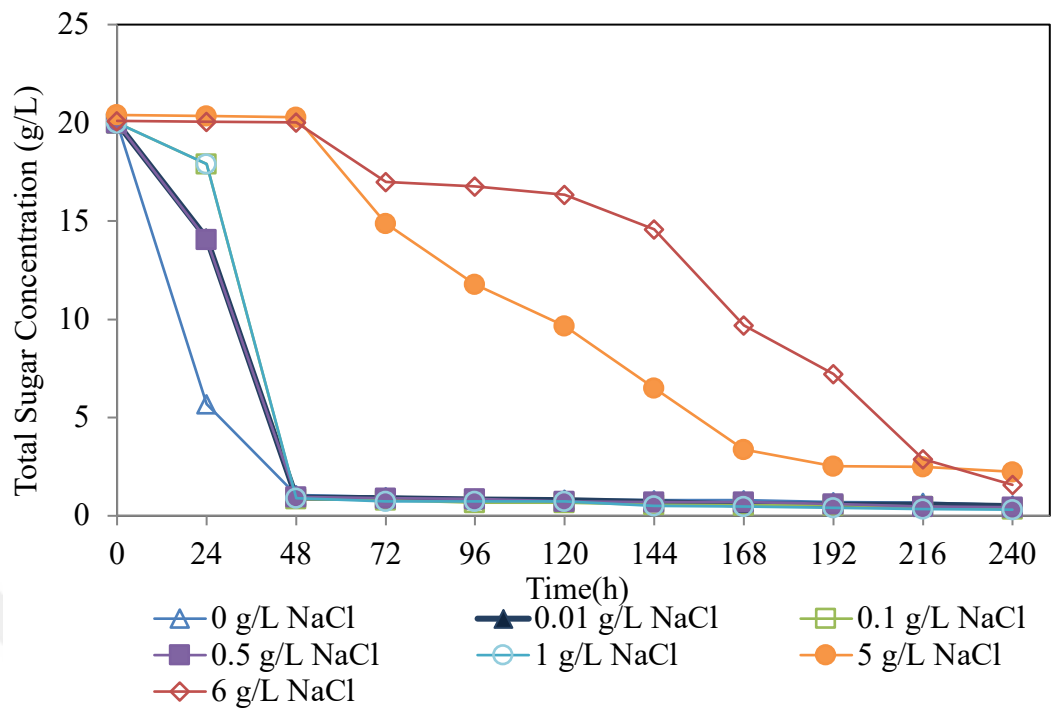


Figure 3 Variation of the total sugar concentrations with time at different NaCl concentrations with anaerobic sludge

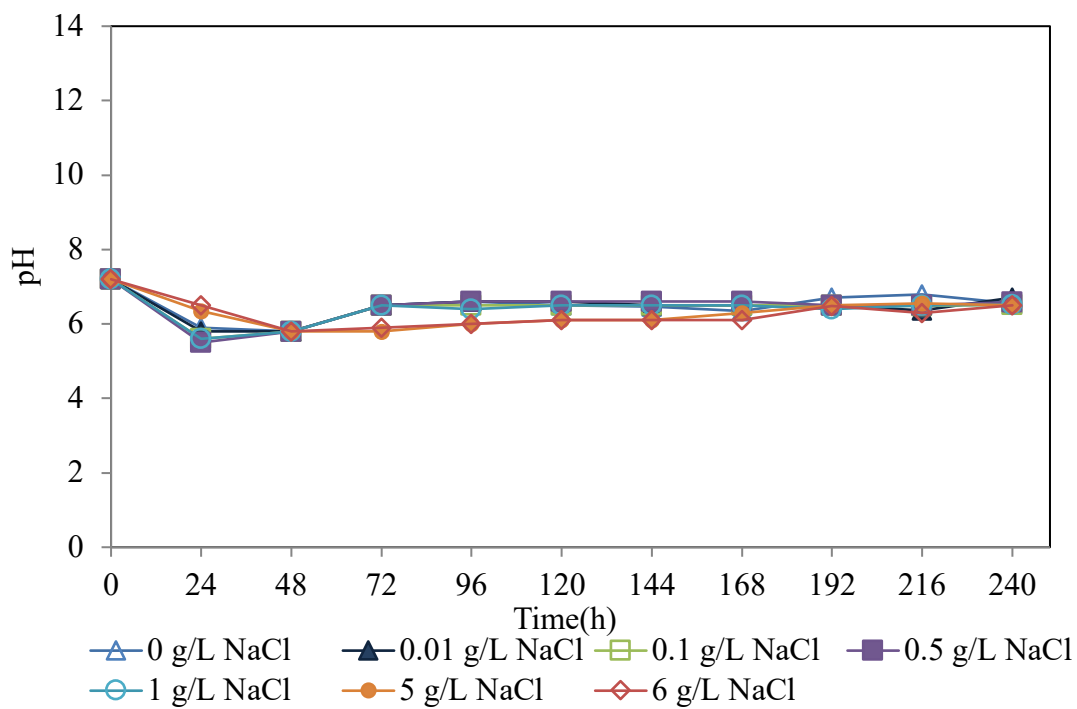


Figure 4 Variation of pH with time at different NaCl with anaerobic sludge

Figure 4 represents pH with time at range of 0 to 6 g/L NaCl concentrations. The lowest pH was observed as 5.6 in most of the tested vessels. The pH was adjusted at 6.5 after 48 hours. Figure 5 shows variation of Butyric Acids (BA) at the end of the fermentation time at various of NaCl concentrations with anaerobic sludge. The production of butyric acid increased with the addition of NaCl, up to a concentration of 5 g/L. The highest BA was found to be 5.4 g/L BA at the NaCl concentration of 5 g/L. The other experimental vessels produced around 2 g/L BA.

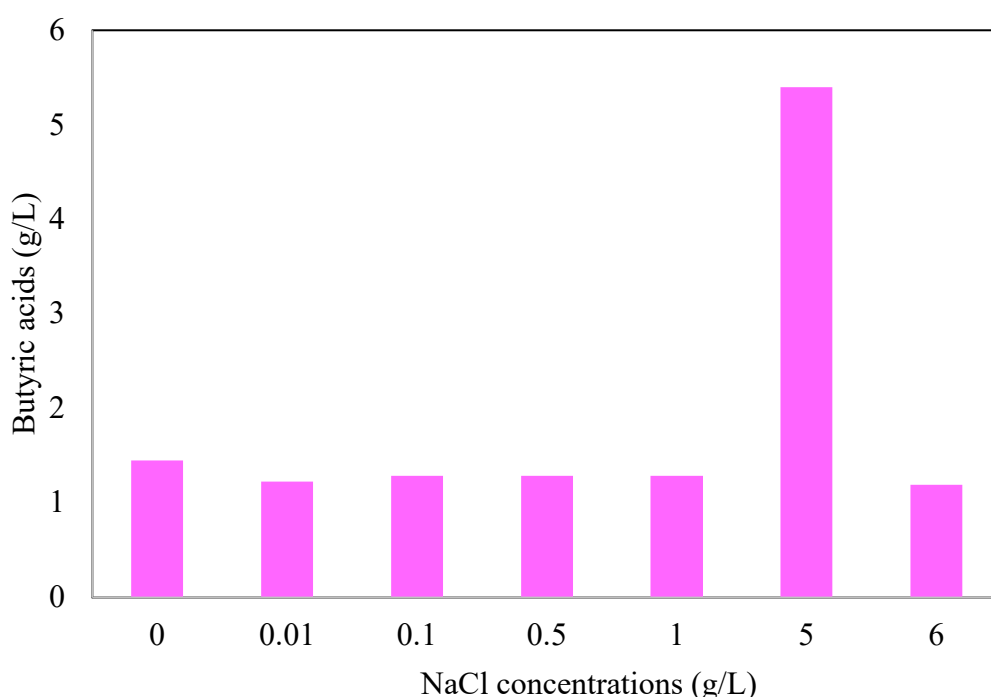


Figure 5 Variation of butyric acids (BA) at the end of the fermentation time at different NaCl concentrations with anaerobic sludge

Figure 6 shows the change in the Butyric acid to Acetic acid ratios (BA/AA) at different NaCl concentrations. The maximum BA/AA ratio of 2.6 g BA/g AA was obtained in the experimental vessel with 5 g/L NaCl concentration. The BA/AA ratio increased with increasing amounts of NaCl until 5 g/L NaCl concentration, after which it decreased by half at 6 g/L NaCl. Figure 7 indicates the variation of butyric acid and hydrogen production yields with various NaCl concentrations.

The highest BA production yield was obtained with 1.3 g BA/g TS at 5 g/L NaCl. The other BA production yields were found below 0.65 g BA/g TS.

No trend increases were obtained with increasing NaCl concentrations. The maximum hydrogen production yield of 6706.9 mL H₂/g TS was observed at 0.01 g/L NaCl concentration. The hydrogen production yield increased up to 0.01 g/L NaCl and then remained below 3500 mL H₂/g TS. Figure 8 represents variation of Butyric Acid Production Rate (BAPR) and Hydrogen Production Rate (HPR) at 0 to 6 g/L NaCl concentrations. The highest BAPR of 0.0136 g BA/g TS h was found at 5 g/L NaCl, the following BAPR of 0.130 g BA/g TS h was found at 0.01 g/L NaCl. The maximum HPR was obtained as 280 mL H₂/g TS h with 0.01 g/L.

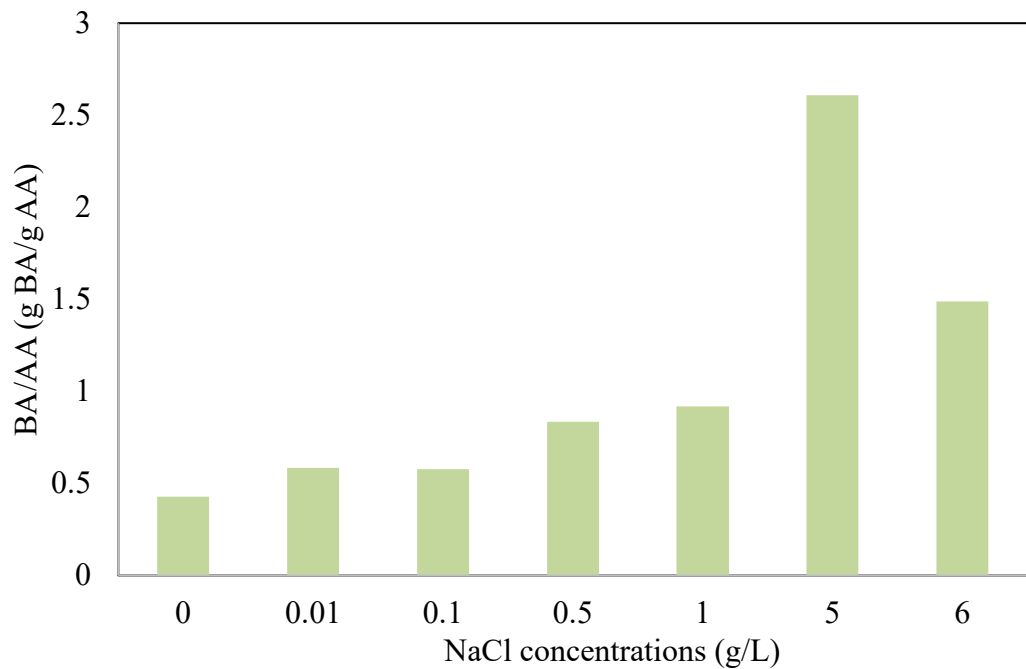


Figure 6 Variation of BA/AA (Butyric acid/Acetic acid) ratio with different NaCl concentrations in anaerobic sludge

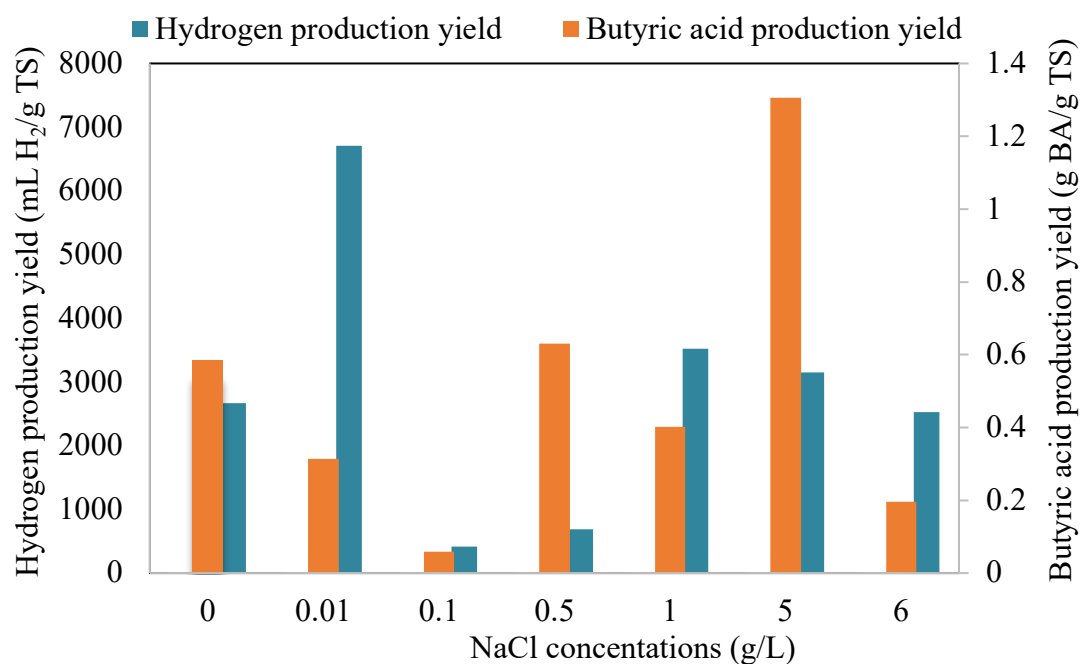


Figure 7 Variation of butyric acid and hydrogen production yields with different NaCl concentrations with anaerobic sludge

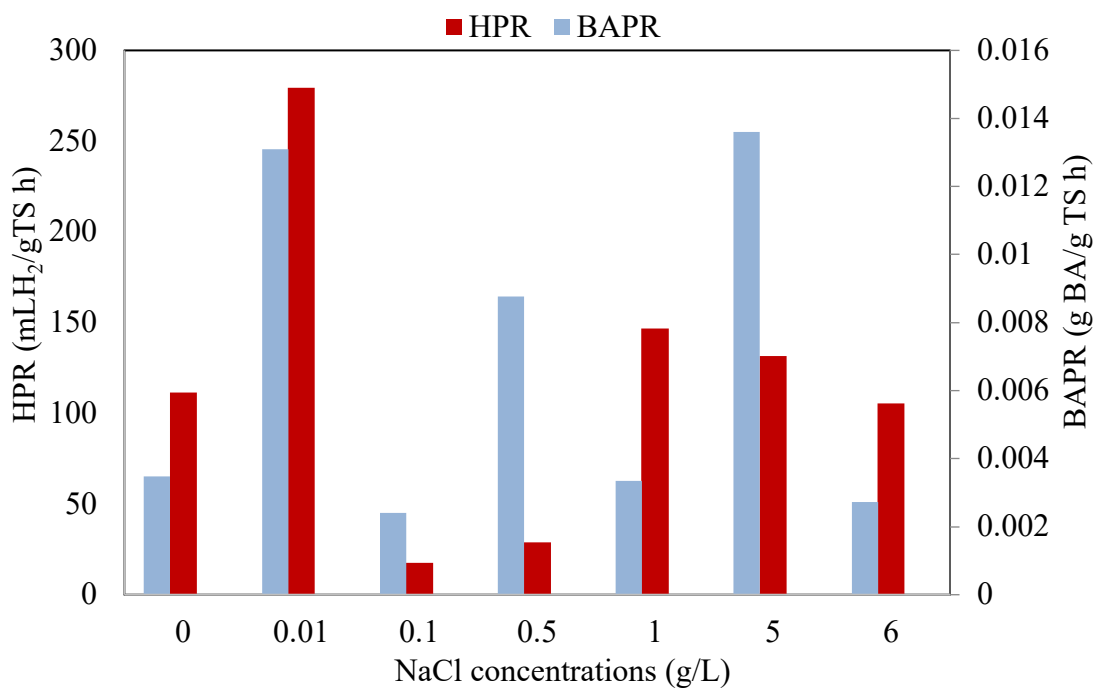


Figure 8 Variation of Butyric Acid Production Rate (BAPR) and Hydrogen Production Rate (HPR) with different NaCl concentrations in anaerobic sludge

The aim of the first experiment was to understand the impact of NaCl on butyric acid production. As a result, the optimum NaCl concentration of 5 g/L was chosen due to the highest amount of butyric acid, BA/AA ratio, CHF, butyric acid production yield, and BAPR. However, due to fluctuations between NaCl concentrations, the optimum NaCl concentration was searched with different parameters in the following experiment.

4.1.2 Evaluation of Lactose Concentration on Biohydrogen and Butyric Acid Production with Anaerobic Sludge

The second experiment was aimed to enhance butyric acid production with evaluation of lactose concentration from 5 to 100 g/L via a constant concentration of 5 g/L NaCl. Anaerobic sludge was used as a microorganism in this experiment at 37°C for 269 hours. Figure 9 displays the Cumulative Hydrogen Formation (CHF) over time for various lactose concentrations. The maximum CHF of 62 mL was observed at a lactose concentration of 10 g/L, while the lowest CHF of 18 mL was found at the highest lactose concentration of 100 g/L.

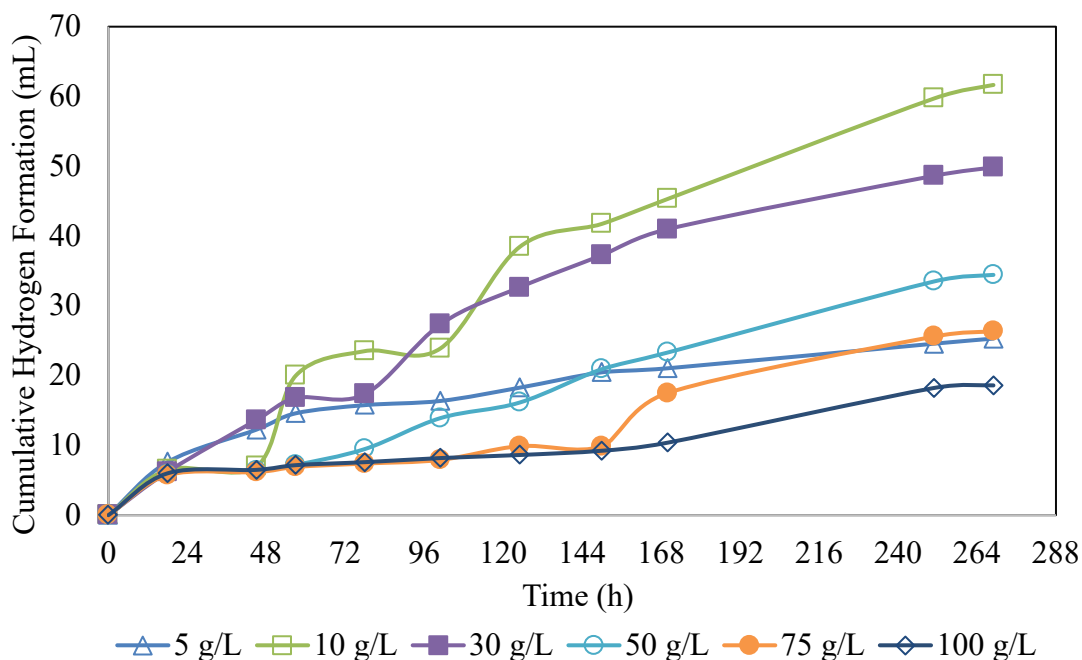


Figure 9 Cumulative Hydrogen Formation (mL) at time with different lactose concentrations with anaerobic sludge

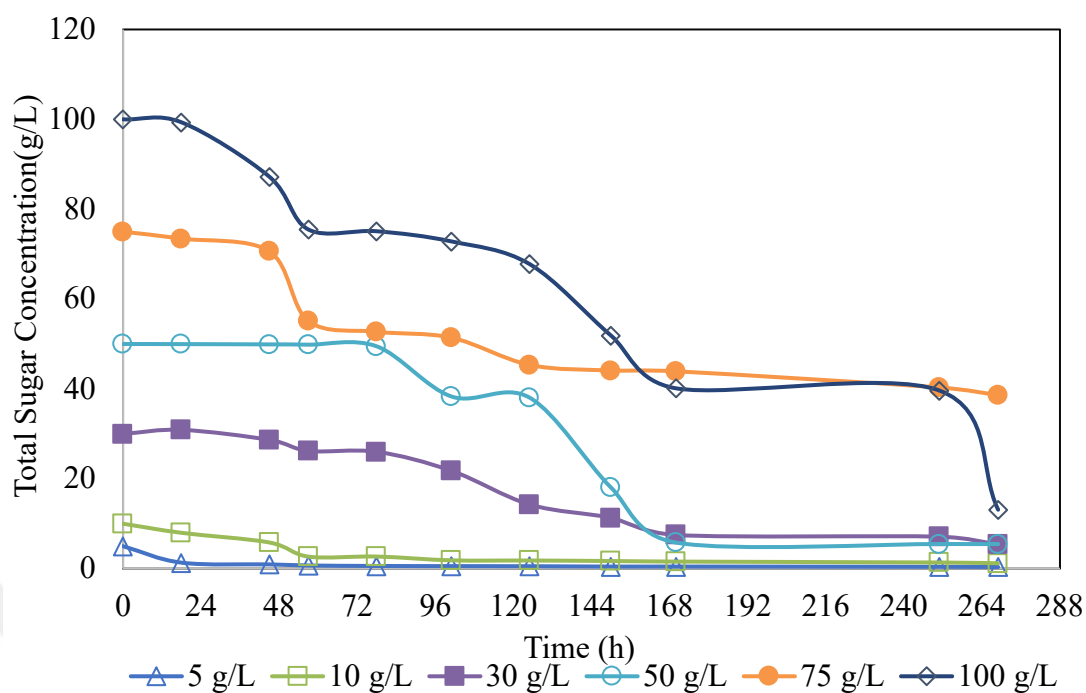


Figure 10 Variation of the total sugar concentrations with time at different lactose concentrations with anaerobic sludge

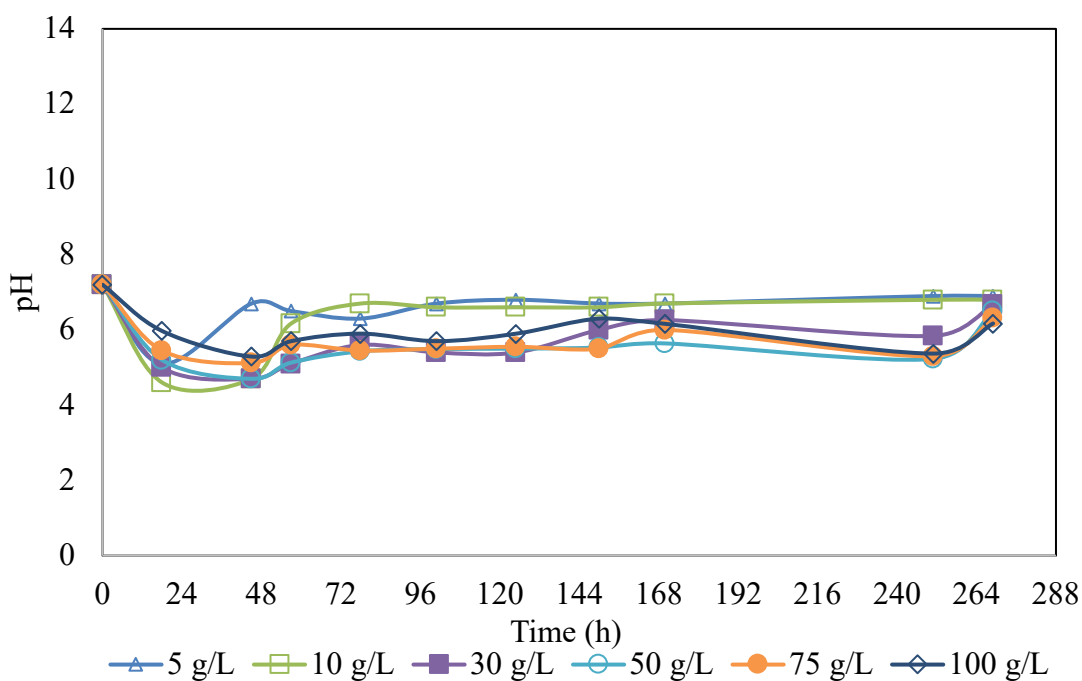


Figure 11 Variation of pH with time at different lactose concentrations with anaerobic sludge

Figures 10 and 11 show the variation of total sugar concentrations and the variation of pH over time with different lactose concentrations from 5 to 100 g/L. Below 30 g/L lactose concentration, all sugars were consumed. However, at 30, 50, 75 and 100 g/L lactose, not all sugars were consumed. All experimental vessels were adjusted to pH 7.2 at beginning of the experiment. The lowest pH was 4.6 at 10 g/L lactose. The pH fluctuated around 5.5 to 6.8. Figure 12 shows the variation of butyric acids at the end of the fermentation time with different lactose concentrations. The highest level of BA was found with 3.8 g/L BA in 30 g/L of lactose.

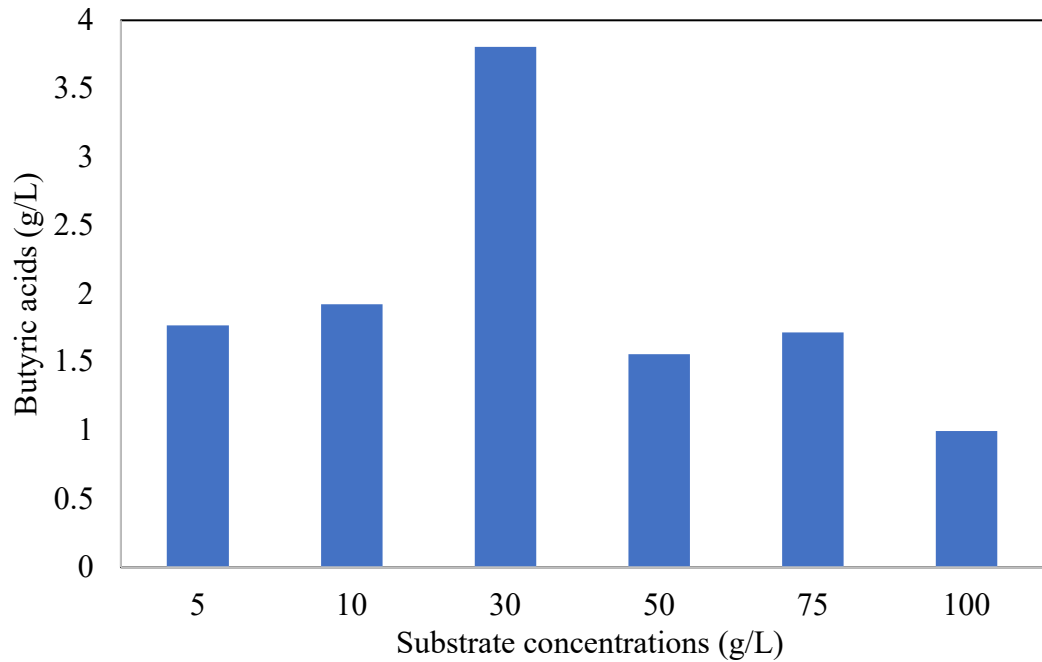


Figure 12 Variation of butyric acids at the end of the fermentation time at different lactose concentrations with anaerobic sludge

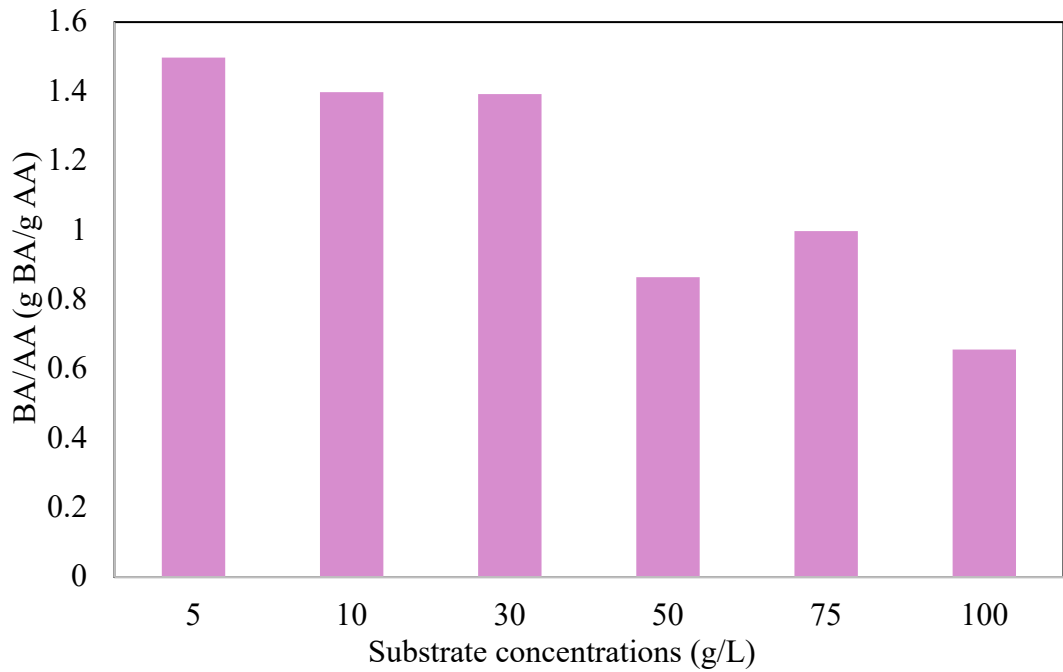


Figure 13 Variation of BA/AA ratio with different lactose concentrations in anaerobic sludge

Figure 13 indicates the variation of the Butyric Acid to Acetic Acid (BA/AA) ratio with various lactose concentrations. The maximum BA/AA ratio was obtained at 5 g/L lactose concentration, which was 1.5 g BA/g AA. The following BA/AA ratios were 1.40 g BA/g AA at 10 and 30 g/L lactose.

Figures 14 and 15 show the variation of hydrogen and butyric acid production yields and the variation of Hydrogen Production Rate (HPR) and Butyric Acid Production Rate (BAPR) via different lactose concentrations. The butyric acid and hydrogen production yields at 5 g/L lactose were maximum at 8.85 g BA/g TS, and 621.5 mL H₂/g TS, respectively. Butyric acid production yields were decreased with increasing amount of lactose. The highest HPR was found to be 7.67 mL H₂/g TS h at 5 g/L lactose. Also, the maximum BAPR was obtained 0.087 g BA/g TS h at 5 g/L lactose.

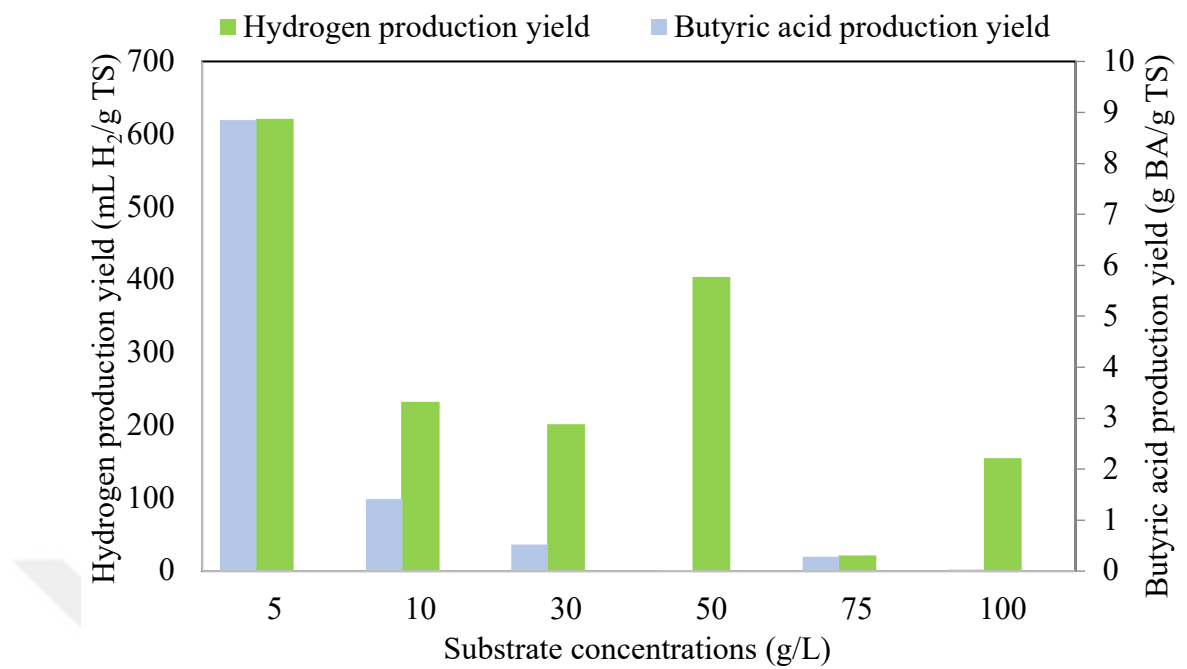


Figure 14 Variation of hydrogen and butyric acid production yields with different lactose concentrations with anaerobic sludge

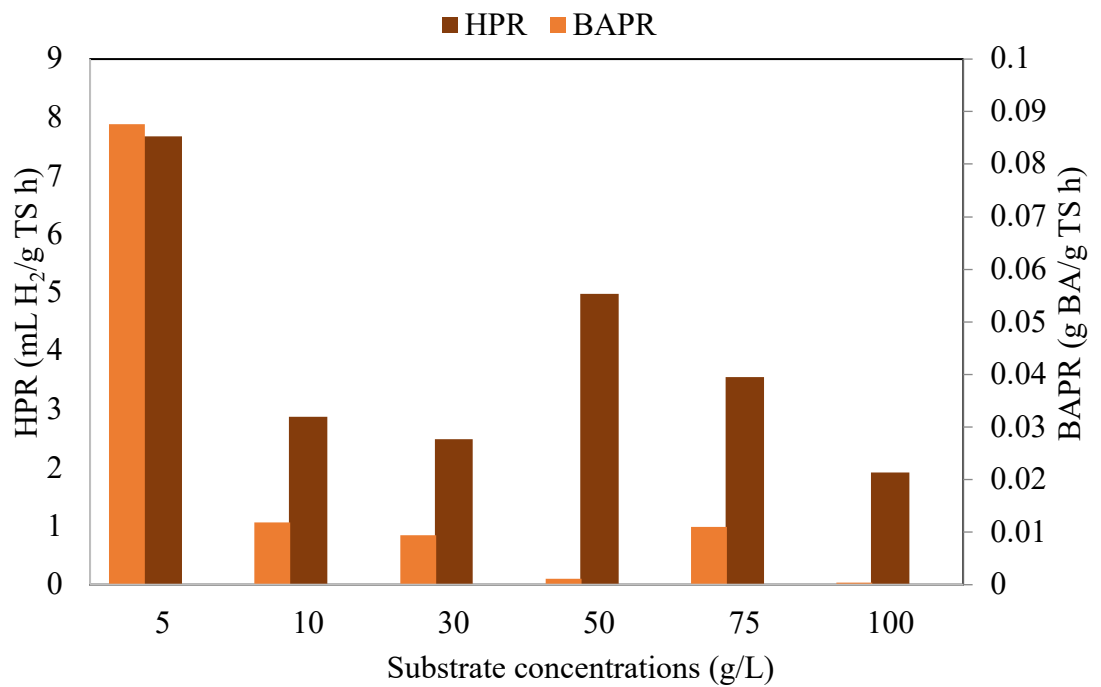


Figure 15 Variation of Hydrogen Production Rate (HPR) and Butyric Acid Production Rate (BAPR) with different lactose concentrations in anaerobic sludge

4.1.3 Optimization of mineral addition to the Fermentation Media for Enhancement of Biohydrogen and Butyric Acid Production with *C. acetobutylicum*

The impact of NaCl, Na₂S₂O₃, and mineral concentrations (Ni, Fe, Cu, Zn) on butyric acid and hydrogen gas production was investigated using the Box-Behnken Design (BBD) (Design Expert 13.0.5.0 Software) in this experiment. *Clostridium acetobutylicum* was used as the organism, and lactose was used as the substrate at 37 °C for 10 days. The mineral concentrations in the fermentation media consisted of 4 elements: nickel (Ni⁺² in the form of NiCl₂), zinc (Zn⁺² in the form of ZnSO₄), iron (Fe⁺² in the form of FeSO₄), and copper (Cu⁺² in the form of CuCl₂). Each mineral was added as a mineral complex in amounts ranging from 0-0.1-0.2 mM. The total concentration of trace minerals in the fermentation media varied between 0-0.4-0.8 mM.

4.1.3.1 Analysis of the Model Based on Input Parameters

The BBD statistical model was used in this experiment to optimize the concentrations of chosen minerals and trace elements for butyric acid production through dark biohydrogen fermentation. Seventeen experimental runs were planned and carried out in duplicate. The conditions applied in each run are summarized in Table 5.

Table 5 Box-Behnken Design

Run	A: NaCl conc. (g/L)	B: Na₂S₂O₃ conc. (mg/L)	C: Mineral conc. (mM)
1	5	100	0.8
2	2.505	100	0.4
3	2.505	200	0.8
4	5	0	0.4

Table 5 Box-Behnken Design (continued)

5	0.1	100	0
6	0.1	200	0.4
7	2.505	200	0
8	2.505	100	0.4
9	2.505	100	0.4
10	2.505	100	0.4
11	2.505	100	0.4
12	2.505	0	0.8
13	5	200	0.4
14	0.1	0	0.4
15	5	100	0
16	2.505	0	0
17	0.1	100	0.8

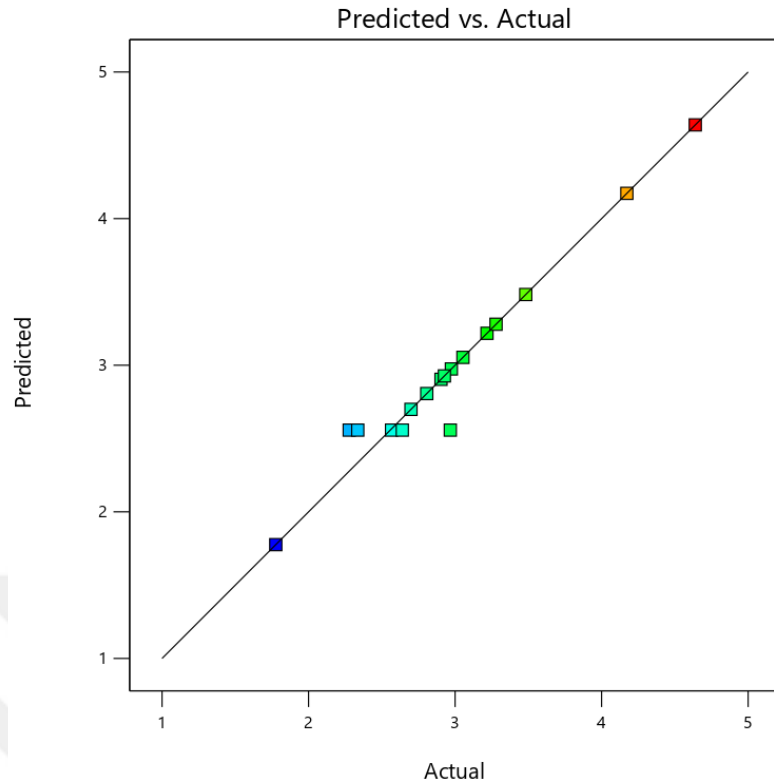


Figure 16 Actual vs. predicted produced butyric acid values

The production model for butyrate and biohydrogen, based on three factors, was fitted to a cubic model. Multiple regression analysis was applied to experimental test data to obtain the third-degree polynomial equation (15).

$$Y = 2.56 + (-0.0653A) + 0.1504B + (-0.2958C) + (-0.7669AB) + (-0.1491AC) + (-0.3268BC) + 0.0636A^2 + 0.3836B^2 + 0.4584C^2 + (-0.8151A^2B) + 0.4844A^2C + 0.2684AB^2 \quad (15)$$

The predicted response of butyrate production (Y) was determined using A, B, and C as coded values. The model was found to be highly significant, as indicated by the high values of the adjusted coefficient of determination (adjusted $R^2 = 0.8353$) and coefficient of determination ($R^2 = 0.9588$).

Figure 16 depicts the predicted butyric acid concentrations using equation (15), which varied between 1.78-4.64 g/L in the experimental design. The model's standard deviation was 0.2749, with a mean value of 2.98 g/L for butyric acid concentration. Table 6 shows the results of the analysis of variance (ANOVA) for butyrate production using a third-order polynomial. The table highlights the significant data, with P values less than 0.05 indicating a significant model (W. Q. Guo et al., 2009; C. H. Zhang et al., 2009). A P value less than 0.01 indicates a highly significant model, while a P value higher than 0.05 indicates an insignificant one (Taherdanak et al., 2015). For significance, the F value must be higher than the P value. The statistical analysis showed that the model was highly significant, as indicated by the F value (7.76) and P value (0.03). The linear terms (A, B, C) and the interactive terms of AC, BC, A², A²C, AB² were found to be insignificant for butyric acid production in this study, with a P value greater than 0.05. However, the interactive terms of AB, B², C² and A²B were found to be significant (P < 0.05), as shown in ANOVA Table 6.

Table 6 ANOVA (Analysis of Variance) table for cubic model of butyric acid with three factor Box-Behnken Design (BBD)

Source	Sum of Squares	Df	Mean Square	F-value	P-value
Model	7.04	12	0.5866	7.76	0.031
A-NaCl conc.	0.017	1	0.017	0.2255	0.6596
B-sodium thiosulfate	0.0905	1	0.0905	1.2	0.3351
C-Minerals	0.35	1	0.35	4.63	0.0977
AB	2.35	1	2.35	31.14	0.0051
AC	0.089	1	0.089	1.18	0.3389
BC	0.4271	1	0.4271	5.65	0.0762
A²	0.0171	1	0.0171	0.2258	0.6595
B²	0.6196	1	0.6196	8.2	0.0457
C²	0.8847	1	0.8847	11.71	0.0267
A²B	1.33	1	1.33	17.59	0.0138
A²C	0.4693	1	0.4693	6.21	0.0673
AB²	0.1441	1	0.1441	1.91	0.2394

Table 6 ANOVA (Analysis of Variance) table for cubic model of butyric acid with three factor Box-Behnken Design (BBD) (continued)

Pure Error	0.3022	4	0.0755		
Cor Total	7.34	16			

To ensure a highly adequate model, the R^2 (0.9588) and (adj) R^2 (0.8353) should be close to 1. According to previous research by Myers et al. (2016), an R^2 value over 0.75 is necessary for an accurate model. The mathematical model generated using RSM was found to be highly acceptable, as the observed and predicted values were close. This suggests that the BBD model was acceptable for optimizing the process. Table 6 displays the ANOVA results, indicating that the interactive effects of sodium chloride and minerals, as well as most third-order interactions (excluding A^2B), have a contrary impact on butyric acid enhancement. Table 7 presents the model's coefficient estimates and their corresponding confidence intervals.

Table 7 Coefficients of estimates and their confidence intervals for Box-Behnken Design

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	2.56	1	0.1229	2.22	2.9	
A-NaCl conc.	-0.0653	1	0.1374	-0.4468	0.3163	2
B- Na ₂ S ₂ O ₃ conc.	0.1504	1	0.1374	-0.2311	0.532	2
C- Minerals	-0.2958	1	0.1374	-0.6774	0.0858	2
AB	-0.7669	1	0.1374	-1.15	-0.3853	1

Table 7 Coefficients of estimates and their confidence intervals for Box-Behnken Design (continued)

AC	-0.1491	1	0.1374	-0.5307	0.2324	1
BC	-0.3268	1	0.1374	-0.7083	0.0548	1
A ²	0.0636	1	0.1339	-0.3083	0.4355	1.01
B ²	0.3836	1	0.1339	0.0117	0.7555	1.01
C ²	0.4584	1	0.1339	0.0865	0.8303	1.01
A ² B	-0.8151	1	0.1943	-1.35	-0.2755	2
A ² C	0.4844	1	0.1943	-0.0552	1.02	2
AB ²	0.2684	1	0.1943	-0.2712	0.808	2

AB: Interaction between NaCl and Na₂S₂O₃ concentration, AC: interaction between NaCl concentration and mineral, BC: interaction between Na₂S₂O₃ concentration and mineral, A²: quadratic value for NaCl concentration, B²: quadratic value for Na₂S₂O₃ concentration, C²: quadratic value for interaction between mineral, A²B: interaction between Na₂S₂O₃ concentration and quadratic value for NaCl concentration, A²C: interaction between mineral and quadratic value for NaCl concentration, AB²: interaction between NaCl concentration and quadratic value for Na₂S₂O₃ concentration, df: degrees of freedom, CI: 95% Confidence Intervals, VIF: Variance Inflation Factor.

Figure 17 displays the cumulative hydrogen formation (in mL) over time at varying concentrations of NaCl, Na₂S₂O₃, and minerals (Cu, Fe, Ni, Zn) within 233 hours. The maximum CHF amount was 190 mL in the 2nd bottle, which contained 0.01 g/L NaCl and 100 mg/L Na₂S₂O₃. The following CHF amount was 179 mL in the 12th bottle, which contained 2.505 g/L NaCl and 200 mg/L Na₂S₂O₃. The CHF amounts in the other fourteen experimental vessels were mostly around 90 to 155 mL. The lowest CHF was 33 mL in the 15th experimental vessel, which contained 5 g/L NaCl and 100 mg/L Na₂S₂O₃.

These results suggest that an increase in NaCl amount has a negative effect on hydrogen gas production, but not on butyric acid production. However, several studies have shown that the addition of 0.01-6 g/L NaCl can enhance butyric acid production (Bevilacqua et al., 2021; H. Fu et al., 2017; Luo et al., 2020; Oh et al., 2019a, 2019b).

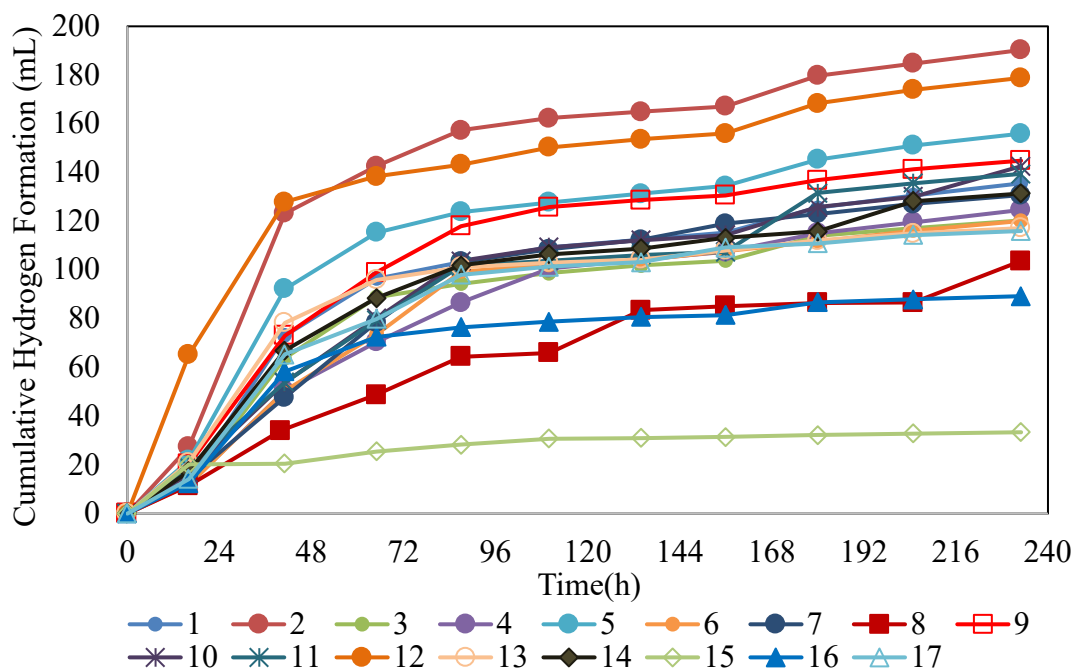


Figure 17 Cumulative Hydrogen Formation (mL) with time at different NaCl, Na₂S₂O₃ and minerals concentrations (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum*

1: 0.01 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **2:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, **3:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **4:** 0.01 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **5:** 2.505 g/L NaCl, **6:** 2.505 g/L NaCl, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn) **7-8-9-10-11:** 2.505 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **12:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, **13:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **14:** 5 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **15:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, **16:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **17:** 5 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 mM minerals conc. (Ni, Fe, Cu, Zn).

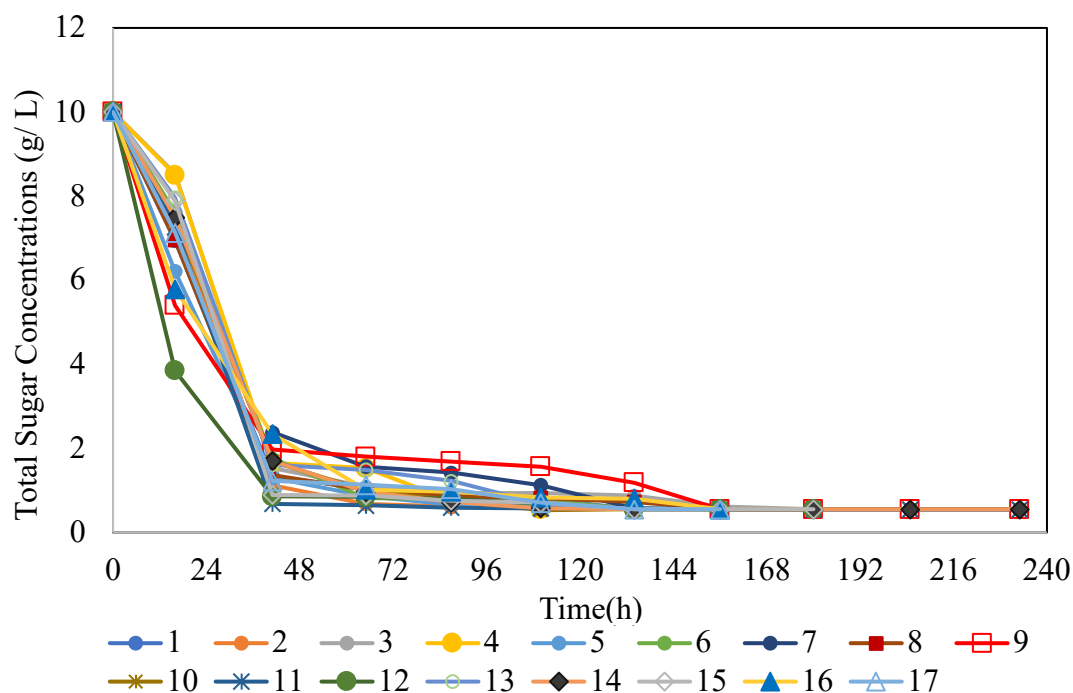


Figure 18 Variation of the total sugar concentrations with time at different NaCl, Na₂S₂O₃ and minerals (Ni, Fe, Cu, Zn) concentrations with *Clostridium acetobutylicum*

1: 0.01 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **2:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, **3:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **4:** 0.01 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **5:** 2.505 g/L NaCl, **6:** 2.505 g/L NaCl, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn) **7-8-9-10-11:** 2.505 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **12:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, **13:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **14:** 5 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **15:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, **16:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **17:** 5 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 mM minerals conc. (Ni, Fe, Cu, Zn).

In Figure 18 shows sugar consumption over time at varying concentrations of NaCl, Na₂S₂O₃, and minerals (Cu, Fe, Ni, Zn) within 233 hours. The initial substrate amount was 10 g/L lactose, and after 48 hours, each experimental vessel had consumed 75% of the lactose. Figure 19 represents the variation of pH over time at different NaCl, Na₂S₂O₃ and mineral concentrations with *Clostridium acetobutylicum* at 37°C. Before dark fermentation started, all runs were adjusted to pH of 7.2 with NaOH.

Then, each experimental vessel's pH dropped to around 4-5. The optimal pH for hydrogen and butyric acid production varied among microorganisms due to its influence on bacterial growth and the acidogenic or solventogenic pathways the bacteria follow. The literature indicates that a pH around 5-7 triggers the formation of VFAs (Jaiswal et al., 2022; Masset et al., 2012). The pH was stabilised at approximately 6.5 within 72 hours.

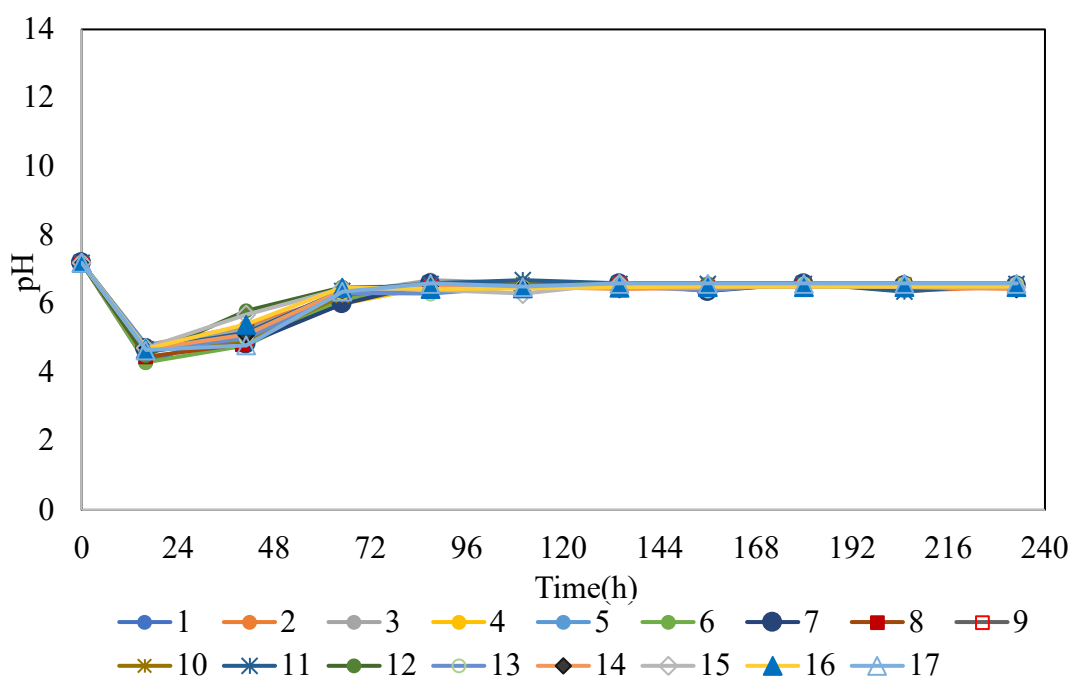


Figure 19 Variation of pH with time at different NaCl, Na₂S₂O₃ and minerals (Ni, Fe, Cu, Zn) concentrations with *Clostridium acetobutylicum*

1: 0.01 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **2:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, **3:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **4:** 0.01 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **5:** 2.505 g/L NaCl, **6:** 2.505 g/L NaCl, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn) **7-8-9-10-11:** 2.505 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **12:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, **13:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **14:** 5 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **15:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, **16:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **17:** 5 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 mM minerals conc. (Ni, Fe, Cu, Zn).

Figure 20 displays the variation of butyric acids (BA) at the end of the fermentation time under different NaCl, Na₂S₂O₃, and minerals concentrations (Ni, Fe, Cu, Zn). At the 14th experimental vessel, which contained 5 g/L NaCl and a mineral concentration of 0.4 mM (Ni, Fe, Cu, Zn), the highest BA recorded was 4.64 g/L BA.

Our findings are consistent with previous studies that have shown the effectiveness of NaCl in enhancing butyric acid production (Bevilacqua et al., 2021; H. Fu et al., 2017; Luo et al., 2020; Oh et al., 2019a, 2019b). The lowest BA concentration was obtained 1.77 g/L BA at 17th experimental vessel that included 5 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 Mm (Ni, Fe, Cu, Zn). However, for this result, it can be said that NaCl can cause inhibition with a high amount of sodium thiosulphate and mineral complex.

Figure 21 represents the variation of Butyric Acid to Acetic Acid ratios on various concentrations of NaCl, Na₂S₂O₃ and mineral. The maximum BA/AA ratio was found 2.64 g BA/g AA at 12th experimental vessel which included 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃.

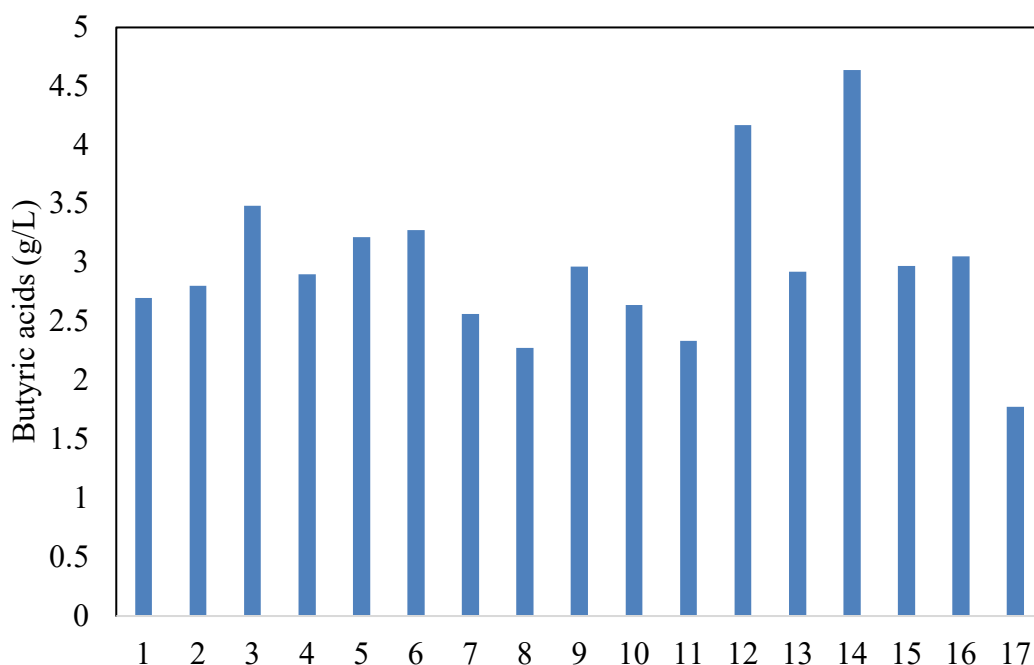


Figure 20 Variation of butyric acids at the end of the fermentation time at different NaCl, Na₂S₂O₃ and minerals (Ni, Fe, Cu, Zn) concentrations with *Clostridium acetobutylicum*

1: 0.01 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **2:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, **3:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **4:** 0.01 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **5:** 2.505 g/L NaCl, **6:** 2.505 g/L NaCl, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn) **7-8-9-10-11:** 2.505 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **12:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, **13:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **14:** 5 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **15:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, **16:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **17:** 5 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 mM minerals conc. (Ni, Fe, Cu, Zn).

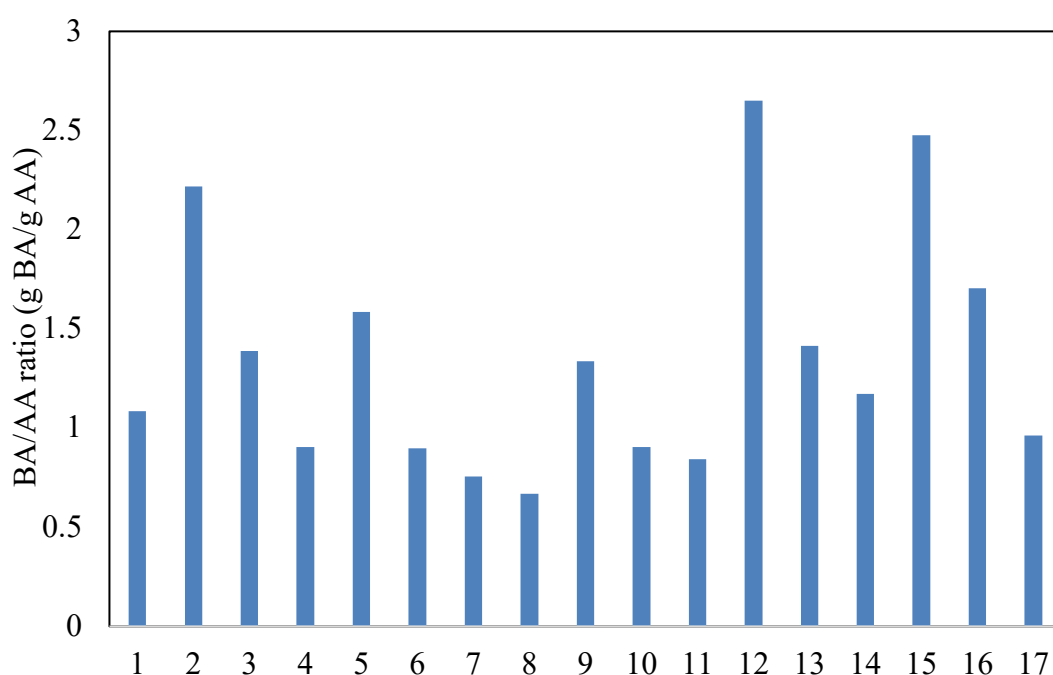


Figure 21 Variation of BA/AA ratio with different NaCl, Na₂S₂O₃ and minerals (Ni, Fe, Cu, Zn) concentrations with *Clostridium acetobutylicum*.

1: 0.01 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **2:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, **3:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **4:** 0.01 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **5:** 2.505 g/L NaCl, **6:** 2.505 g/L NaCl, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn) **7-8-9-10-11:** 2.505 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **12:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, **13:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **14:** 5 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **15:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, **16:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **17:** 5 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 mM minerals conc. (Ni, Fe, Cu, Zn).

Figures 22 and 23 show the variation of hydrogen and butyric acid production yields, and the variation of Hydrogen Production Rate (HPR) and butyric acid production rate (BAPR) with different NaCl, Na₂S₂O₃ and minerals concentrations, respectively. The highest BA yield and BAPR was observed 197.9 g BA/ g TS and 1.6 g BA/g TS h, respectively, in the 14th experimental vessel, which contained 5 g/L NaCl and 0.4 Mm mineral concentrations. In the 14th experimental vessel, there was a significant difference in both BA yield and BAPR compared to the other runs.

For hydrogen production yield, the highest value was obtained 243.6 mL H₂/g TS at 12th experimental vessel which involved 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃. The maximum HPR was found 2.6 g H₂/g TS h in 2nd experimental vessel, the following HPR values were around 2.5 g H₂/g TS h in 9th,12th,15th experimental vessels.

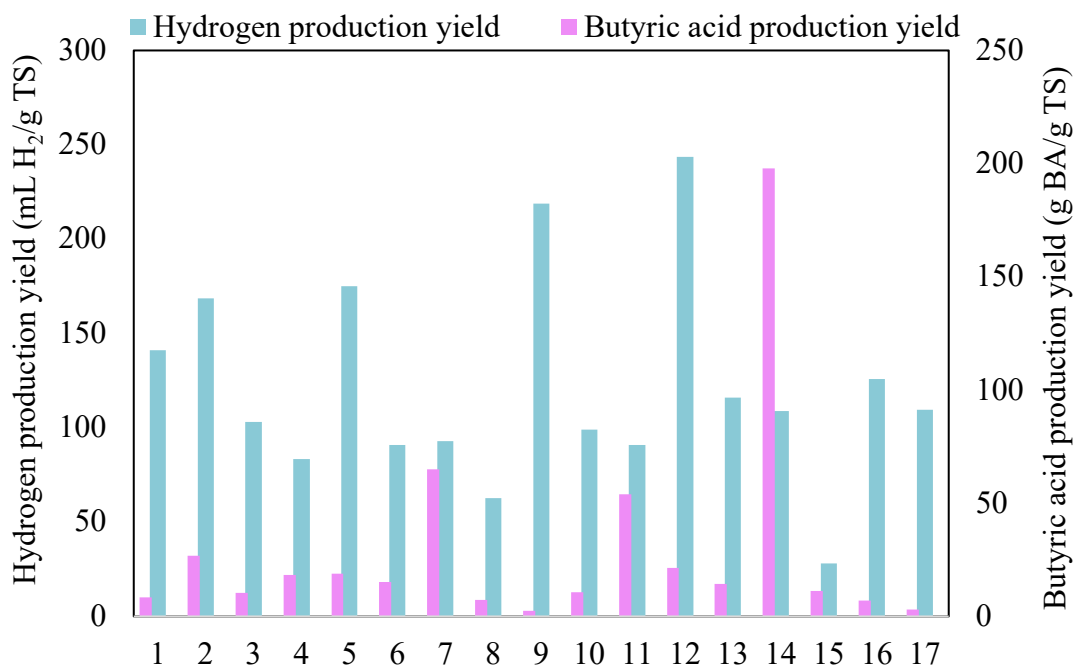


Figure 22 Variation of hydrogen and butyric acid production yields with different NaCl, Na₂S₂O₃ and minerals (Ni, Fe, Cu, Zn) concentrations with *Clostridium acetobutylicum*

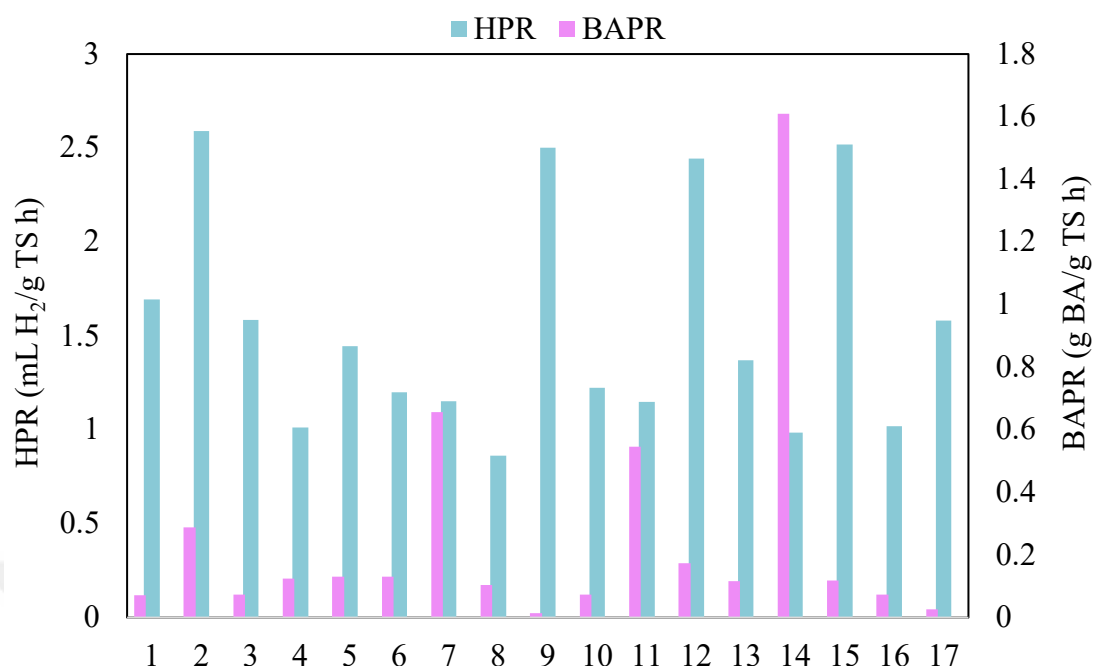


Figure 23 Variation of hydrogen production rate (HPR) and butyric acid production rate (BAPR) with different NaCl, Na₂S₂O₃ and minerals (Ni, Fe, Cu, Zn) concentrations with *Clostridium acetobutylicum*

1: 0.01 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **2:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, **3:** 0.01 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **4:** 0.01 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **5:** 2.505 g/L NaCl, **6:** 2.505 g/L NaCl, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn) **7-8-9-10-11:** 2.505 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **12:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, **13:** 2.505 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **14:** 5 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn), **15:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, **16:** 5 g/L NaCl, 100 mg/L Na₂S₂O₃, 0.8 Mm minerals conc. (Ni, Fe, Cu, Zn), **17:** 5 g/L NaCl, 200 mg/L Na₂S₂O₃, 0.4 mM minerals conc. (Ni, Fe, Cu, Zn).

4.1.3.2 Impact of Parameter Interaction on Butyric Acid Production Response

Figure 24-26 depicts the interactive effects of variables using response surface methodology (RSM). Specifically, it shows the effect of sodium chloride (A) concentration (g/L) and sodium thiosulphate (B) concentration (mg/L) on butyrate production. The interaction effect of AB was negative, the concentration of butyrate initially decreased until it reached the mean value of B, and then increased above it.

Meanwhile, as B values increased, the effect of A values on butyric acid production also increased. The model indicated that both AB and A²B were significant factors with negative effects, while B² had a positive effect on butyrate production. Contrary to the model, the literature shows that testing NaCl amounts enhances butyrate production. Additionally, it is known that high concentrations of NaCl shift the pathway into the solventogenic phase with the presence of butyric acid (Taheri et al., 2018; Singh et al. 2016). Trace amounts of sodium thiosulphate act as donors of hydrogen sulphate in the pathway and enhance hydrogen and butyrate production (Feng et al., 2005; Y. Wang et al., 2013). This finding is parallel to that of the study conducted by Chen et al., (2008). The authors reported an increase in hydrogen production under high sulphate concentrations and low HRTs. They found that the metabolic fractions were 77.3% butyrate, 15.6% acetate, 4.4% ethanol and 2.0% propionate (Chen et al., 2008). The model results indicate that an increase in sodium chloride concentrations may cause a metabolic shift towards butanol production, with butyric acid being produced throughout the fermentation period.

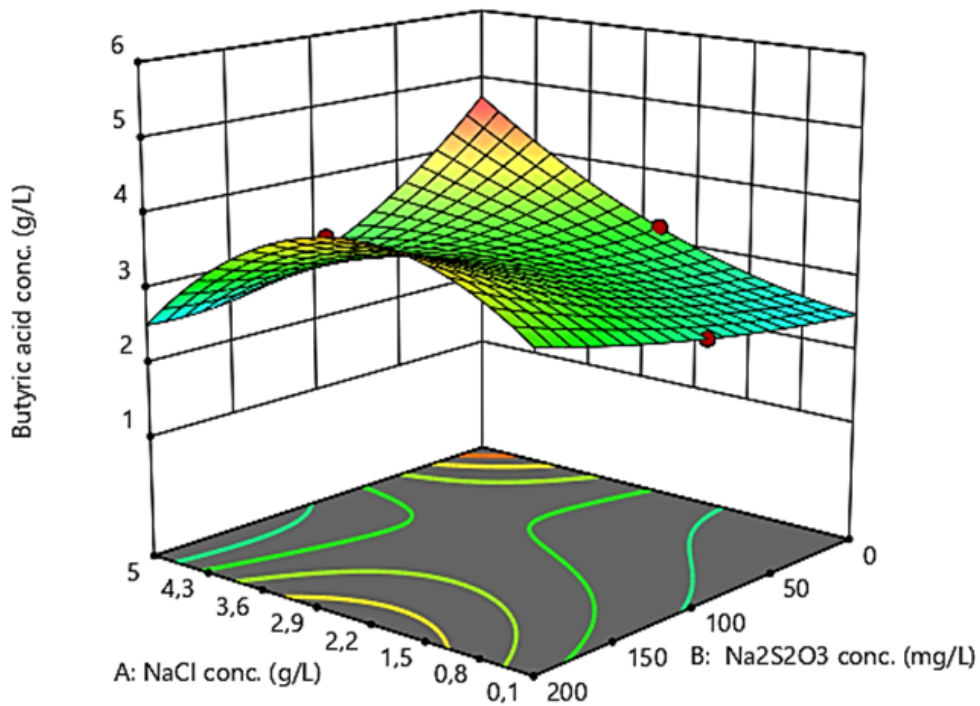


Figure 24 Response surface plot showing the effect of sodium chloride concentration (g/L) and sodium thiosulphate concentration (mg/L) on butyric acid production

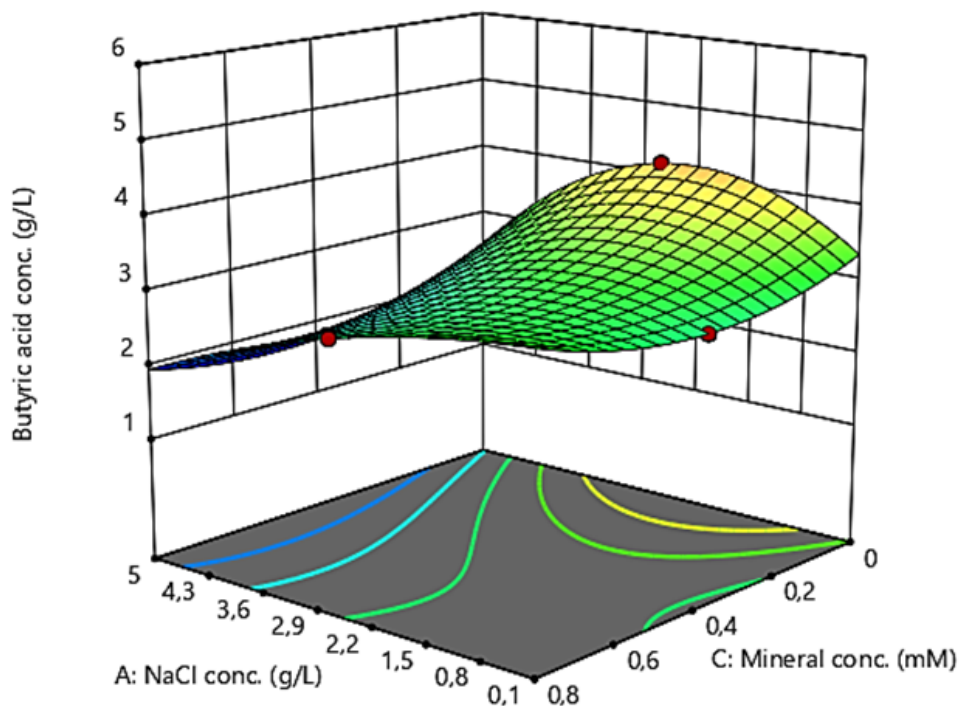


Figure 25 Response surface plot showing the effect of sodium chloride concentration (g/L) and mineral concentration (mM) on butyric acid production

Figure 25 shows that the interactions between sodium chloride (A) and mineral concentration (C). High levels of butyric acid production were achieved at higher mineral concentrations. The coefficient of the square term C^2 was positive. Figure 26 illustrates the interaction effects of mineral complex (C) and sodium thiosulphate (B) with butyrate. Consistent with previous research, increasing mineral concentrations were found to increase hydrogen and butyric acid levels (X. Fu et al., 2019; Zheng & Yu, 2004). However, Taherdanak et al., (2016) and Suanon et al., (2016) discussed the results and found that different substrates react differently to mineral addition. Additionally, high concentrations of trace elements may inhibit metabolic activities (Taherdanak et al., 2016).

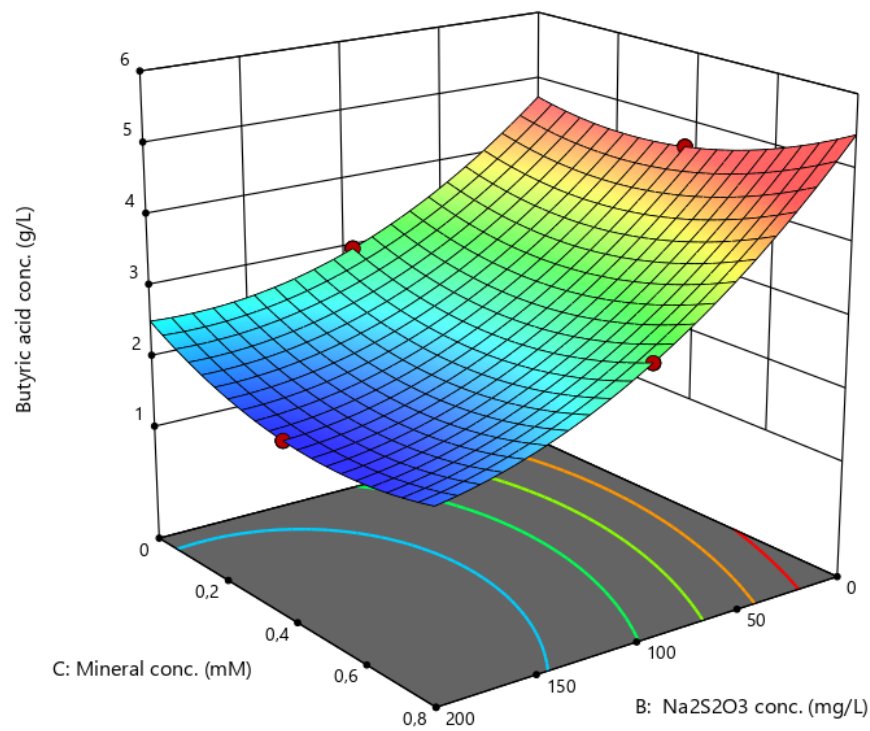


Figure 26 Response surface plot showing the effect of sodium thiosulphate concentration (mg/L) and mineral concentration (mM) on butyric acid production

The model optimising the conditions indicates that maximum butyrate production could be achieved with low NaCl, high mineral concentrations, and high sodium thiosulphate concentrations at 10 g/L lactose concentration and 37 °C using *C. acetobutylicum*.

4.1.3.3 Evaluation of CHF, BA/AA, $Y_{P/S}$ and HPR for key variables

The trial results of Cumulative Hydrogen Formation (CHF), Butyric Acid/Acetic Acid (BA/AA) ratio, hydrogen production yield ($Y_{P/S}$) and hydrogen production rate (HPR) for each run were given in Table 8. CHF, BA/AA, $Y_{P/S}$, and HPR are key parameters in butyrate based biohydrogen fermentation processes. (All of these parameters have been taken as a response in the BBD model. The P values for all models were very close to 0.05. However, they were higher than the P value considered acceptable. For this reason, the BBD model was optimised according to the concentrations of butyric acid). The high values obtained from the experimental design are highlighted in Table 8.

The 5th run with the lowest NaCl concentration and Na₂S₂O₃ concentration in the middle (0) with no minerals gave the highest CHF (190.2 mL). This was followed by the 7th run (178.8 mL) with 200 mg/L Na₂S₂O₃ and 2.5 g/L NaCl. The butyric acid to acetic acid ratios bigger than two were obtained with runs 5, 7 and 15. In all these runs there were no minerals in the experimental media. These results contradict the model obtained. When evaluating the yields, the highest yields were obtained with the 2nd, 5th, 7th, 9th, 15th, and 16th runs with different concentrations. The hydrogen production rates of the 5th, 7th, 9th, and 15th runs were the highest among the experimental runs.

Furthermore, the results showed that HPR, BA/AA, and $Y_{P/S}$ reached to their topmost values at the mean values of the independent variables of 0.4 mM mineral concentrations, 100 mg/L Na₂S₂O₃, and 2.5 g/L NaCl. These results didn't fully meet the model. This might be due to a metabolic shift to produce different end products. Yet, it is critical to increase these parameters to get excessive hydrogen production potentials. In biohydrogen production based on butyric acid, the concentration of butyric acid is widely discussed in the literature as an important parameter (Table 8).

Table 8 Experimental Results of CHF, BA/AA, $Y_{p/s}$ and HPR

Run	CHF (mL)	BA/AA (g/g)	$Y_{p/s}$ (mmol H_2 /mmol lactose)	HPR (mL/g h)
1	89.2	1.7	1.8	1.0
2	130.7	0.7	2.4	1.1
3	117.1	1.4	1.6	1.3
4	131.3	1.1	1.8	0.9
5	190.2	2.2	2.3	2.6
6	124.6	0.9	1.5	1.0
7	178.8	2.6	3.6	2.4
8	103.7	0.6	1.6	0.8
9	144.8	1.3	3.7	2.5
10	142.5	0.9	1.8	1.2
11	139.4	0.8	1.7	1.1
12	120.1	0.8	1.8	1.2
13	116	0.9	1.8	1.6
14	135.5	1.0	2.0	1.7
15	33.4	2.4	3.0	2.5
16	155.9	1.5	2.6	1.4
17	120.3	1.3	1.4	1.5

4.2 Experiments Performed with CWP as Substrate

Cheese Whey (CW) produced as a by-product of cheese and dairy production carries a high organic load, making it a significant pollutant. Due to the large volume of this waste, it poses a threat to the environment. Therefore, CW is an ideal source for use as a substrate for the generation of hydrogen, not only because it is abundant and widely accessible, but also due to its high biodegradability (Carrillo-Reyes et al., 2012). It can be used as a low-cost raw material to produce biofuels and chemicals through fermentation, thanks to its high levels of carbohydrates, lactose, protein, and fat (Buchanan et al., 2023). In addition, CW makes the separation and purification of these products very costly, and its large volume coating makes it difficult to store (Almeida et al., 2022). For this reason, after spray drying the CW to produce cheese whey powder (CWP), obtaining a highly concentrated, low volume and at the same time more durable raw material contributes economically (Khanna et al., 2011; Lovato et al., 2021; Policastro et al., 2023; Venetsaneas et al., 2009). Cheese whey or Cheese Whey Powder (CWP) can be used as a substrate for fermentation. Also, CW produced the hydrogen production yields ranging from 1-4 mol H₂/mol lactose depending on operation variables such as temperature, pH, and organic loading rate (Davila-Vazquez et al., 2009; Gannoun et al., 2008; J. Xia et al., 2021). Cheese whey fermentation can produce VFAs, primarily acetic, propionic, and butyric acid, up to 20-30 g/L depending on the working parameters, particularly pH, in addition to hydrogen production (Ferraz et al., 2021; Ferreira Rosa et al., 2014; X. Guo et al., 2017).

The experiments with CWP aimed:

- I. To investigate the impacts of CaCO₃ concentrations with *Clostridium acetobutylicum* on butyric acid and bio-hydrogen gas production.
- II. To select organisms for enhancing butyric acid and hydrogen gas production potentials.
- III. Effect of organism (biomass) concentration with *Clostridium pasteurianum* on butyric acid and bio-hydrogen gas production.
- IV. To investigate the impact of substrate (CWP) concentrations on butyric acid and bio-hydrogen gas production potentials.

4.2.1 Effects of CaCO_3 on Biohydrogen and Butyric Acid Production with *Clostridium acetobutylicum*

The stimulatory influence of CaCO_3 (0-4 g/L) on the growth and metabolism of *Clostridium* species known from the literature was tested in this experiment to enhance butyric acid and biohydrogen production. 20 g/L cheese whey powder was used in the fermentation vessels. Figure 23 depict the variation of cumulative biohydrogen formation volumes (CHF) with time and Figure 24 gives the depletion of total sugar with time. The highest CHF was obtained with 0.1 g/L CaCO_3 concentration. The following concentration was 0.5 g/L CaCO_3 giving nearly 2/3 of the concentration obtained at 0.1 g/L. It can be concluded from CHF that trace amounts of CaCO_3 enhances hydrogen production however further increases decrease the CHF amounts. At the end of the fermentation time the total sugar consisted of mainly lactose was mostly consumed in all tested vessels. The total sugar was consumed within 24 hours in the tested vessels, except the vessels without CaCO_3 addition and the one containing 0.1 g/L CaCO_3 .

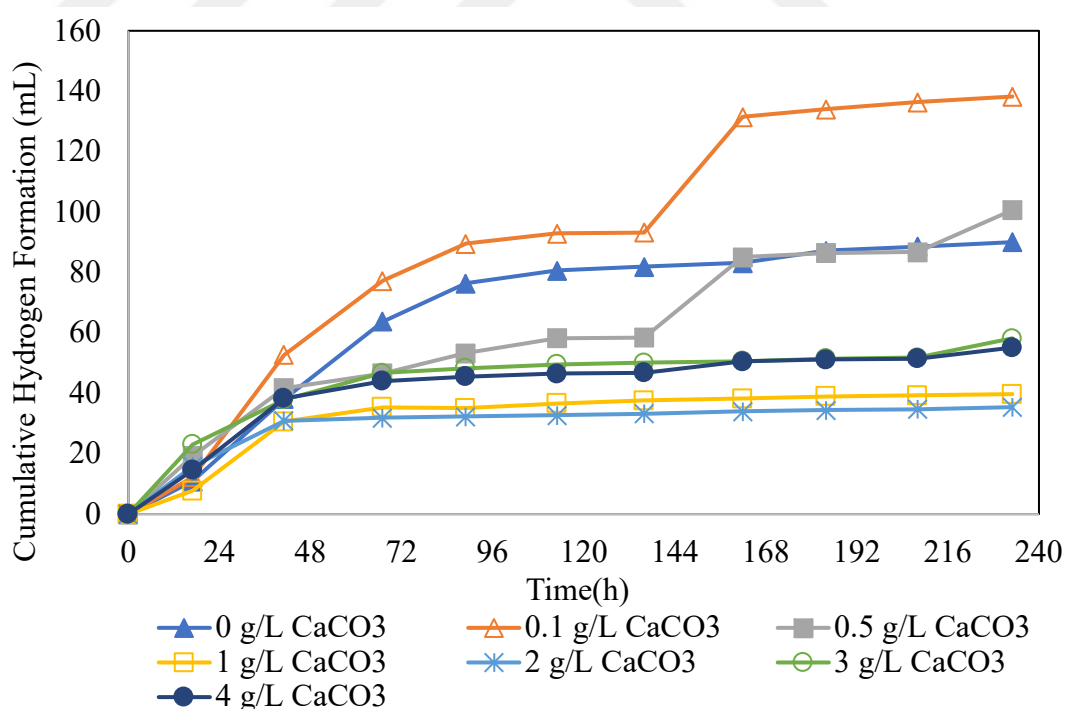


Figure 27 Cumulative Hydrogen Formation (mL) with time at different CaCO_3 concentrations with *Clostridium acetobutylicum*

In these fermentation media, the total sugar was depleted in 41 hours. These results showed that while the highest CHF_s were obtained with 0.1 g/L CaCO₃ and with the vessel without CaCO₃; the others shifted to different pathways producing end products other than hydrogen and related organic acids (Su et al., 2020).

It was assumed that the pH buffering effect might contribute to the enhancement. In the experiments, also phosphate buffer was used to control pH. The measurements of the pH values during the fermentation period were not lower than pH of 5. The optimum pH for hydrogen and butyric acid production varies between species and strains, as pH plays a very important role in microbial catalysis and bacterial growth.

For *Clostridium species* it varies between 5-7 (Jaiswal et al., 2022; Masset et al., 2012). For example, T. Guo et al., (2012) reported a two-step pH control strategy maintained at pH 5.5 during acidogenesis, where solventogenesis was favored and almost 40% increase in acetone-butanol-ethanol production was achieved with *Clostridium acetobutylicum*. In the present experiment, it is not wanted to have shift to solventogenic phase due to the aim focusing on butyric acid production.

The pH of the experimental vessels was adjusted daily to 6.5 during the fermentation period. During the first two days, the pH droplets were less than the control. Increasing CaCO₃ concentrations higher than 0.1 g/L resulted with pH between 5.5-6 at 24 hours while the pH of 0.1 g/L CaCO₃ amount was 5 within 24 hours. There was no lag phase in any of the vessels, while the degradation of CWP and the production of metabolites started immediately. After two days the pH was over 6.5 in all tested vessels throughout the fermentation period (Figure 29). The results showed a clear buffering effect of carbonate over a concentration 0.5 g/L.

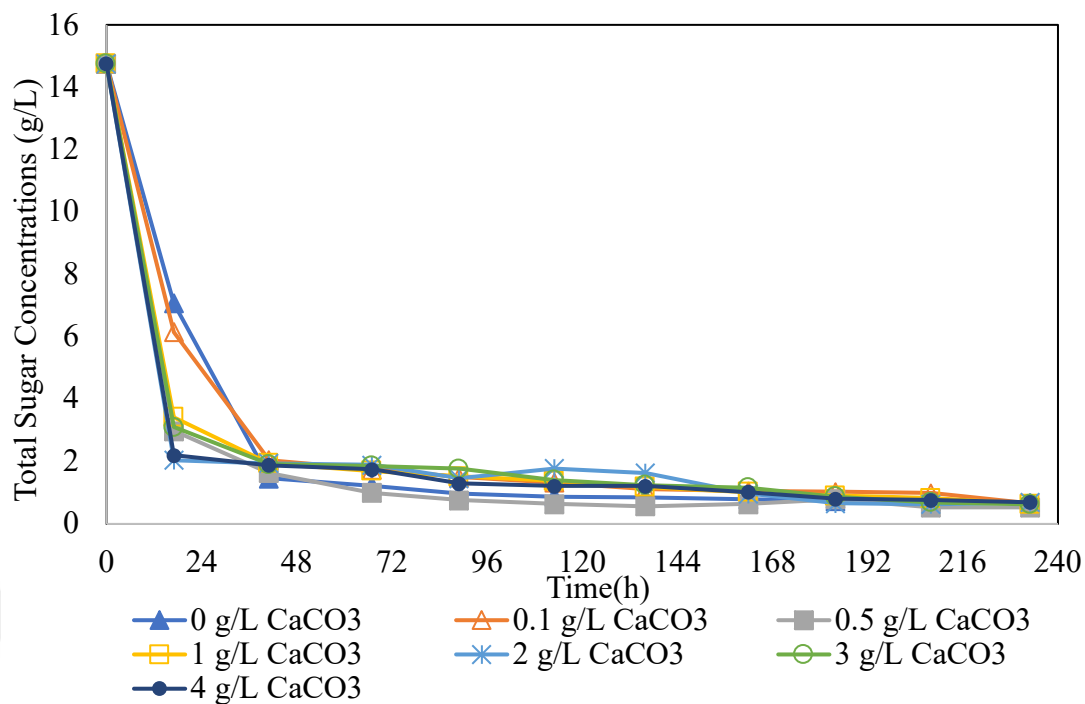


Figure 28 Variation of the total sugar concentrations with time at different CaCO₃ concentrations *Clostridium acetobutylicum*

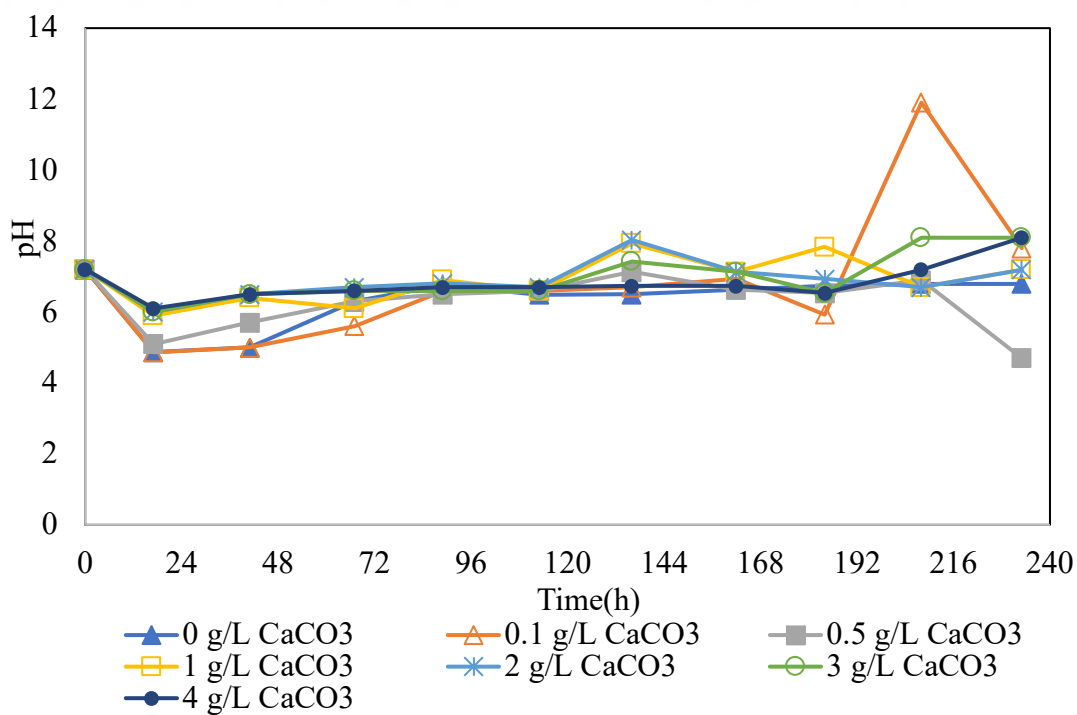


Figure 29 Variation of pH with time at different CaCO₃ concentrations *Clostridium acetobutylicum*

Figure 30 depicts the total organic acid and the maximum butyric acid concentrations obtained from the bottles where different CaCO_3 concentrations were experienced. The main organic acids obtained from the tested bottles were acetic, butyric, propionic, and lactic acid. The lactic and propionic acid concentrations were low in all bottles. The butyric acid concentrations were about 40-45% of the total organic acid concentrations when the CaCO_3 concentration was above 0.1 g/L. More than half of the total organic acid concentration was butyric acid in the control bottle. The highest concentration of butyric acid (84% of total organic acids, 2.4 g/L) was obtained in the experimental condition where 0.1 g/L CaCO_3 was added. Figure 31 shows the BA/AA (Butyric Acid/Acetic Acid) concentrations at different CaCO_3 concentrations. The highest BA/AA ratio (7.7) was obtained at 0.1 g/L CaCO_3 concentration, as was the case with CHF production, where it was followed by the control experiment (6.32).

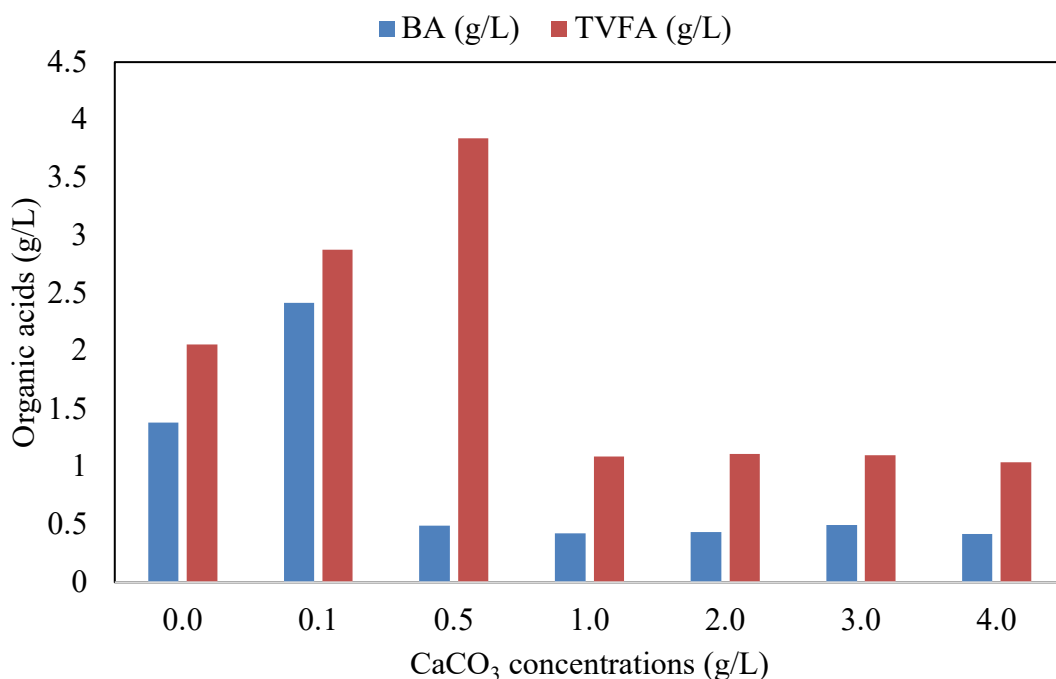


Figure 30 Variation of butyric acids and total volatile fatty acids at the end of the fermentation time at different CaCO_3 concentrations with *Clostridium acetobutylicum*

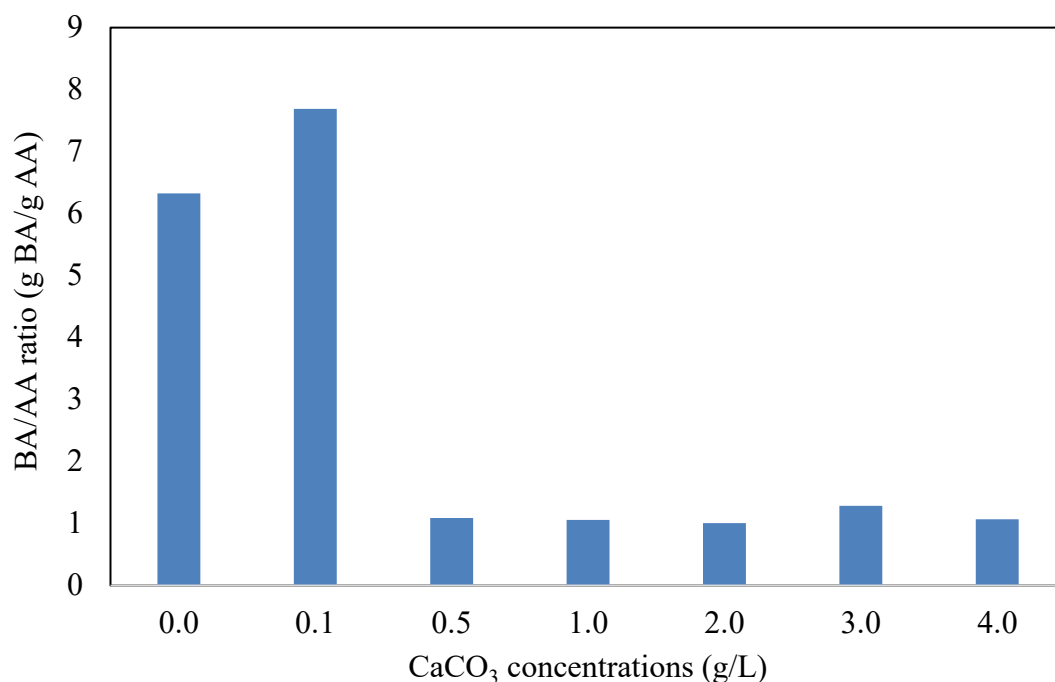


Figure 31 Variation of BA/AA ratio with different CaCO₃ concentrations with *Clostridium acetobutylicum*

Different studies reveal higher CaCO₃ concentrations where 4-5 g/L CaCO₃ was found optimum in solventogenic *Clostridium* species producing ethanol or butanol and lower pH such as pH 5.5 (Jaiswal et al., n.d.; Tian et al., 2015). While the main goal is to enhance organic acid concentrations without shifting to solventogenic phase the results obtained in our study were acceptable with no ethanol or butanol as a metabolite at the end of the fermentation period. The buffering capacity of CO₃²⁻ has not a sole effect in CaCO₃ concentrations up to 2 g/L. The buffering capacity of CO₃²⁻ until 2 g/L may not be seen due to the phosphate buffering capacity used in the experiments. However, at 3 and 4 g/L after some time the pH rose up to pH 8. It was stated that MgCO₃ improved the consumption of saccharides by *C. acetobutylicum* (Kanouni et al., 1998), and carbonate groups such as (NH₄)₂CO₃, NH₄HCO₃, K₂CO₃, NaHCO₃, Na₂CO₃ and CaCO₃ augmented the total acetone-butanol-ethanol amounts with *C. beijerinckii* (Han et al., 2013). The same trend was also given by Tian et al., (2015) revealing that only the buffering effect of the CaCO₃ was effective when compared with Na₂CO₃.

They conclude the discussions as CaCO_3 increase protein synthesis, upregulated proteins may involve in glucose uptake and utilization, increases the levels of different heat shock proteins (GrpE and Dnak), stimulates the changes in the electron transport system, and reduces the polarization resistance.

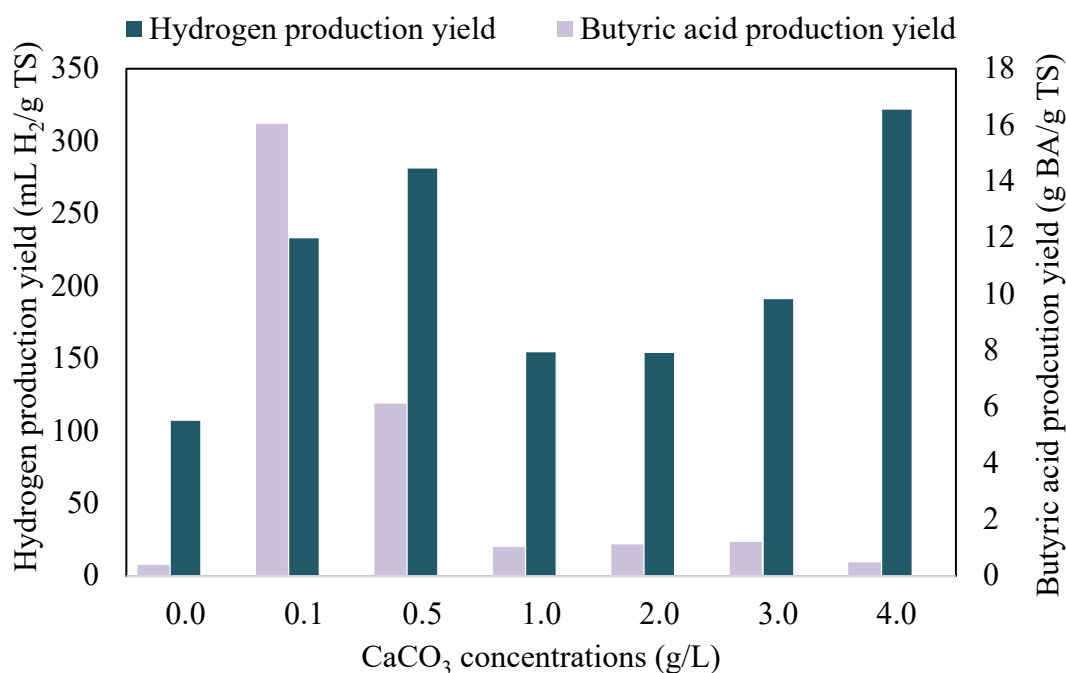


Figure 32 Variation of hydrogen and butyric acid production yields with different CaCO_3 concentrations with *Clostridium acetobutylicum*

Figure 32 gives the hydrogen production and butyric acid production yields at different calcium carbonate concentrations. The hydrogen and butyric acid production yields increased up to 0.1 g/ L calcium carbonate. Further hydrogen production yields varied and were lower than in this concentration. The butyric acid production yields at CaCO_3 concentrations higher than 0.1 g/ L were constant at 0.03 g BA/g TS. Probably solvent production was enhanced with increasing calcium carbonate concentrations. Figure 33 represents the HPR and VBPR at different CaCO_3 concentrations with *Clostridium acetobutylicum*. The maximum butyric acid production rate was found to be 0.226 g butyrate/g TS h at 0.1 g/L CaCO_3 concentration. The other VBPRs are below the 0.1 g butyrate/g TS h.

However, the highest HPR values were found at 1 g/L and 2 g/L CaCO_3 concentrations, i.e. 6.43 and 6.42 mL H_2 /g TS h, respectively.

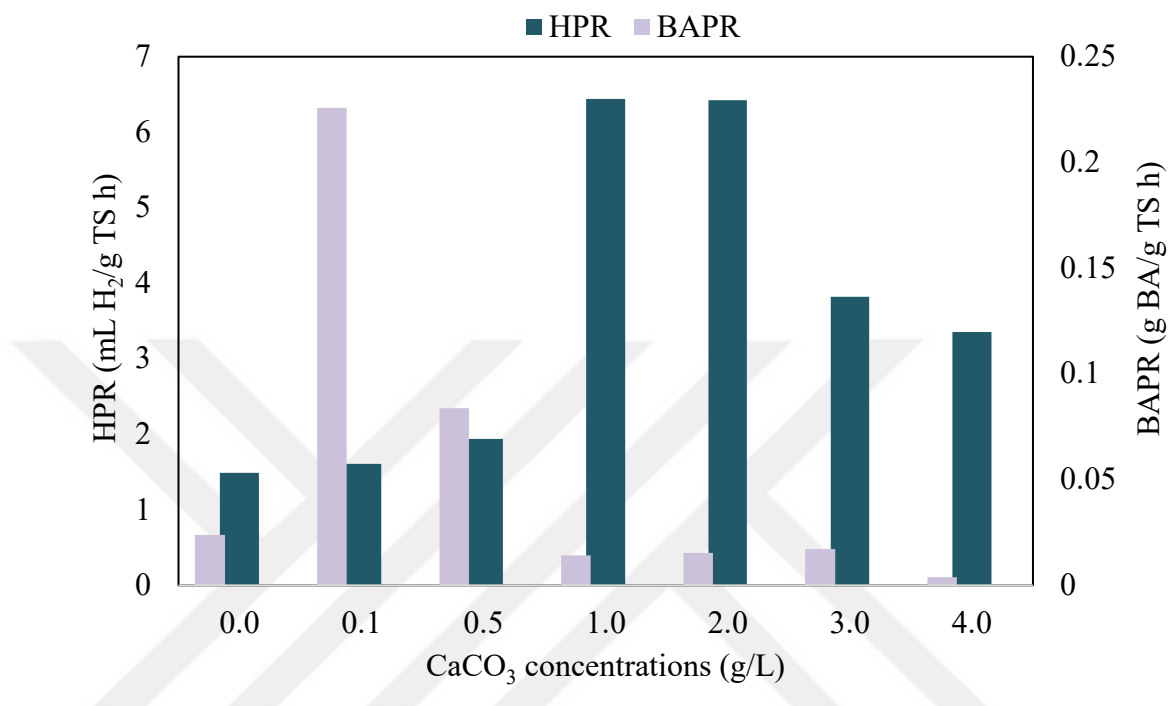


Figure 33 Variation of Hydrogen Production Rate (HPR) and Butyric Acid Production Rate (BAPR) at time with different CaCO_3 concentrations with *Clostridium acetobutylicum*

The effects of non-buffering CaCl_2 on CWP fermentation by *C. acetobutylicum* were investigated as control experiments. The main motivation for using CaCl_2 lied on that the effects of CaCO_3 on hydrogen fermentation may be due to its buffering capacity or Ca^{2+} mediated effects. In the presented study, CO_3^- at the CaCO_3 concentration maximizing butyric acid and hydrogen gas was not the sole effect of enhancement. Ca^{2+} plays an important role in bacterial cell division, structural maintenance, chemotactic activity, spore creation, and DNA reproduction (Han et al., 2013; Holland et al., 1999). For this reason, the effects of Ca^{+2} were examined to prove the mediated effects of calcium.

As shown in Figure 34, the addition of CaCl_2 obviously proved that Ca^{2+} plays an important role in hydrogen production. When 5 mM (0.56 g/L) CaCl_2 was added to the fermentation medium, the hydrogen production was close to the control and with 10 mM (1.11 g/L) CaCl_2 , a significant difference was observed in the hydrogen production when compared with the control vessel. Further increases in CaCl_2 concentrations were not tested, as the literature does not suggest a further increase in metabolites and links this to potential toxic effects of Ca^{2+} on the organism (Tian et al., 2015).

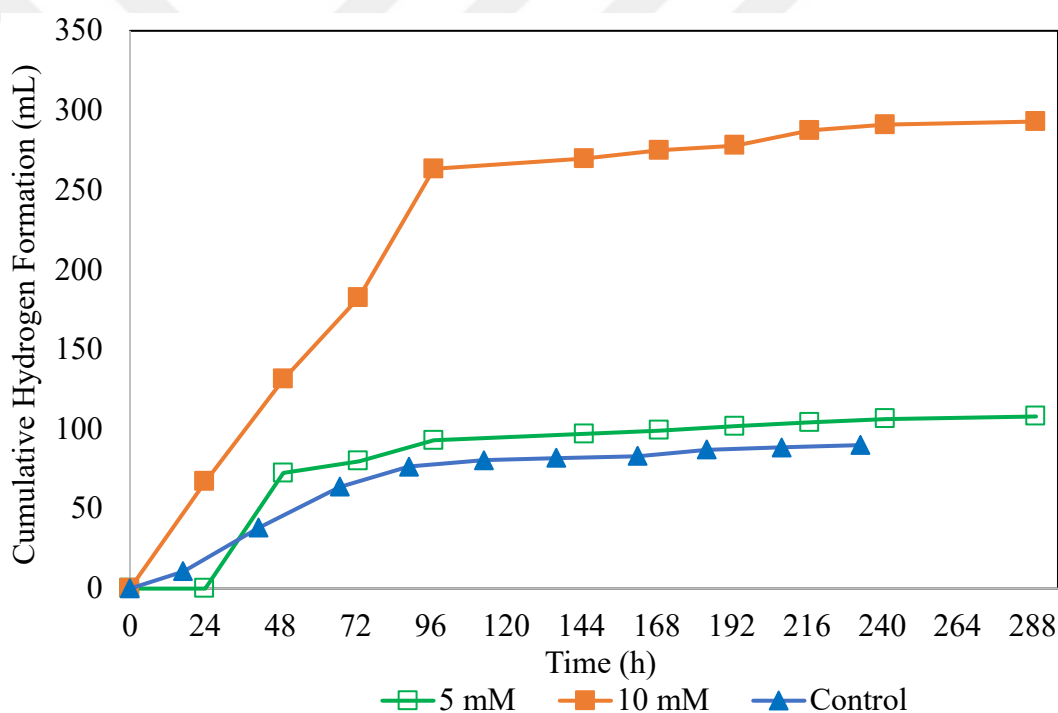


Figure 34 Variation of Cumulative Hydrogen Formation (CHF) with time at different CaCl_2 concentrations *Clostridium acetobutylicum*

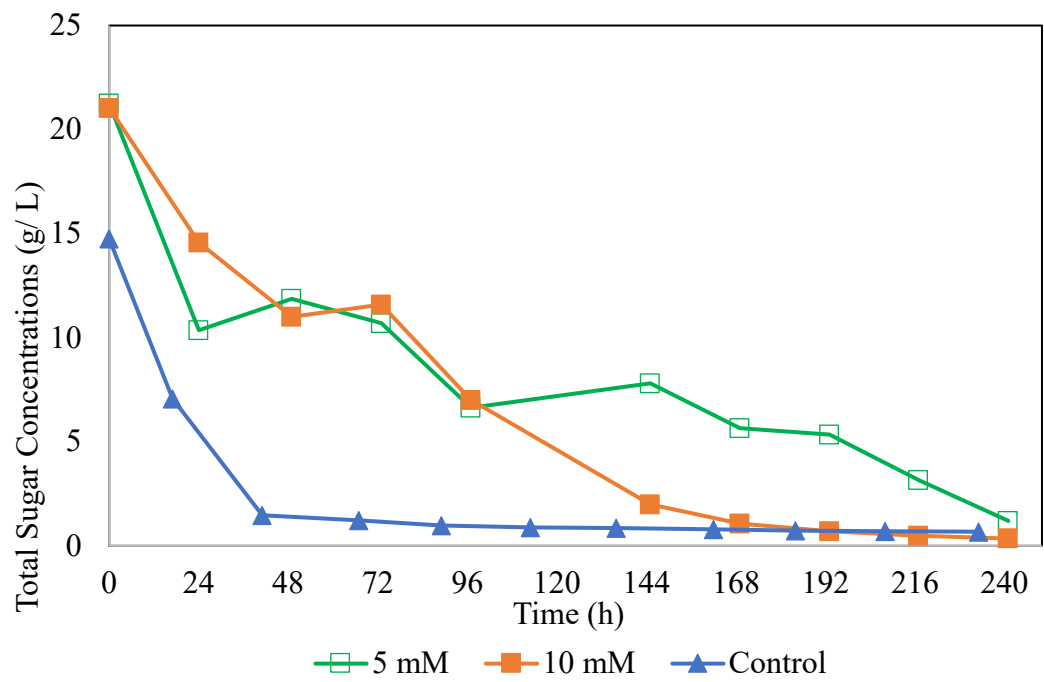


Figure 35 Variation of total sugar with time at different CaCl_2 concentrations with *Clostridium acetobutylicum*

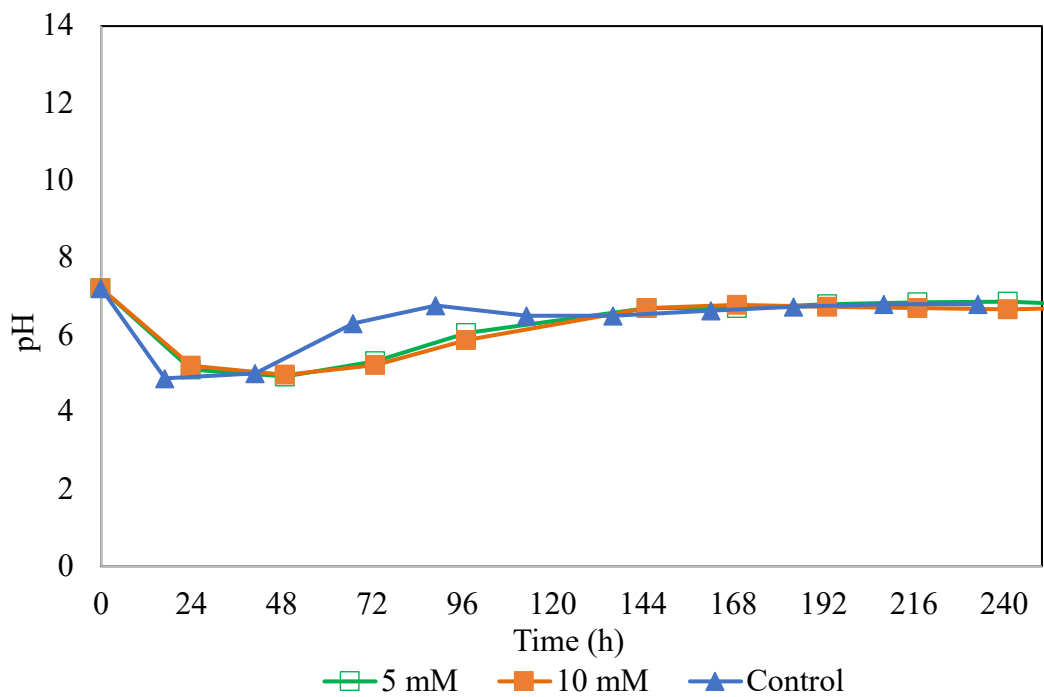


Figure 36 Variation of pH with time at different CaCl_2 concentrations *Clostridium acetobutylicum*

Figure 35 depicts the total sugar concentration at different CaCl_2 concentrations with time. Interestingly while 90% of the total sugar was consumed immediately in the control experiment, the others had 52% remaining total sugars in 48 hours. It took 240 and 192 hours to consume all the total sugar at 5 and 10 mM CaCl_2 concentrations, respectively. Also, it should be noted that the pH in the tested vessels decreased to 5 for the first three days in vessels with CaCl_2 and then increased slightly to 6 the next day and reached to 6.7 throughout the remaining fermentation period (Figure 36). In the control experiment for the first two days the pH was around 5 and the next day it increased to 6.3 and reached to an approximate steady pH (6.7) after 90 hours. The different behavior of the pH than the control refers to the difference of the metabolic activity of the organism. The presence CaCl_2 enhanced the activity of different enzyme groups that caused a lack time in consuming sugars. However, the slow consumption helped to enhance cumulative hydrogen formation. In the literature it is been said that CaCl_2 at high concentrations may have a bacteriostatic and bactericidal action on microbes, and it is suggested to have less concentration than 5 mM CaCl_2 due to its probable toxic effects on *C. acetobutylicum* (Tian et al., 2015).

Also, previous studies showed that Ca^{2+} promotes acetone-butanol-ethanol fermentation, which is linked with stabilizing effects of Ca^{2+} on membrane proteins such as glucose permeases. It increases the activity of coenzyme A transferase, acetate kinase and acetoacetate decarboxylase (Han et al., 2013; Holland et al., 1999; Kanouni et al., 1998.; Tian et al., 2015; S. Y. Wu et al., 2012). As like the literature hydrogen production was enhanced 69% when compared with the control vessel in 10 mM CaCl_2 . There was no toxic effect in this fermentation vessel, as the literature mentioned, while total sugar was consumed, and end products were produced. As a result, using trace amounts of CaCO_3 played an important role on enhancing butyric acid production along with hydrogen formation by *C. acetobutylicum*.

4.2.2 Selection of Organism for Enhancing Hydrogen Production via Butyric Acid Pathway

This section of the thesis aims to select the best organism enhancing butyrate and biohydrogen gas potential. The microorganisms used were anaerobic sludge, *Clostridium acetobutylicum*, *C. beijerinckii*, and *C. pasteurianum*. The experiment was carried out under mesophilic conditions (37°C) for 263 hours. Based on the previous experiments, the fermentation media was supplemented with NaCl (2.505 g/L,) Na₂S₂O₃ (100 mg/L,) NiCl₂ (0.1 mM), FeSO₄ (0.1 mM), ZnSO₄ (0.1mM), CuCl₂ (0.1mM), and CaCO₃ (0.7 g/L). CWP (20 g/L) was used as the carbon source.

Figure 37 represents the cumulative hydrogen formation (CHF) with time via various microorganisms. The highest CHF was observed with *C. pasteurianum* at 334 mL. In 47 hours, *C. pasteurianum* organism reached to 274 mL. The following CHFs were found as 170 and 160 mL with anaerobic sludge and *C. beijerinckii*, respectively. The lowest amount of CHF was observed at 74 mL with *Clostridium acetobutylicum*. Figure 38 shows the sugar consumption with different microorganisms over time. The fastest rate of sugar consumption (64 % of the total sugar) was observed in the anaerobic sludge within 19 hours.

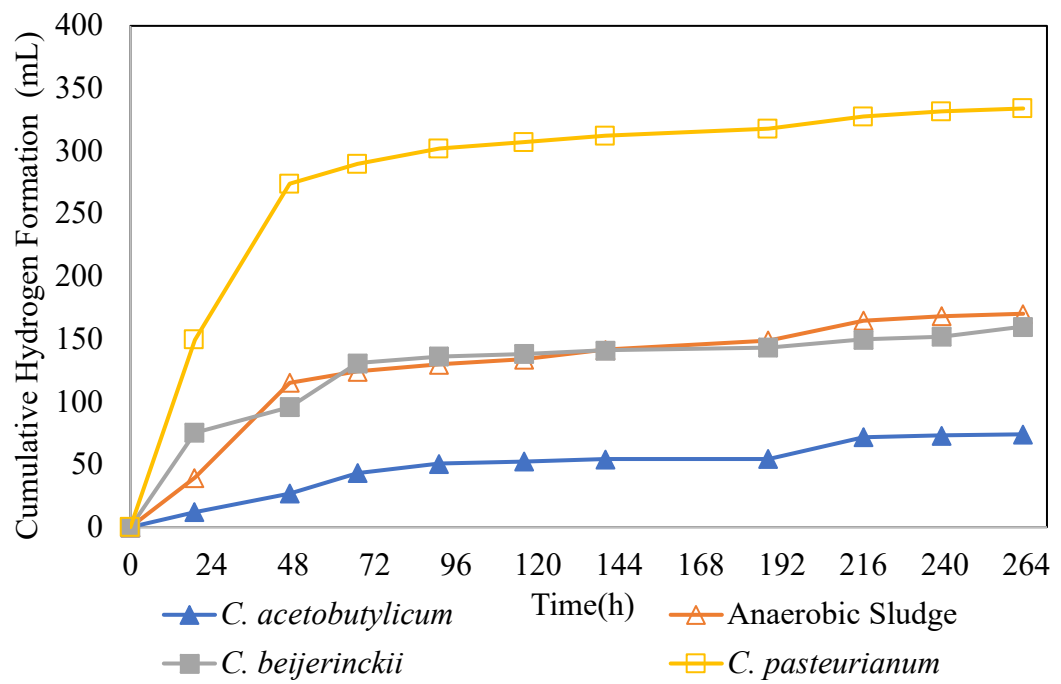


Figure 37 Cumulative Hydrogen Formation (mL) with time at different microorganisms

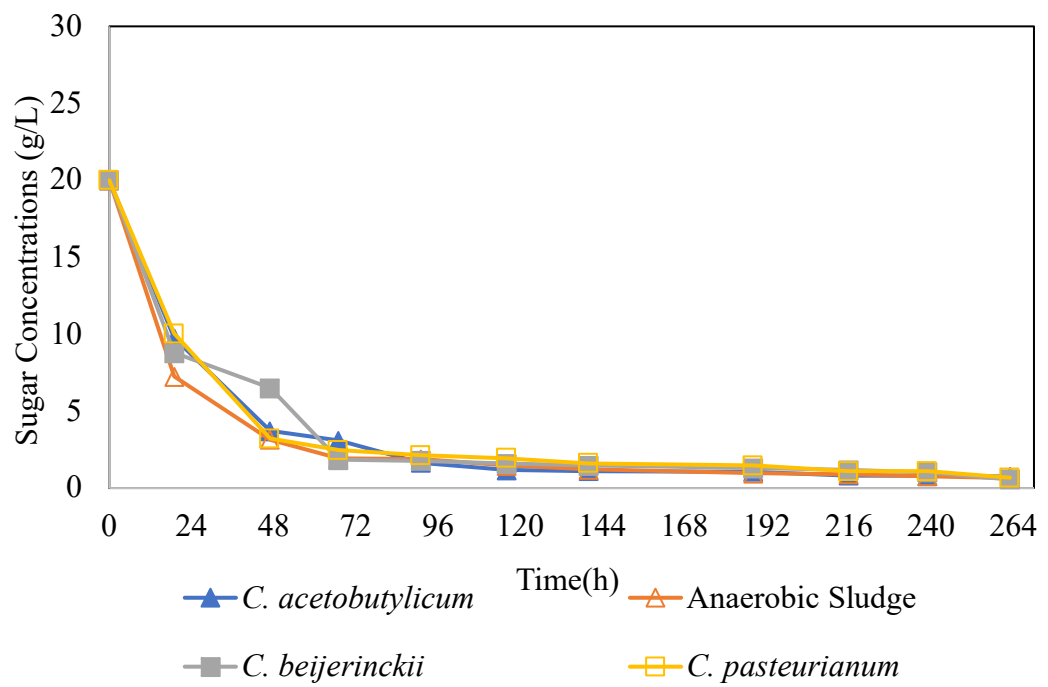


Figure 38 Variation of the total sugar concentrations with time at different microorganisms

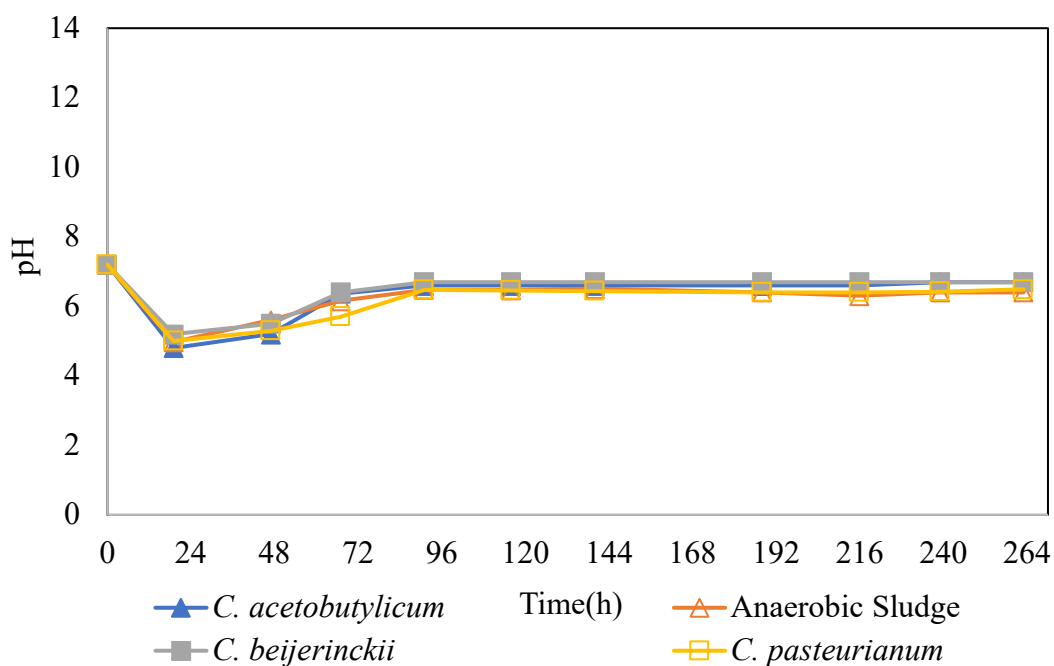


Figure 39 Variation of pH at time with different microorganisms

Figure 39 indicates the variation of pH with time at different organisms. The pH was adjusted to 7.2 at the beginning of the fermentation period and dropped to around 5 within 19 hours. The decline of pH indicates to VFA production (Calli et al., 2008; Tsai et al., 2014; H. Wu et al., 2017; X. Zhang et al., 2021). After 91 hours, pH was nearly stable around 6.5. Figure 40 shows the variation of butyric acids (BA) at the end of the fermentation time via different organisms. *C. pasteurianum* produced the maximum amount of BA at 6.8 g/L, whereas the other microorganisms produced BA at concentrations below 4 g/L. Based on these results, *C. pasteurianum* did not produce propionic, lactic acid, and acetic acid. The literature reveals that *C. pasteurianum* is a good butyric acid and butanol producer, while butanol is produced via a pathway called hot pathway where butanol can be produced from butyric acid in the solventogenic phase (Chen et al, 2016, Ahn et al., 2011a, 2011b; Krasňan et al., 2018; Raganati et al., 2015; Schwarz et al., 2017).

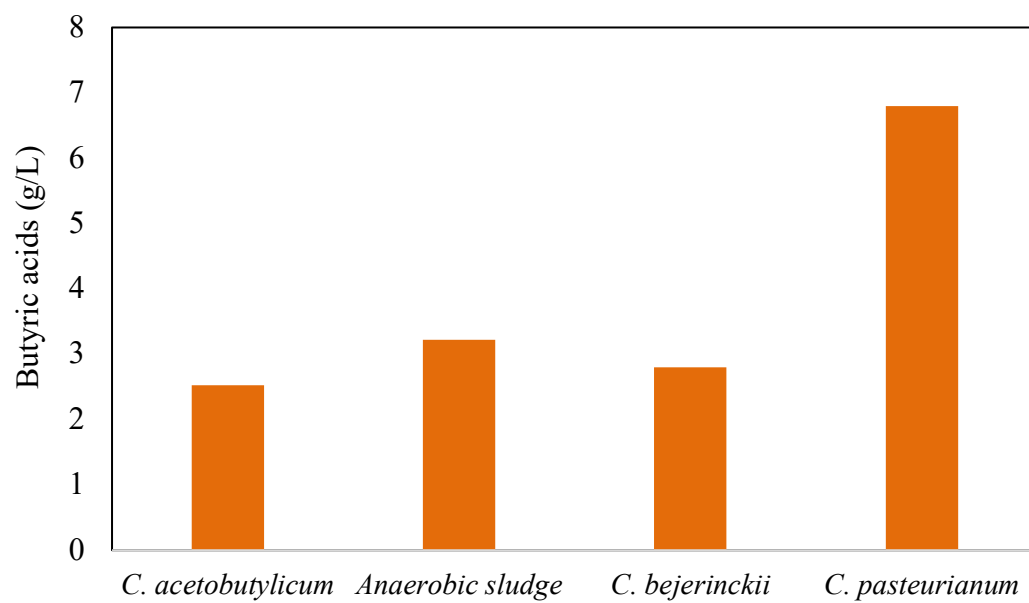


Figure 40 Variation of butyric acids at the end of the fermentation time at different microorganisms

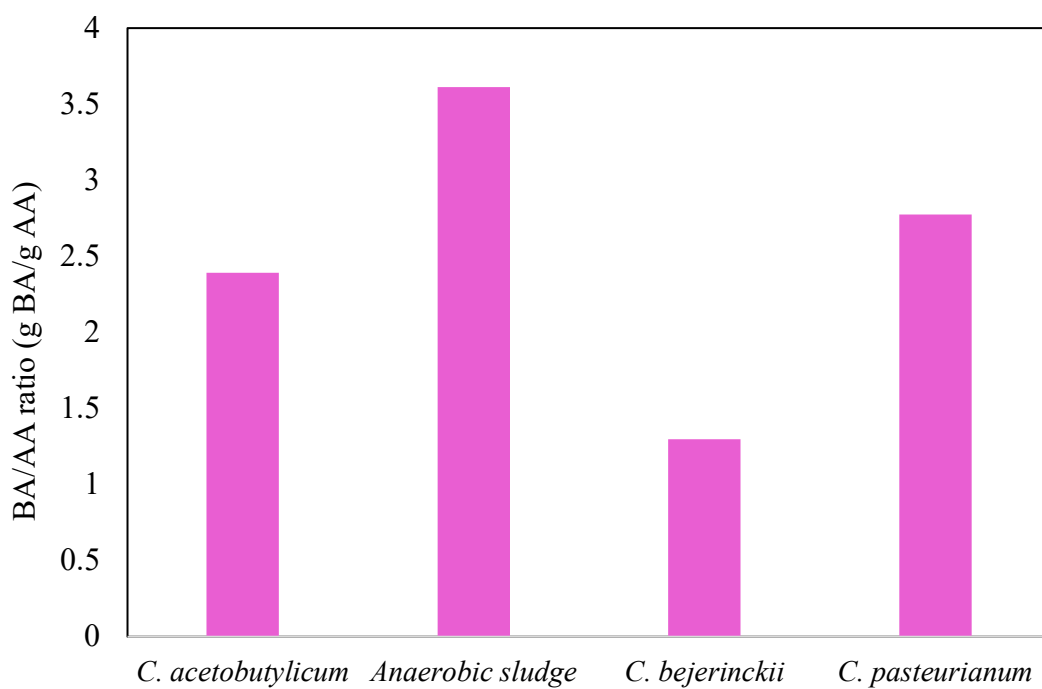


Figure 41 Variation of BA/AA ratio with different microorganisms

Figure 41 represents the variation of BA/AA via various organisms. The highest BA/AA ratio was 3.6 g BA/g AA obtained at anaerobic sludge. The following BA/AA ratio was 2.8 g BA/g AA at *C. pasteurianum*. The lowest ratio of 1.3 g BA/g AA was observed in the *C. beijerinckii*.

Figures 42 and 43 show the variation in hydrogen and butyric acid production yields, as well as the HPR and BAPR with different microorganisms. The highest yields of hydrogen and butyric acid were found to be 467 mL H₂/g TS and 16.44 g BA/g TS, respectively, with *C. pasteurianum*. The second-highest BA yield was obtained at 15 g BA/g TS with *C. beijerinckii*. The H₂ yield was observed to be 345 mL H₂/g TS with *C. acetobutylicum*, while the maximum H₂ production rate was 17 mL H₂/g TS h, also with *C. acetobutylicum*. The HPR for *C. pasteurianum* was found to be 9 mL H₂/g TS h, and the maximum BAPR of 0.33 g BA/g TS h was obtained with *C. pasteurianum* as well. Based on the results of the experiments, *C. pasteurianum* was selected due to its production of butyric acid, cumulative hydrogen formation, butyric and hydrogen production yields, and butyric acid production rate.

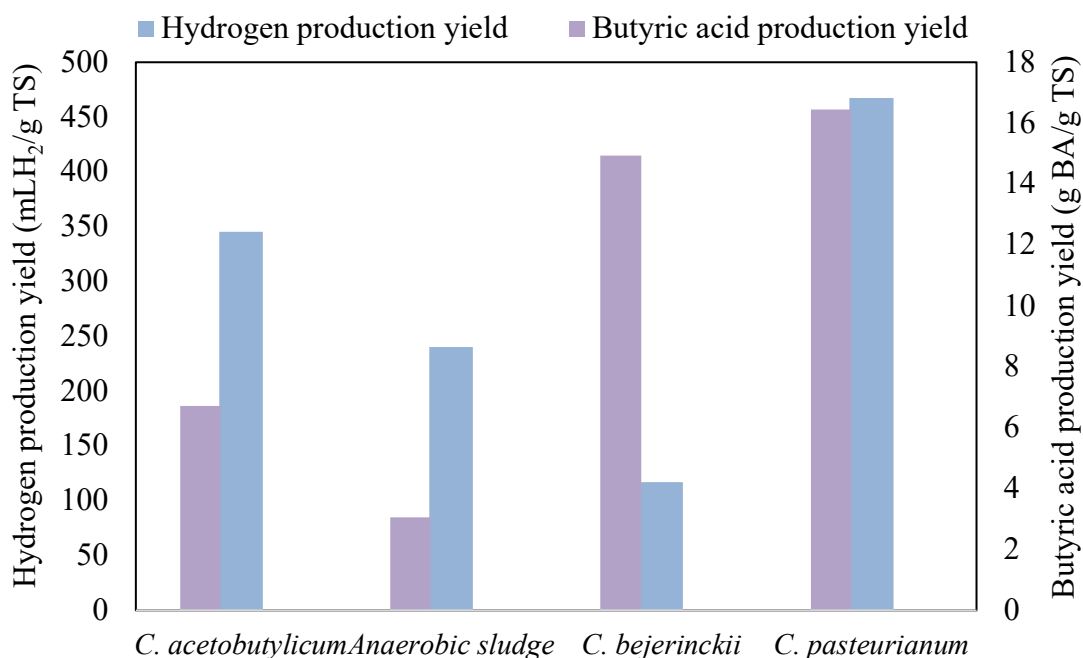


Figure 42 Variation of hydrogen and butyric acid production yields with different microorganisms

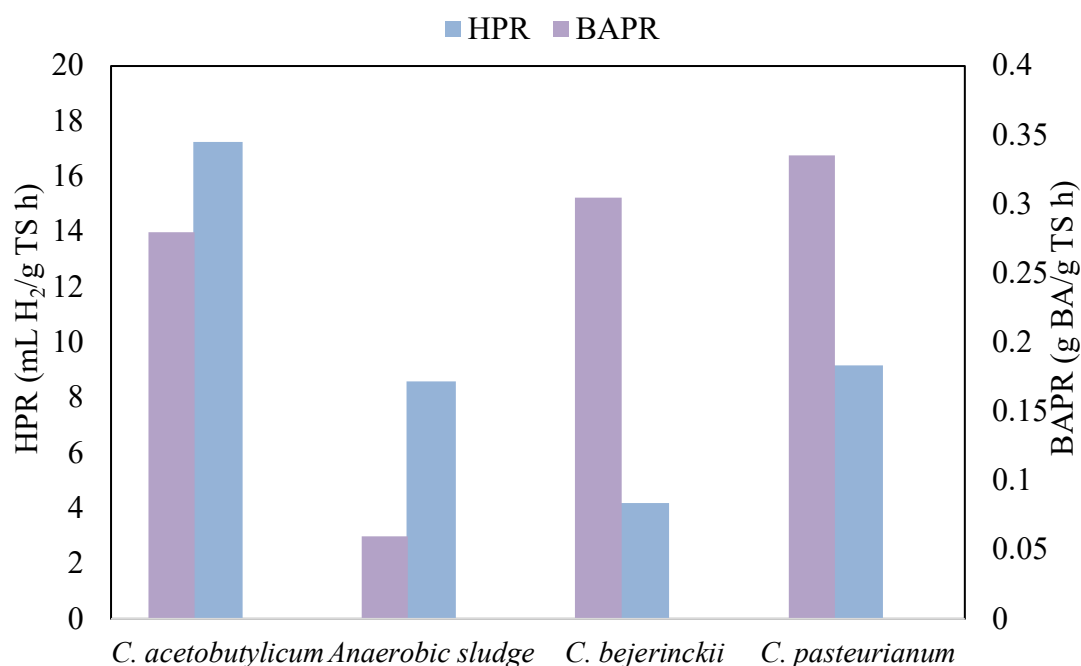


Figure 43 Variation of Hydrogen Production Rate (HPR) and Butyric Acid Production Rate (BAPR) at time with different microorganisms

4.2.3 Effects of Organism Concentration Enhancing Butyric Acid Production via Hydrogen Production

In this experiment different *C. pasteurianum* DSM 525 inoculum concentrations (0.5-3 g/L) with 45 g/L CWP concentration were tried to enhance butyric acid production along with hydrogen gas production at mesophilic dark fermentation conditions. Figure 44 shows the amount of Cumulative Hydrogen Formation (CHF) on different initial microorganism concentrations at 37°C. The CHF raised up with increasing initial microorganism concentration until 1.5 g/L and then decreased with increasing amounts. According to this figure, 1.5 g/L (11% v/v) initial microorganism concentration gave the highest CHF amount as 458 mL within 283 h. The second highest was with 2 g/L (CHF:397 mL) initial organism concentration. In the literature, higher concentrations of CHF were obtained with mixed cultures, however not focusing on enhancing butyric acid production.

The literature gives similar results to our study depicting an inoculum concentration of 10% v/v enhancing hydrogen production (Kargi et al., 2012, Kriswantoro &, 2024).

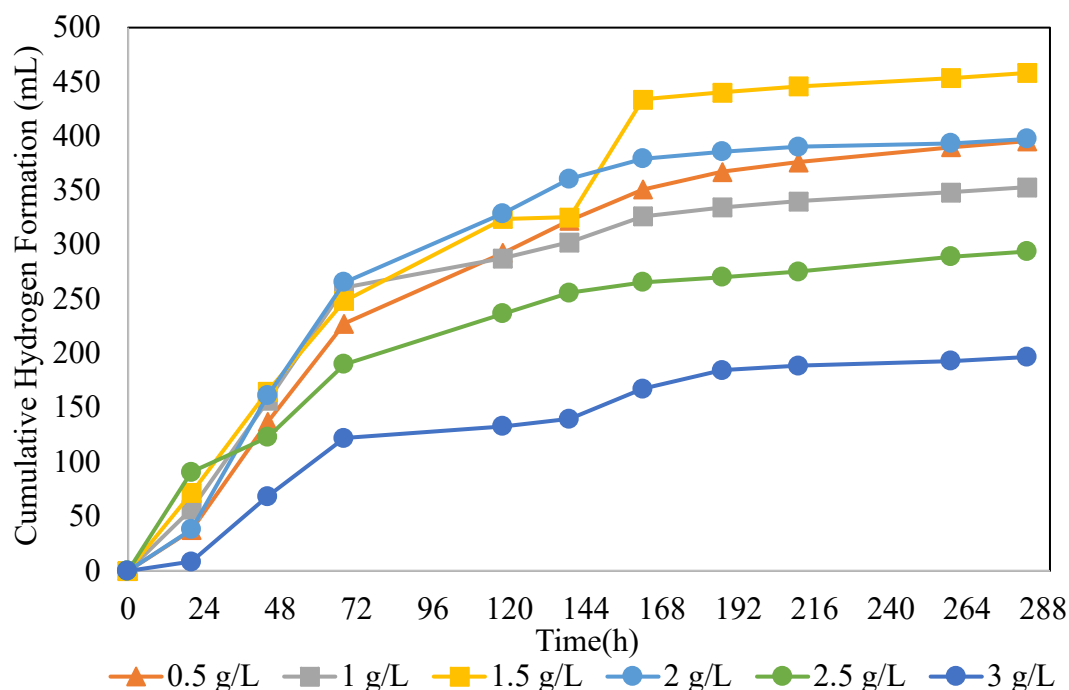


Figure 44 Cumulative Hydrogen Formation (mL) with time at different microorganism concentrations via *C. pasteurianum*

Figure 45 indicates the variations of sugar consumption with time. The initial substrate concentration was constant at 45 g/L CWP. Within 24 hours, the sugar started to be consumed by the organism with no lag phase. The sugar in all the experimental vessels were consumed completely in approximately 192 hours. Figure 46 plots the variations of pH with time. The initial pH in the experimental set up was 7.2 and dropped to around 4.5-5.5 within 24 hours. The lowest pH was observed at an initial microorganism concentration of 3 g/L, while the highest was observed at a concentration of 1 g/L. Between 48 and 144 hours, the pH remained around 5 to 5.5. After 144 hours, the pH did not decrease despite adjustments.

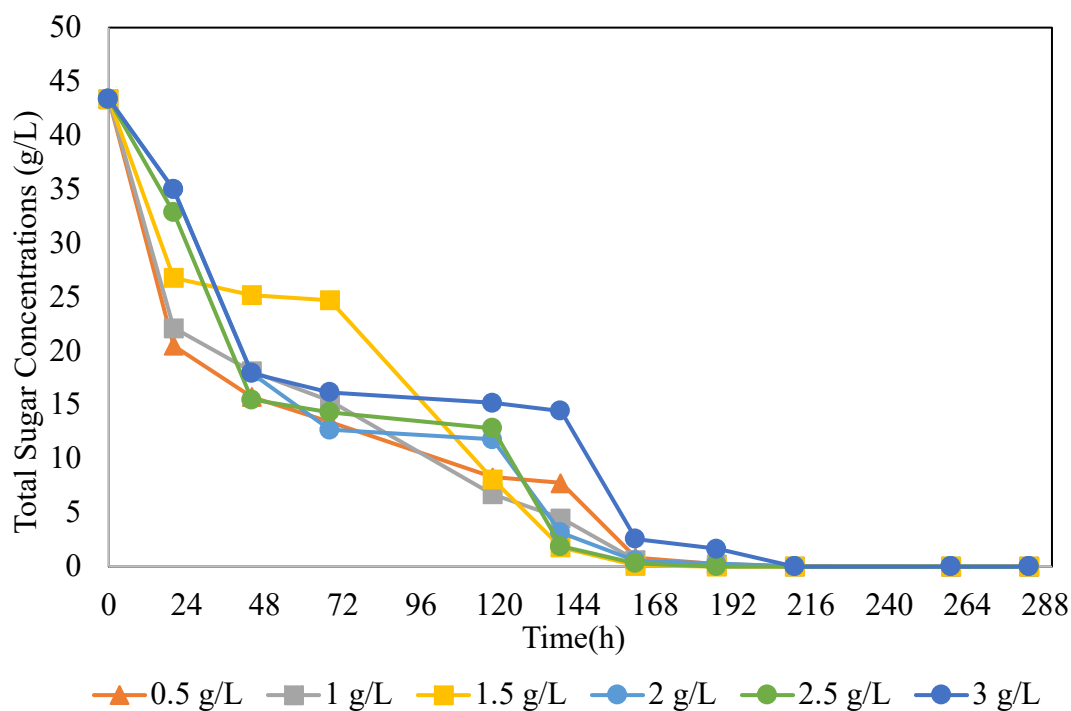


Figure 45 Variation of the total sugar concentrations with time at different microorganism concentrations via *C. pasteurianum*

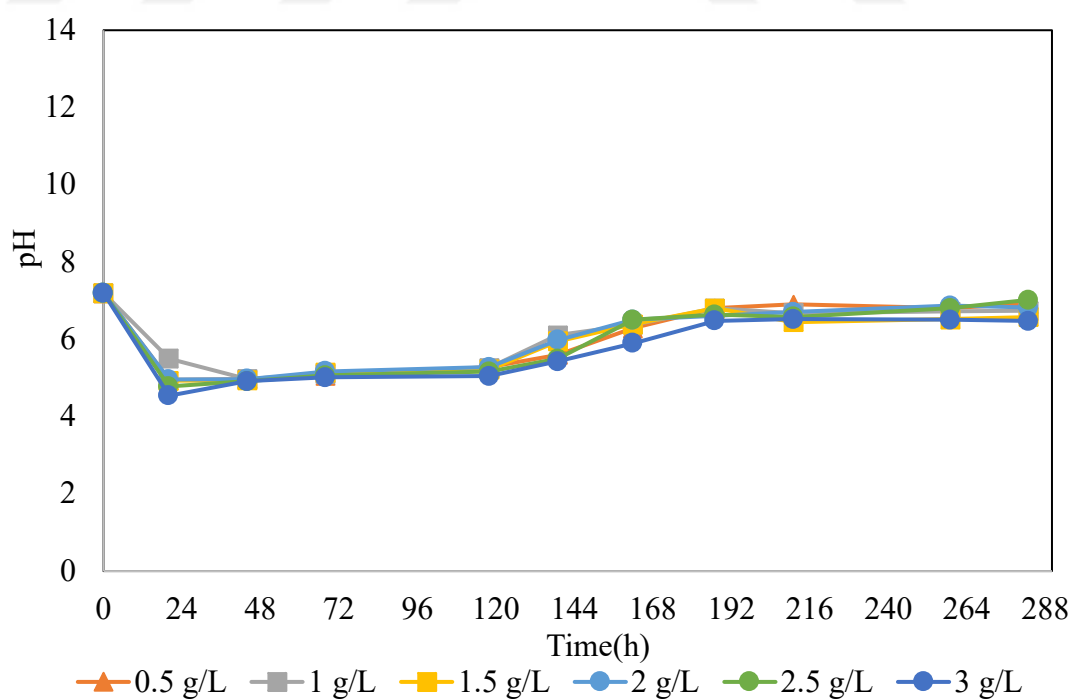


Figure 46 Variation of pH with time at different microorganism concentrations via *C. pasteurianum*

In Figure 47, the production of Butyric acid (BA) and Total Volatile Fatty Acid (TVFA) were depicted in different initial microorganism concentrations. The highest BA as 1.523 g/L and TVFA production as 5.947 g/L, was obtained at an initial microorganism concentration of 1.5 g/L. Figure 48 shows the Butyric Acid to Acetic Acid ratios (BA/AA). BA/AA ratios increased up to 1 g/L microorganism concentration and then decreased with increasing concentrations. Based on Figure 2b, the initial microorganism concentration of 1 g/L had the highest BA/AA ratio of 3.346 g BA/g AA, followed by a concentration of 1.5 g/L with a ratio of 3.071 g BA/ g AA. At concentrations above 1.5 g/L, TVFA concentrations decreased with increasing organism concentrations. Although, the TVFA production was expected to increase at these concentrations due to the decrease in pH, no TVFA increase was observed. This may be due to the transition of the pathway to the solventogenic phase, while the microorganism used (*C. pasteurianum* DSM 525) is known as a butanol producer (R. Gallardo et al., 2014; Gallazzi et al., 2015; Sarchami et al., 2016).

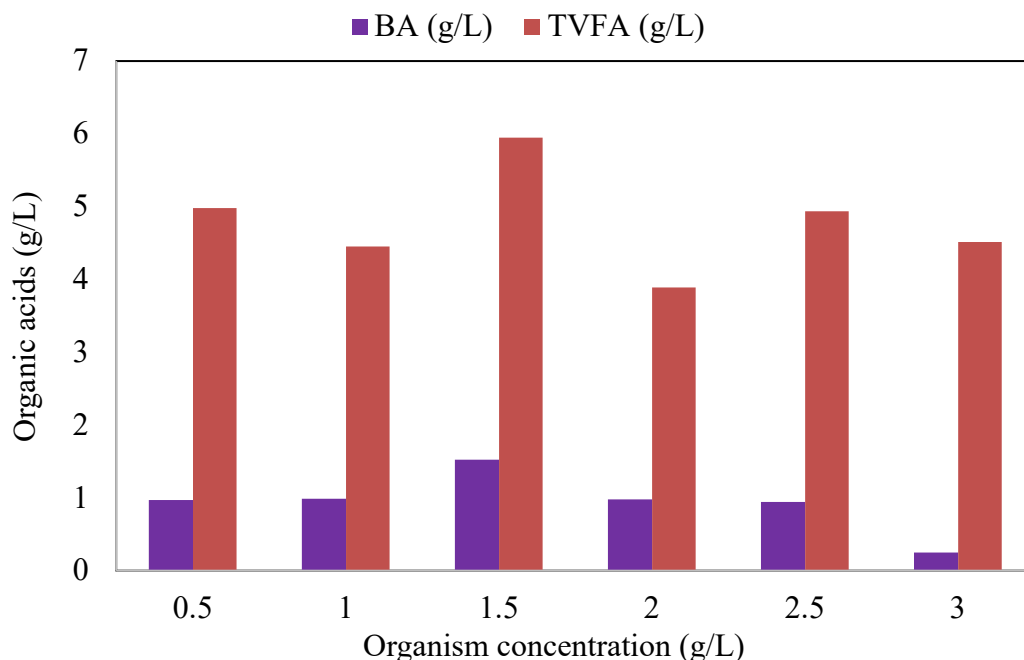


Figure 47 Variation of butyric acids and total volatile fatty acids at the end of the fermentation time at different microorganism concentrations via *C. pasteurianum*

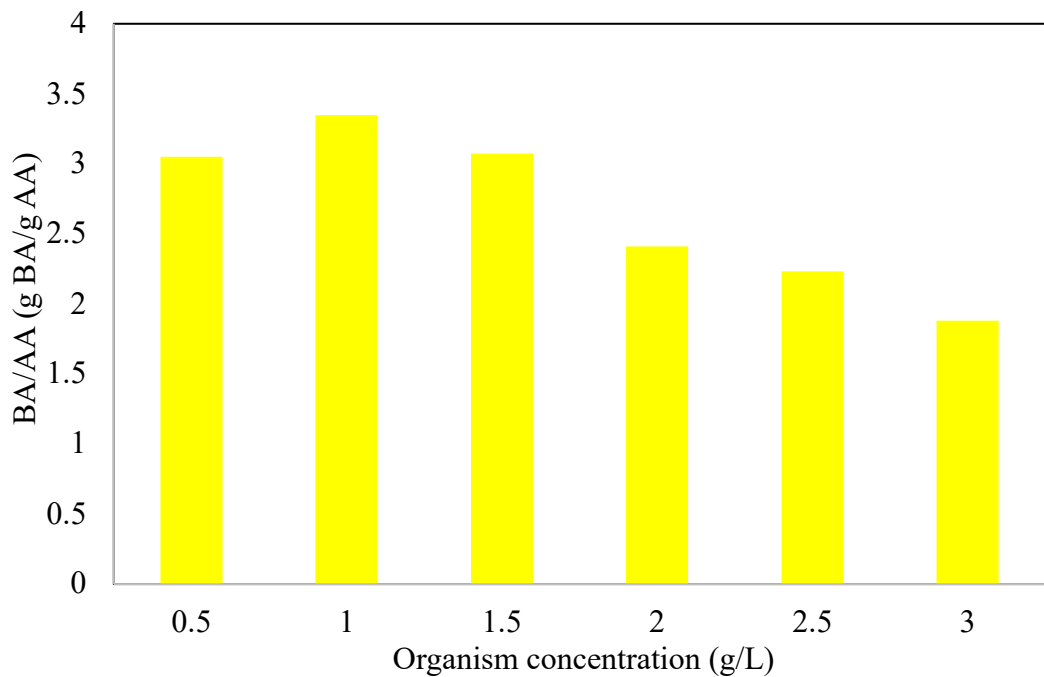


Figure 48 Variation of BA/AA ratio with different microorganism concentrations via *C. pasteurianum*.

Figure 49 indicates the hydrogen and butyric acid production yields with different initial organism concentrations. Based on the results, the maximum hydrogen production yield of 1278.63 mLH₂/g TS and butyric acid production yield of 0.37 g BA/g TS were achieved at an initial organism concentration of 1.5 g/L. The yield of butyric acid increased with an increase in the initial concentration of microorganisms up to 1.5 g/L. However, with further increases of initial organism concentrations the yields decreased, and the amount of the yields were close to each other. The results revealed that there is no need for high organism concentrations to enhance hydrogen and butyric acid production while the optimum biomass amount was 1.5 g/L. Figure 50 signifies the Hydrogen Production Rate (HPR) and Butyric Acid Production Rate (BAPR), with initial organism concentrations. The highest HPR and BAPR were obtained at the same initial organism concentration (1.5 g/L) and values were 111.88 mLH₂/g TS h and 0.0176 g BA/g TS h, respectively. Specific Butyric Acid (SBAPR) and Specific Hydrogen (SHPR) Production Rates were depicted in Figure 51. The maximum SHPR was found in 2 g/L while the maximum SBAPR was observed in 1 g/L organism concentration.

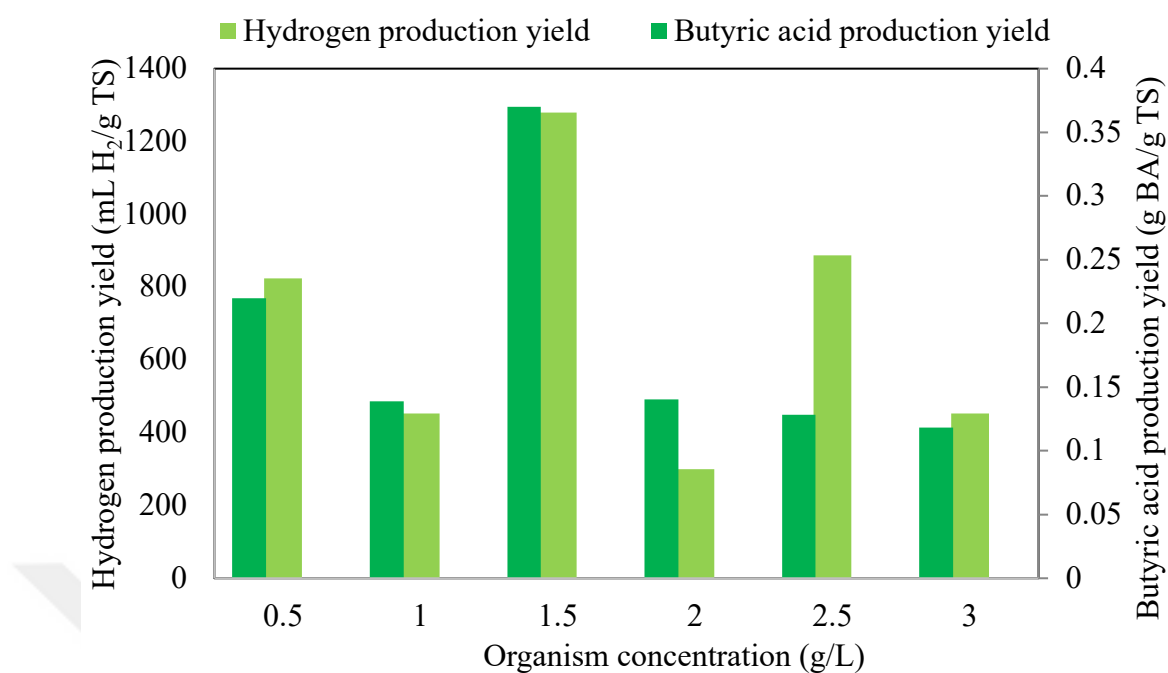


Figure 49 Variation of hydrogen and butyric acid production yields with different microorganism concentrations via *C. pasteurianum*

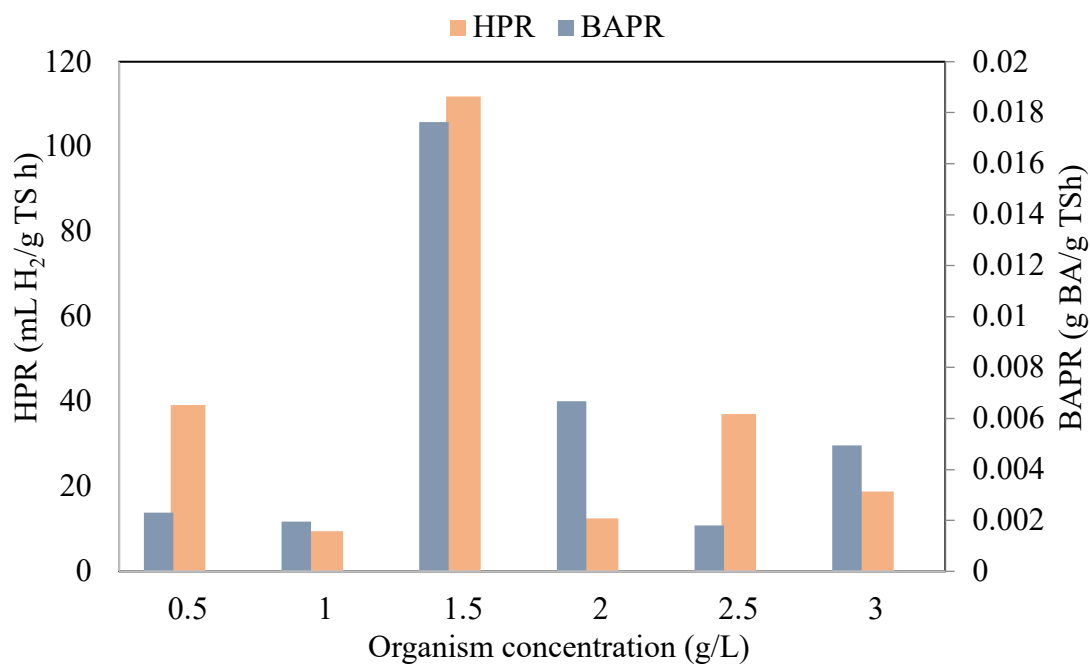


Figure 50 Variation of Hydrogen Production Rate (HPR) and Butyric Acid Production Rate (BAPR) at time with different microorganism concentrations via *C. pasteurianum*

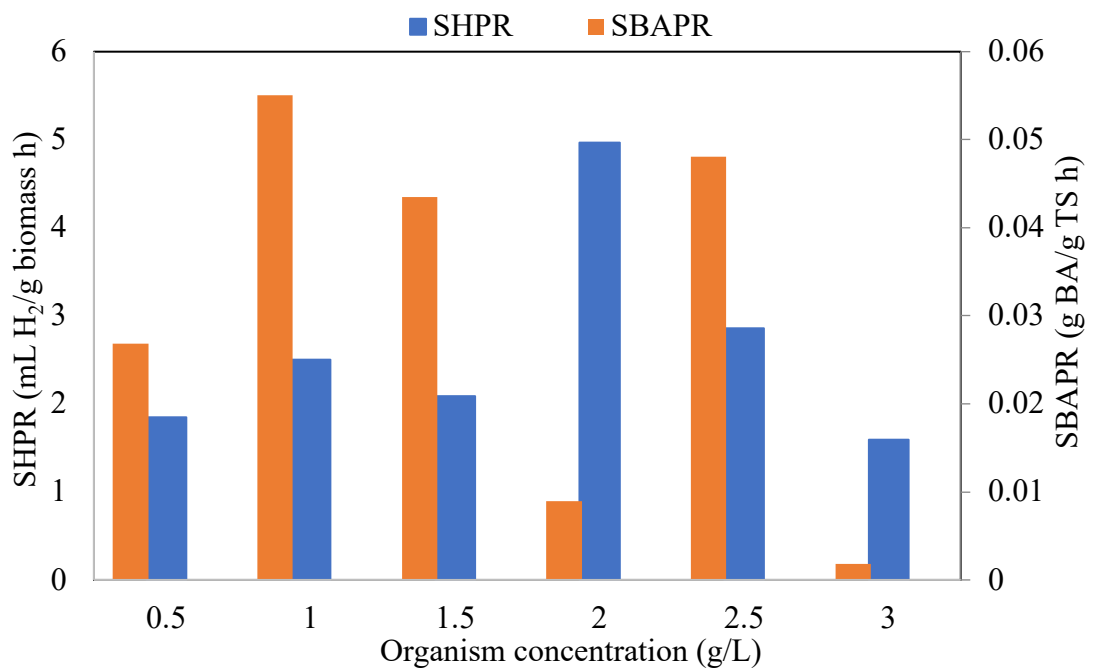


Figure 51 Variation of Specific Hydrogen Production Rate (SHPR) and Specific Butyric Acid Production Rate (SBAPR) at time with different microorganism concentrations via *C. pasteurianum*

4.2.4 Effects of Substrate Concentration Enhancing Hydrogen Production via Butyric Acid Production

A range of 40-200 g/L CWP concentrations were used to investigate the effects of substrate concentration on hydrogen and butyric acid production. The trials were performed as in previous experiments and the organism concentration improving hydrogen and butyric acid were used. Figures 51, 52, and 53 represent variation of CHF, sugar concentration, and pH with time, respectively. As shown in Figure 51, 80 g/L CWP gave the highest CHF which was 651.1 mL within 259 h (10 days). The second highest CHF which was close to 80 g/L CWP was obtained with 100 g/L CWP as 611.5 mL 259 h (10 days). The lowest CHF volume was 136.7 mL (200 g/L CWP). Figure 52 represents the consumption of different substrate concentrations. It can be observed that at CWP concentrations of 40 g/L, 80 g/L, and 100 g/L CWP the total sugars were nearly consumed.

In contrast, at CWP concentrations of 150 g/L and 200 g/L CWP, 25% and 44% of total sugars remained in the media at the end of the fermentation period. Figure 53 the variation of pH with time depicts that, in all tested vessels the pH dropped below 5.5 within 24 hours. The pH was manually adjusted with NaOH periodically to 6.5. After 24 hours the pH varied between 5 to 6.5 in different trials.

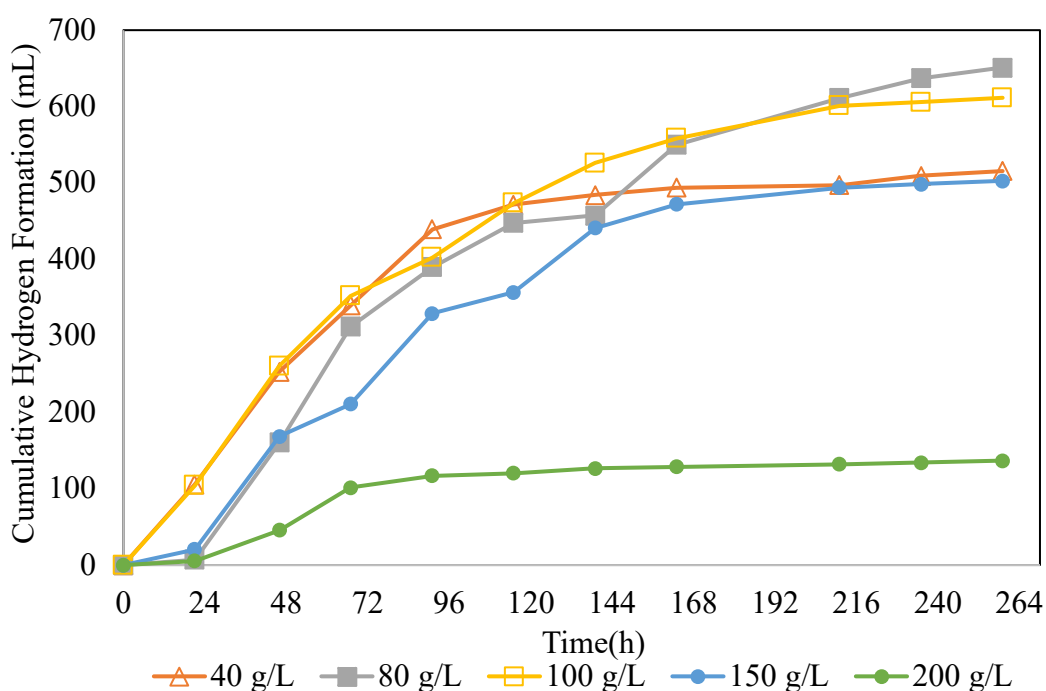


Figure 52 Cumulative Hydrogen Formation (mL) with time at different CWP concentrations via *C. pasterianum*

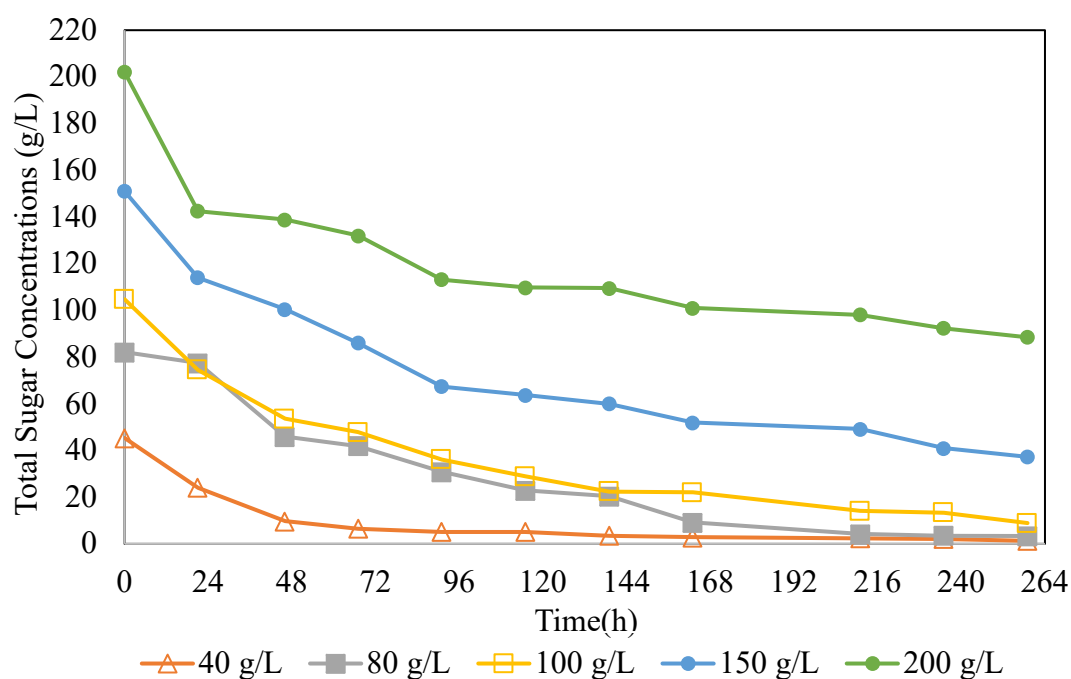


Figure 53 Variation of the total sugar concentrations with time at different CWP concentrations via *C. pasterianum*

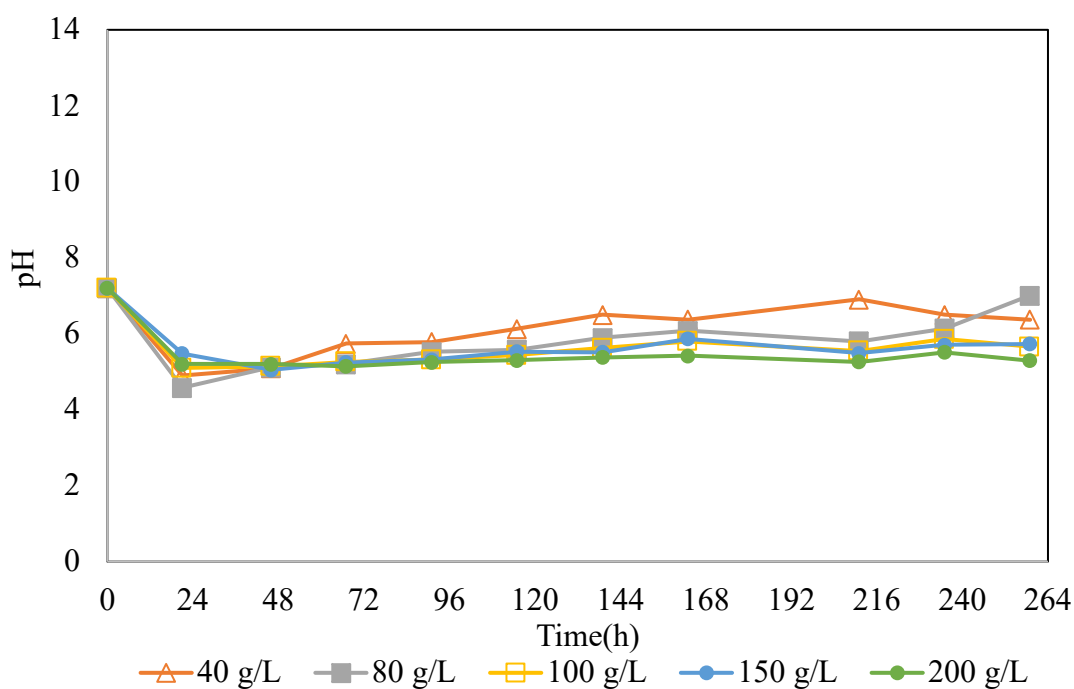


Figure 54 Variation of pH with time at different CWP concentrations via *C. pasterianum*

Figures 54 and 55 represent butyric acid (BA) and total volatile fatty acid (TVFA), and the ratio of BA/AA with different substrate concentrations. In Figure 54, 1.17 g/L and 1.10 g/L BA was produced at 150 g/L and 80 g/L CWP, respectively. The BA concentrations were close to each other in all experimental media. However, the TVFA concentrations increased with increasing substrate concentrations up to 150 g/L CWP. The highest total volatile fatty acid production was 5.02 g/L from 150 g/L CWP, followed by 200 g/L CWP as 4.17 g/L. In Figure 55, BA/AA ratio elevated from 2.11 (40 g/L CWP) to 3.23 g BA/g AA at 80 g/L CWP. There was no significant difference in BA/AA ratios in further increases of substrate concentrations. Different studies in the literature demonstrated higher substrate concentrations enhancing hydrogen production potentials (Q. Wang et al., 2023). However, there was no data revealing BA/AA ratio improvements except our previous study giving a BA/AA ratio of 2.6 g BA/g AA with the hydrogen production yield of 3.7 mmol H₂/mmol lactose (Karakaya & Özmihçi, 2024).

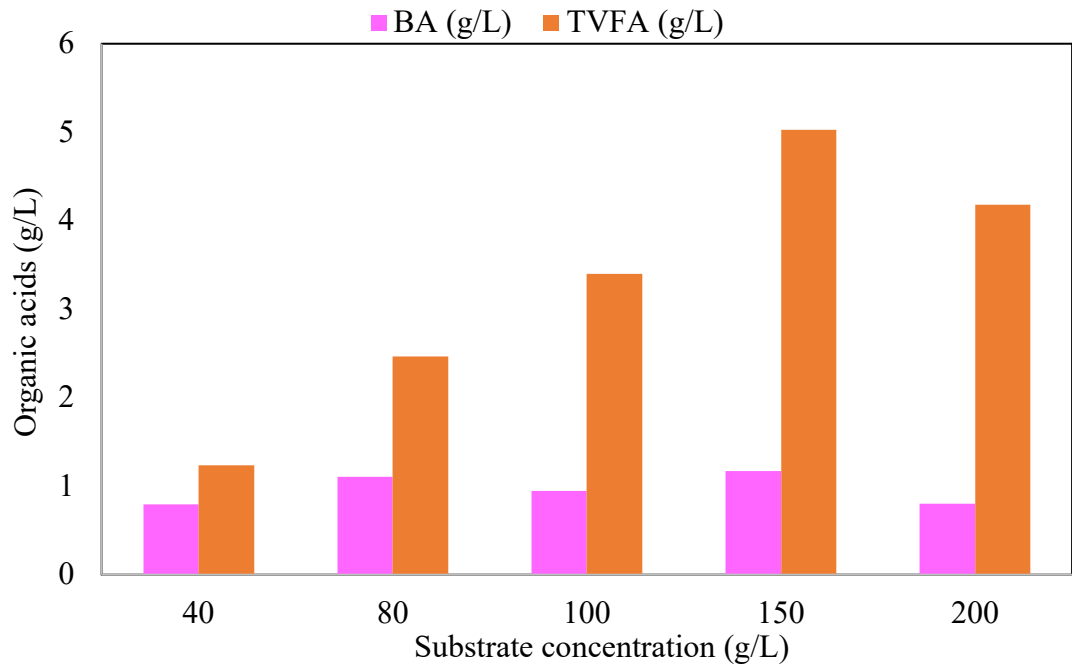


Figure 55 Variation of butyric acids and total volatile fatty acids at the end of the fermentation time at different CWP concentrations via *C. pasteurianum*

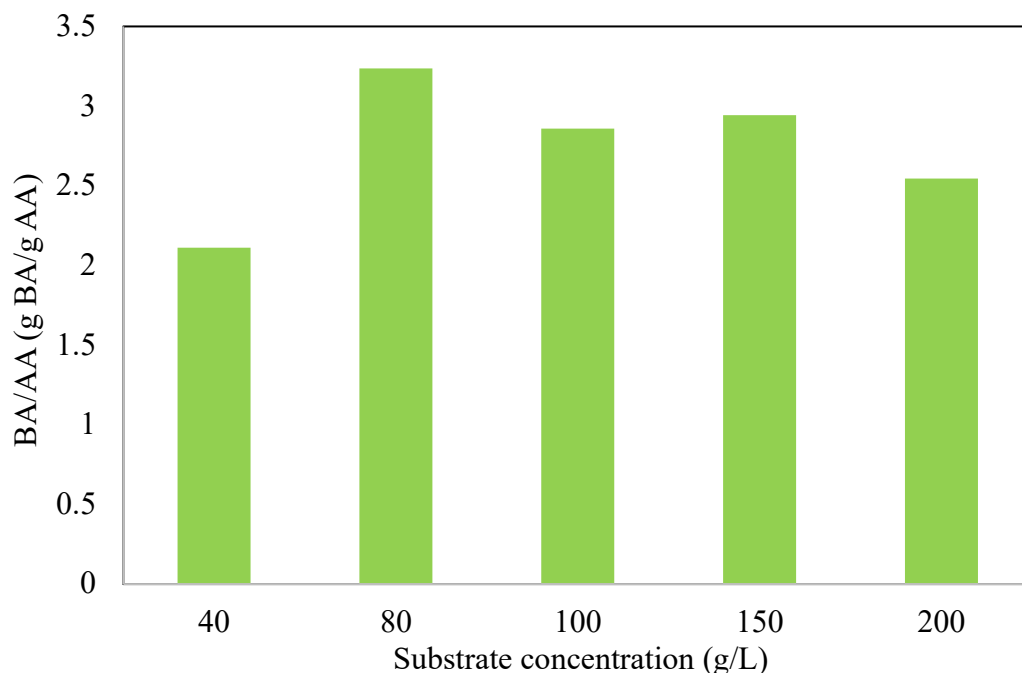


Figure 56 Variation of BA/AA ratio with different CWP concentrations via *C. pasteurianum*

Figures 56 and 57 indicate hydrogen and butyric acid production yields, and HPR-BAPR at different CWP concentrations. The hydrogen production yields and rates declined with rising substrate concentrations. The BA production yield and rates increased up to 80 g/L and 100 g/L CWP amounts and then decreased with additional substrate concentrations, respectively. The highest hydrogen production yield and HPR were achieved with 40 g/L CWP (1163.3 mL H₂/g TS and 48.5 mL H₂/g TS h, respectively). The maximum butyric acid production yield was 0.365 g BA /g TS h at 80 g/L CWP and the BAPR was 0.005 g BA/g TS h, at 100 g/L CWP.

In the Figure 58 shows the variation of Specific Hydrogen Production Rate (SHPR) and Specific Butyric Acid Production Rate (SBAPR) at time with different CWP concentrations. The highest SHPR and SBAPR were observed at substrate concentration of 40 g/L as 175 mL H₂/g biomass h and 0.63 g BA/g biomass h, respectively: due to the absence of substrate inhibition on the organism in the fermentation media.

The present experiment found similar results to Q. Wang et al. (2023) regarding TVFA production, which was better at 150-200 g/L. However, for butyric acid production, butyric acid production yield, BA/AA ratio, and cumulative hydrogen formation, the optimal substrate concentration was found to be 80 g/L. Therefore, it can be concluded that 80 g/L CWP is the optimum substrate concentration. Based on these data, it can be said that substrate inhibition occurred at 200 g/L CWP and therefore biohydrogen production was very low compared to the others. J. Wang & Wan, (2008), similar to our finding, concluded that hydrogen production and hydrogen production capability improved with decreasing substrate amount and reaching optimal levels. The summary of the designed experiment is given in the Table 9.

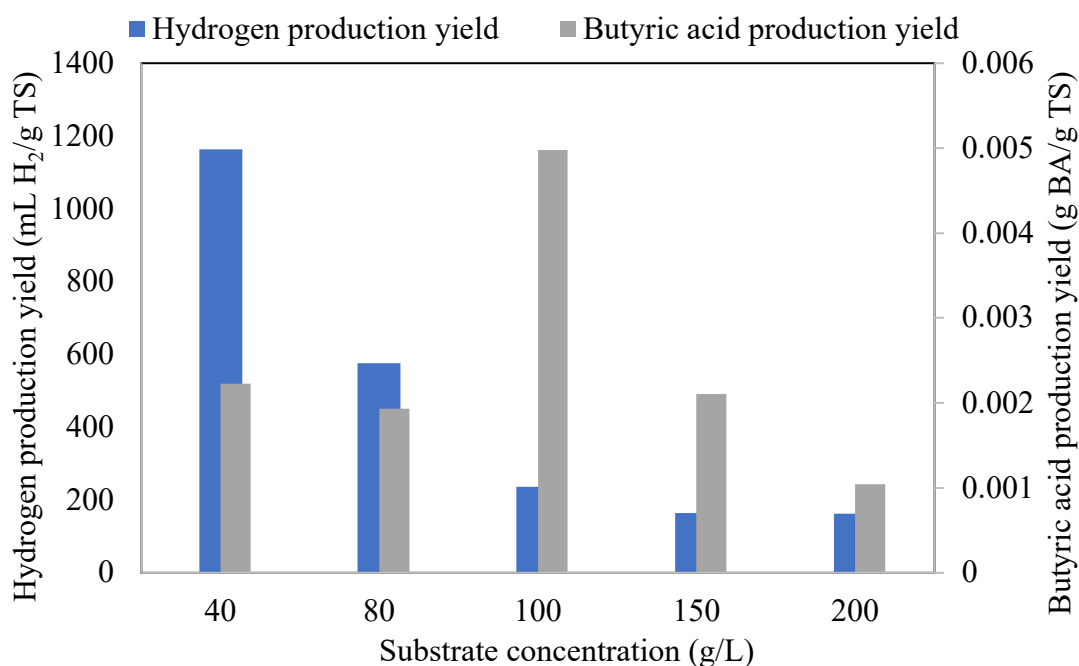


Figure 57 Variation of hydrogen and butyric acid production yields with different CWP concentrations via *C. pasterianum*

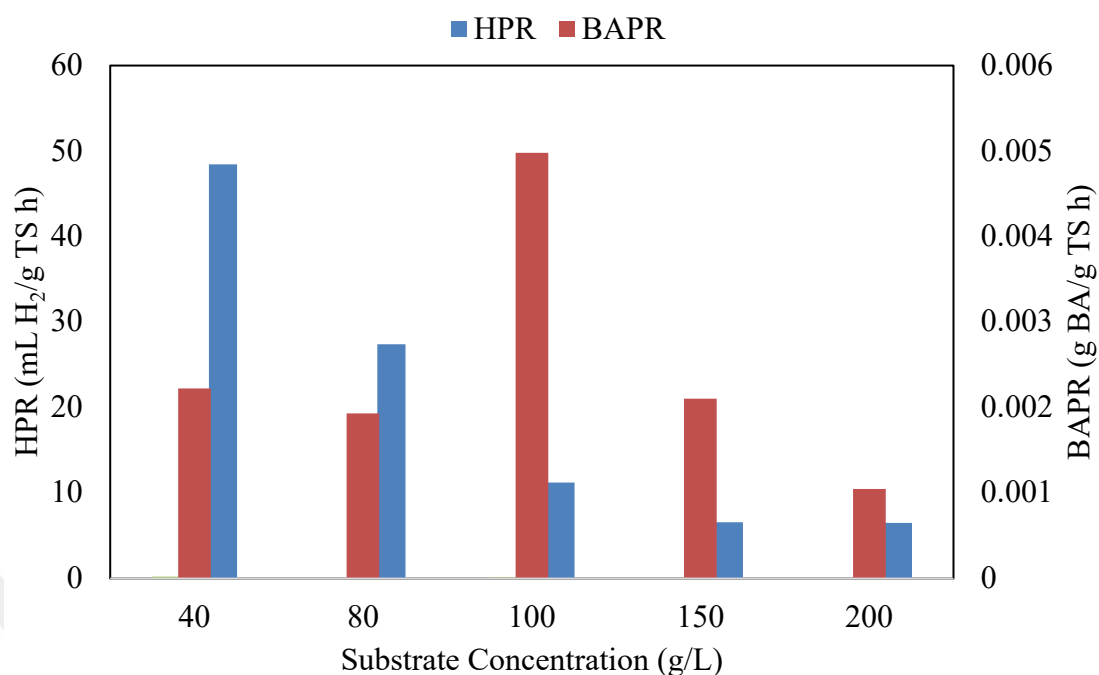


Figure 58 Variation of Hydrogen Production Rate (HPR) and Butyric Acid Production Rate (BAPR) at time with different CWP concentrations via *C. pasteurianum*

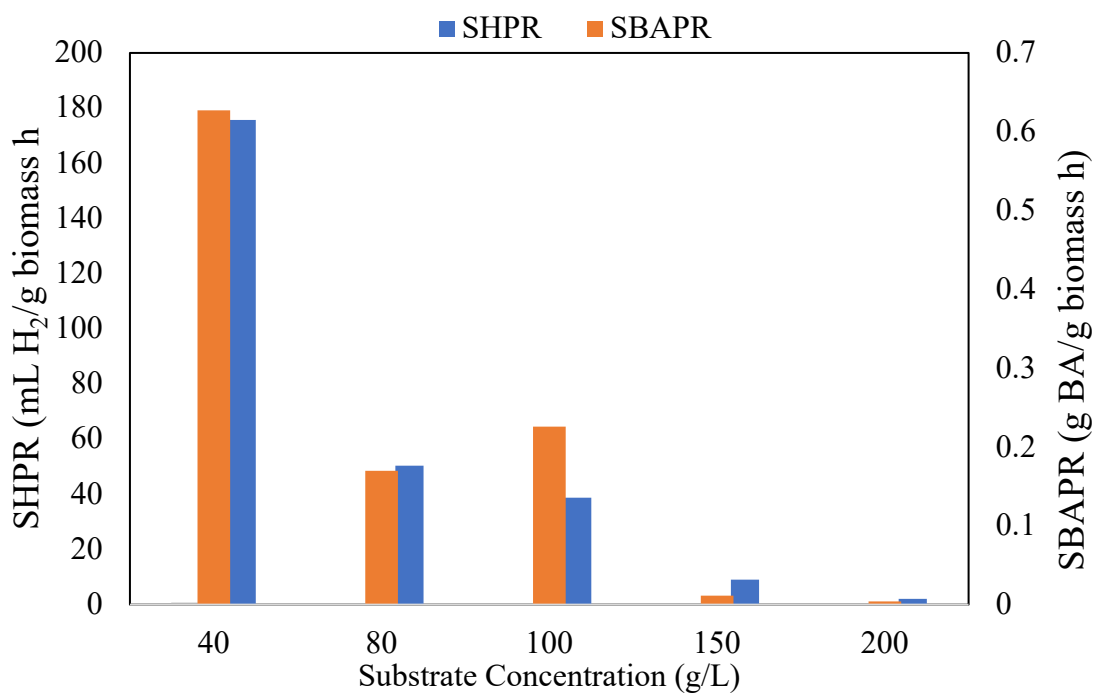


Figure 59 Variation of Specific Hydrogen Production Rate (SHPR) and Specific Butyric Acid Production Rate (SBAPR) at time with different CWP concentrations via *C. pasteurianum*

Table 9 The summary of the designed experiments

Aim of the experiment	BA (g/L)	BA/AA (g/g)	Y _{BA} (g/g)	VBAPR (g/g/h)	CHF (mL)	Y _{H2} (mL/g)	VHPR (mL/g/h)	TVFA (g/L)	Microorganism	Substrate
Impact on NaCl	5.4 (5 g/L NaCl)	2.6 (5 g/L NaCl)	1.3 (5 g/L NaCl)	0.0136 (5 g/L NaCl)	130.7 (5 g/L NaCl)	6706.7 (0.01 NaCl)	279.45 (0.01 NaCl)	10.1 (5 g/L NaCl)	Anaerobic Sludge	20 g/L Lactose
Impact on lactose conc.	3.8 (30 g/L lactose)	1.5 (5 g/L control lactose)	8.85 (5 g/L control lactose)	0.154 (5 g/L lactose)	61.65 (10 g/L lactose)	621.45 (5 g/L control lactose)	7.67 (5 g/L control lactose)	32.046 (50 g/L lactose)	Anaerobic Sludge	(5-100 g/L) Lactose
Effect on trace elements with BBD	4.64 (5 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn))	2.64 (2.505 g/L NaCl, 200 mg/L Na ₂ S ₂ O ₃)	197.91 (5 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn))	1.6 (5 g/L NaCl, 0.4 Mm minerals conc. (Ni, Fe, Cu, Zn))	190.3 (0.01 g/L NaCl, 100 mg/L Na ₂ S ₂ O ₃)	243.62 (2.505 g/L NaCl, 200 mg/L Na ₂ S ₂ O ₃)	2.6 (0.01 g/L NaCl, 100 mg/L Na ₂ S ₂ O ₃)	10 (2.505 g/L NaCl, 100 mg/L Na ₂ S ₂ O ₃)	<i>C. acetobutylicum</i>	10 g/L Lactose
Effect on CaCO ₃	2.41 (0.1 g/L CaCO ₃)	7.68 (0.1 g/L CaCO ₃)	16.04 (0.1 g/L CaCO ₃)	0.226 (0.1 g/L CaCO ₃)	138.25 (0.1 g/L CaCO ₃)	321.94 (4 g/L CaCO ₃)	6.43 (1 g/L CaCO ₃)	3.84 (0.5 g/L CaCO ₃)	<i>C. acetobutylicum</i>	15 g/L CWP
Selection of organisms	6.8 (C. <i>pasteurianum</i>)	3.61 (Anaerobic sludge)	16.44 (C. <i>pasteurianum</i>)	0.335 (C. <i>pasteurianum</i>)	334 (C. <i>pasteurianum</i>)	467.42 (C. <i>pasteurianum</i>)	17.25 (C. <i>Acetobutylicum</i>)	15.54 (C. <i>Acetobutylicum</i>)	Anaerobic sludge, <i>C. acetobutylicum</i> , <i>C. beijerinckii</i> , <i>C. pasteurianum</i>	20 g/L CWP
Effect of organism concentrations	1.52 (1.5 g/L org. kons.)	3.34 (1 g/L org. kons.)	0.37 (1.5 g/L org. kons.)	0.0176 (1.5 g/L org. kons.)	458 (1.5 g/L org. kons.)	1278.6 (1.5 g/L org. kons.)	111.8 (1.5 g/L org. kons.)	5.94 (1.5 g/L org. kons.)	<i>C. pasteurianum</i>	45 g/L CWP
Effect of substrate conc.	1.17 (150 g/L CWP)	3.23 (80 g/L CWP)	0.365 (80 g/L CWP)	0.0152 (80 g/L CWP)	651.1 (80 g/L CWP)	1163.34 (40 g/L CWP)	48.47 (40 g/L CWP)	5.028 (150 g/L CWP)	<i>C. pasteurianum</i>	(40-200 g/L) CWP

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

The aim of this thesis is to improve butyric acid along with hydrogen gas production potential. Studies were separated for the use of substrate as lactose and Cheese Whey Powder (CWP). The initial experiments were conducted using lactose while CWP contains a considerable amount of lactose, with a concentration of approximately 45 to 50 g/L (Siso et al., 1996). Furthermore, lactose was utilised as a carbon source to eliminate the adverse effects of the proteins and lipids present in CWP, as well as to ascertain whether the mineral salts in CWP interfere with the fermentation of end products. Lactose used as a substrate in first three experiments: the effect of NaCl (0-6 g/L), the variation of lactose concentrations (5-100 g/L), and the impact of NaCl, Na₂S₂O₃, and mineral concentrations with Box-Behnken Design (BBD) (Design Expert 13.0.5.0 Software) on production potential of butyric acid and hydrogen gas. All the experiments were carried out under mesophilic conditions in batch dark fermentation with a fermentation period of 10 days.

Different minerals (NaCl, Na₂S₂O₃, NiCl₂, FeSO₄, ZnSO₄, CuCl₂, CaCO₃), microorganisms (anaerobic sludge, *Clostridium acetobutylicum*, *Clostridium pasteurianum*, *Clostridium beijerinckii*), and amount of substrate and organism concentrations were tried.

First of all, the impact of NaCl was investigated. From this perspective, the highest BA, BA/AA, Y_{BA/TS}, BAPR, and CHF were 5.4 g/L, 2.6 g BA/g AA, 1.3 g BA/g TS, and 130.7 mL H₂ found at 5 g/L NaCl concentration, respectively. 20 g/L lactose, and anaerobic sludge, was used in these experiments. In the literature, sodium chloride was used to enhance butyric acid along with hydrogen (Kajal G Singh, 2016; Taheri et al., 2018; Y. Wang et al., 2013). However, due to fluctuations between NaCl concentrations, the optimum NaCl concentration was searched with different parameters in the following experiment.

The effect of lactose concentration was studied at the range of 5-100 g/L lactose and anaerobic sludge. As a result of this experimental design, the maximum BA was 3.8 g/L at 30 g/L lactose. The highest BA/AA, $Y_{BA/TS}$, $Y_{H_2/TS}$, and HPR were 1.5 g BA/ g AA, 8.85 g BA/ g TS, 621.5 mL H_2 /g TS, and 7.7 mL H_2 /g TS h at 5 g/L lactose, respectively. The best lactose concentration was observed at 5 g/L, indicating that substrate inhibition may be the cause of this phenomenon.

The various parameters was investigated with Box-Behnken Design (BBD) at *Clostridium acetobutylicum* with 10 g/L lactose. The influence of NaCl (0.1-2.505-5 g/L), $Na_2S_2O_3$ (0-100-200 mg/L), and mineral concentrations ($NiCl_2$, $FeSO_4$, $ZnSO_4$, $CuCl_2$) (0-0.4-0.8 mM) searched to understand the butyric acid and H_2 gas production potentials. The highest BA, $Y_{BA/TS}$, and BAPR were 4.64 g/L, 198 g BA/ g TS, and 1.6 g BA/g TS h at 5 g/L NaCl, 0.4 Mm minerals concentrations (Ni, Fe, Cu, Zn), respectively. The maximum CHF and HPR were 190 mL H_2 and 2.6 mL H_2 /g TS h at 0.1 g/L NaCl, 100 mg/L $Na_2S_2O_3$. The outputs of the present study, as represented by the ANOVA table, indicated that the model of butyric acid production, for three factors, fit to a modified cubic model with a high R^2 of 96%. The interactive variables ($NaCl \times Na_2S_2O_3$), ($Na_2S_2O_3 \times Na_2S_2O_3$), (minerals x minerals) and ($NaCl \times NaCl \times Na_2S_2O_3$) were found to have a significant effect on butyric acid production. According to the model, the highest butyric acid concentration was observed with the lowest NaCl concentration. There was an increase in butyric acid production with mineral concentrations. The model optimising the conditions indicated that the highest butyric acid concentrations could be achieved with the lowest NaCl concentration, the highest mineral concentration, and the highest sodium thiosulphate concentration. In contrast to the model, the experiments demonstrated that BA/AA, $Y_{P/S}$ and HPR were maximised with 2.5 g/L NaCl, 0.4 mM minerals and 100 mg/L sodium thiosulphate concentrations. The results of the presented study were comparable with the literature, revealing high BA/AA ratios along with high hydrogen production potentials.

An optimisation of calcium carbonate addition to enhance butyrate formation from a perspective of biohydrogen production was carried out for this experiment. *C. acetobutylicum* at 37°C was used with CWP (20 g/L) at different $CaCO_3$ concentrations in dark fermentation conditions. The highest hydrogen and butyric acid

production potential was found with 0.1 g/L CaCO₃ addition. The results of the experiment yielded the following values: 138.25 mL of hydrogen, 2.4 g/L of butyric acid, a BA/AA ratio of 7.68, a hydrogen production yield of 233 mL H₂/g TS, and a butyric acid production yield of 0.17 g BA/g TS. The stimulatory effects of calcium carbonate on hydrogen fermentation by *C. acetobutylicum* are mainly attributed to calcium ions, while control experiments performed with calcium chloride addition showed enhancement of hydrogen production. However, butyric acid was not produced in the control experiments. Further increases of calcium carbonate concentrations gave lower hydrogen and butyric acid potentials with higher buffering capacities of CO₃.

The different microorganisms (anaerobic sludge, *Clostridium acetobutylicum*, *C. beijerinckii*, *C. pasteurianum*) impact on the butyric acid production potential via hydrogen gas. Based on the previous experiments, the fermentation media was supplemented with NaCl (2.505 g/L), Na₂S₂O₃ (100 mg/L), NiCl₂ (0.1 mM), FeSO₄ (0.1 mM), ZnSO₄ (0.1mM), CuCl₂ (0.1mM), and CaCO₃ (0.7 g/L). 20 g/L CWP was used as the carbon source. According to the experiments the highest BA, Y_{BA/TS}, BAPR, CHF, and Y_{H₂/TS} were found 6.8 g/L, 16.44 g BA/ g TS, 0.335 g BA/g TS h, 334 mL H₂, 467.5 mL H₂/ g TS at *C. pasteurianum*, respectively. *C. pasteurianum* was selected due to the results. The literature reveals that *C. pasteurianum* is a good butyric acid and butanol producer, while butanol is produced via a pathway called hot pathway where butanol can be produced from butyric acid in the solventogenic phase (Chen et al, 2016, Ahn et al., 2011; Krasňan et al., 2018; Raganati et al., 2015; Schwarz et al., 2017).

The initial microorganism concentrations were tested for the impact on hydrogen and butyric acid formation. Different *C. pasteurianum* DSM 525 inoculum concentrations (0.5-3 g/L) were tried under the batch mesophilic dark fermentation conditions. According to results, the highest butyric acid concentration, Y_{BA/TS}, BAPR, CHF, Y_{H₂/TS}, HPR were observed with 1.5 g/L (BA:1.523 g/L, Y_{BA/TS}: 0.37 g BA/ g TS, BAPR: 0.0176 g BA/g TS h, CHF: 458 mL, Y_{H₂/TS}: 1278.63 mL H₂/ g TS, HPR:111.8 mL H₂/ g TS h) inoculum concentrations.

These results show that organism concentrations more than 1.5 g/L cause a metabolic shift into different end products. Thus, high organism concentrations are not required to enhance hydrogen and butyric acid production, whereas the optimum biomass level was 1.5 g/L.

The substrate (CWP) concentrations effect was observed with *C. pasteurianum* DSM 525 under the mesophilic conditions. CWP concentrations (40-200 g/L) were tried at batch mode and dark fermentation. Based on the results, the highest BA/AA, $Y_{BA/TS}$, BAPR, and CHF were found at 80 g/L CWP (BA/AA: 3.23 g BA/g AA, $Y_{BA/TS}$: 0.36 g BA/ g TS, BAPR: 0.003 g BA/g TS h, CHF:651 mL). Further increases in biomass and substrate concentrations than 1.5 g /L and 80 g/L CWP gave lower CHFs, organic acids probably due to the shift of metabolic pathway to other end products than organic acids. The effects of the presented research were equivalent to the literature, which showed high butyric acid ratios along with good hydrogen production capacities.

The study demonstrates that in addition to producing hydrogen, the process can also yield commercially available butyrate, resulting in the production of both green hydrogen and green chemicals. This dual output can contribute to the development of a sustainable economy.

5.2 Recommendations for the Future

The production of butyric acid and biohydrogen using lactose and cheese whey powder represents a promising solution for the identification of suitable feedstocks for these energy production processes. Further studies are required to enhance the yields of hydrogen gas production in conjunction with butyric acid. The following steps can be implemented in future studies:

1. The production of lactic acid and propionic acid can be avoided. Additional supplements can be incorporated to direct the production pathway towards butyric acid.
2. It is recommended that fed–batch or continuous processes may be tried in the fermentation medium to increase biohydrogen production by avoiding the accumulation of organic acids other than butyric acid.
3. Adsorbents or simultaneous separation methods may be added to the fermentation media to avoid end product inhibition to reach higher hydrogen and butyric acid formation potentials.
4. Furthermore, butyric acid production should be investigated with different microorganism types (*Clostridium butyricum*, *Clostridium tyrobutyricum*)

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APPENDICES

Appendix-1: Raw Data for NaCl (0-6 g/L) Concentrations Experiments

Table A.1.1 Raw data for 20 g/L lactose with no NaCl concentration at anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	20	0	0	0	0
24	5.90	5.69	1.57	0.3	0.55	0.45
48	5.80	1.07	1.8	0.6	1.40	1.46
72	6.50	0.95	2.2	0.94	0.6	
96	6.60	0.88	2.5	0.95	0	1.22
120	6.50	0.85	3.3	1.3	0	1.97
144	6.46	0.80	5.8	1.4	0	1.93
168	6.35	0.80	5.75	1.38	0	2.09
192	6.70	0.7	6.5	1.45	0	1.95
216	6.80	0.66	5.9	1.3	0	1.74
240	6.56	0.55	5.2	1.41	0	1.68

Table A.1.2 Hydrogen production data for 20 g/L lactose with no NaCl concentration at anaerobic sludge experiment

Time (hour)	V _I (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	100	210	0	0	0	0	0	0	0
24	92	218	0.04	8.50	10.00	0.039	0.39	8.89	0.36
48	84	226	0.064	5.9	6.00	0.064	0.38	15.168	0.61
72	77	233	0.185	28.83	10.00	0.185	1.86	45.848	1.84
96	70	240	0.168	0	10.00	0.168	1.68	47.527	1.91
120	65	245	0.144	0	10.00	0.144	1.44	48.966	1.97
144	60	250	0.118	0	10.00	0.118	1.18	50.144	2.017
168	55	255	0.114	0	10.00	0.114	1.14	51.283	2.06
192	50	260	0.099	0	10.00	0.099	0.99	52.274	2.10
216	45	265	0.094	0	9.00	0.095	0.85	53.123	2.137
240	40	270	0.086	0	10.00	0.087	0.86	53.98	2.171

Table A.1.3 Raw data for 20 g/L lactose with 0.01 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	20	0	0	0	0
24	5.8	14.133	1.439	0.27	0.819	0.25
48	5.8	0.92	0.96	0.56	1.13	0.52
72	6.5	0.9	1.68	0.822	0	0.82
96	6.6	0.845	2.28	0.8	0	1.25
120	6.6	0.80	2.78	1.14	0	1.63
144	6.5	0.70	4.30	1.188	0	1.824
168	6.5	0.573	4.88	1.23	0	1.918
192	6.5	0.555	5.55	1.11	0	1.698
216	6.37	0.555	5.3	1.18	0	2.086
240	6.7	0.49	3.91	1.215	0	1.726

Table A.1.4 Hydrogen production data for 20 g/L lactose with 0.01 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	V _i (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	100	210	0	0	0	0	0	0	0
24	93	217	0.112	24.3	25.00	0.112	2.8	27.104	1.066
48	86	224	0.178	14.157	211.00	0.172	36.23	77.49	3.05
72	79	231	0.21	9.934	30.00	0.21	6.29	93.708	3.7
96	73	237	0.189	0	10.00	0.189	1.89	95.601	3.76
120	68	242	0.161	0	10.00	0.16	1.61	97.214	3.82
144	63	247	0.139	0	10.00	0.139	1.39	98.60	3.87
168	59	251	0.122	0	10.00	0.122	1.22	99.817	3.92
192	54	256	0.105	0	9.00	0.105	0.943	100.76	3.96
216	49	261	0.095	0	9.00	0.095	0.854	101.615	3.99
240	44	266	0.086	0	9.00	0.086	0.772	102.387	4.025

Table A.1.5 Raw data for 20 g/L lactose with 0.1 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	20	0	0	0	0
24	5.60	17.9	1.144	0.283	1.24	0.213
48	5.80	0.88	0.93	0.54	1.023	0.53
72	6.50	0.8	1.762	0.73	0.08	1.44
96	6.50	0.7	2.017	0.73	0	1.56
120	6.50	0.66	2.15	0.97	0	1.636
144	6.50	0.555	2.75	0.94	0	1.753
168	6.50	0.55	4.85	1.29	0.67	1.97
192	6.50	0.53	2.864	1.09	0	1.84
216	6.48	0.48	3.48	0.95	0	2.016
240	6.52	0.343	4.17	1.12	0	2.165

Table A.1.6 Hydrogen production data for 20 g/L lactose with 0.1 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	100	210	0	0	0	0	0	0	0
24	94	216	0.17	36.5	15.00	0.17	2.5335	39.016	1.534
48	87	223	0.195	6.87	10.00	0.195	1.944	47.83	1.88
72	80	230	0.194	1.338	10.00	0.194	1.943	51.11	2.0094
96	74	236	0.165	0	7.00	0.165	1.1571	52.26	2.055
120	69	241	0.138	0	10.00	0.138	1.377	53.64	2.11
144	64	246	0.119	0	10.00	0.119	1.186	54.83	2.157
168	59	251	0.10	0	10.00	0.10	1.011	55.84	2.2
192	54	256	0.09	0	9.00	0.09	0.774	56.615	2.225
216	49	261	0.08	0	9.00	0.08	0.7128	57.33	2.254
240	44	266	0.067	0	9.00	0.067	0.6021	57.93	2.277

Table A.1.7 Raw data for 20 g/L lactose with 0.5 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	20	0	0	0	0
24	5.50	14.055	1.28	0.59	1.255	0
48	5.80	0.97	0.743	0.62	1.01	0
72	6.50	0.91	1.63	0.67	0.17	1.016
96	6.60	0.87	1.97	0.806	0.082	1.45
120	6.60	0.73	2.53	1.138	0	1.69
144	6.60	0.706	2.71	1.09	0	1.83
168	6.60	0.7	4.185	1.13	0.116	1.68
192	6.50	0.61	3.15	1.194	0	1.76
216	6.50	0.46	2.96	1.089	0	2.146
240	6.58	0.44	3.97	1.29	0	1.69

Table A.1.8 Hydrogen production data for 20 g/L lactose with 0.5 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	V_I(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	V_{total} gas (mL)	H₂-1 (%)	V_t x H₂-1	V_{cum.} (mL)	Cumulative H₂ (mmol)
0	100.00	210.00	0	0	0	0	0	0	0
24	93.00	217.00	0.15	31.68	25.00	0.15	3.65	35.33	1.39
48	87.00	223.00	0.18	8.35	70.00	0.18	12.57	56.24	2.21
72	80.00	230.00	0.18	2.34	10.00	0.18	1.84	60.42	2.38
96	74.00	236.00	0.17	0	9.00	0.17	1.51	61.94	2.43
120	69.00	241.00	0.14	0	10.00	0.14	1.43	63.36	2.49
144	64.00	246.00	0.12	0	10.00	0.12	1.20	64.56	2.54
168	59.00	251.00	0.11	0	10.00	0.11	1.07	65.64	2.58
192	54.00	256.00	0.09	0	10.00	0.09	0.94	66.58	2.62
216	49.00	261.00	0.08	0	9.00	0.08	0.75	67.33	2.65
240	44.00	266.00	0.08	0	9.00	0.08	0.68	68.01	2.67

Table A.1.9 Raw data for 20 g/L lactose with 1 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	20.00	0	0	0	0
24	5.60	17.90	1.41	0.28	1.19	0.27
48	5.80	0.90	0.65	0.60	0.85	1.75
72	6.50	0.74	1.27	0.66	0	
96	6.40	0.72	1.96	0.77	0	1.75
120	6.50	0.72	2.62	1.13	0	1.98
144	6.50	0.49	4.42	1.14	0	2.17
168	6.50	0.48	2.78	1.12	0	2.18
192	6.40	0.40	3.79	1.08	0	2.34
216	6.50	0.36	4.34	1.13	0	2.10
240	6.52	0.31	3.97	1.29	0	2.25

Table A.1.10 Hydrogen production data for 20 g/L lactose with 1 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	Vtotal gas (mL)	H₂-1 (%)	Vt x H₂-1	Vcum. (mL)	Cumulative H₂ (mmol)
0	100.00	210.00	0	0	0	0	0	0	0
24	93.00	217.00	0.11	23.83	70.00	0.11	7.69	31.51	1.24
48	86.00	224.00	0.16	12.95	200.00	0.16	32.84	77.31	3.04
72	80.00	230.00	0.16	0	10.00	0.16	1.56	78.86	3.10
96	74.00	236.00	0.16	2.63	10.00	0.16	1.63	83.13	3.27
120	69.00	241.00	0.13	0	10.00	0.13	1.29	84.42	3.32
144	64.00	246.00	0.13	0	10.00	0.13	1.26	85.68	3.37
168	59.00	251.00	0.11	0	10.00	0.11	1.13	86.80	3.41
192	54.00	256.00	0.09	0	10.00	0.09	0.91	87.72	3.45
216	50.00	260.00	0.08	0	9.00	0.08	0.73	88.44	3.48
240	45.00	265.00	0.08	0.34	10.00	0.08	0.81	89.59	3.52

Table A.1.11 Raw data for 20 g/L lactose with 5 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	20.40	0	0	0	0
24	6.35	20.34	0.90	0.61	0	0.46
48	5.80	20.29	1.43	0.23	0.20	0.60
72	5.80	14.87		1.79	0	
96	6.00	11.79	2.38		0	0.50
120	6.10	9.68	3.01	1.78	0	
144	6.10	6.49	2.26	3.61	0	0.82
168	6.30	3.38	2.41	4.22	0	0.79
192	6.50	2.53	2.00	5.22	0	0.80
216	6.55	2.48	3.92	5.40	0	0.75
240	6.50	2.24	3.20	4.81	0	0.76

Table A.1.12 Hydrogen production data for 20 g/L lactose with 5 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	Vtotal gas (mL)	H₂-1 (%)	Vt x H₂-1	Vcum. (mL)	Cumulative H₂ (mmol)
0	100.00	210.00	0	0	0	0	0	0	0
24	94.00	216.00	0.04	9.29	77.00	0.04	3.31	12.60	0.50
48	88.00	222.00	0.13	18.64	20.00	0.13	2.52	33.75	1.33
72	83.00	227.00	0.20	17.88	31.00	0.20	6.26	57.89	2.28
96	79.00	231.00	0.26	13.95	21.00	0.26	5.43	77.28	3.04
120	74.00	236.00	0.29	7.74	22.00	0.29	6.29	91.30	3.59
144	69.00	241.00	0.29	3.17	41.00	0.29	12.02	106.49	4.19
168	64.00	246.00	0.29	1.88	10.00	0.29	2.95	111.32	4.38
192	60.00	250.00	0.30	1.75	12.00	0.30	3.57	116.64	4.59
216	55.00	255.00	0.32	6.54	14.00	0.32	4.44	127.62	5.02
240	50.00	260.00	0.31	0	10.00	0.31	3.10	130.71	5.14

Table A.1.13 Raw data for 20 g/L lactose with 6 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	20.10	0	0	0	0
24	6.50	20.06	0.37	0.55	0	0.33
48	5.80	20.03	1.00	0.72	0	0.44
72	5.90	17.00	1.70	0.87	0	0.55
96	6.00	16.77	2.42	1.19	0	0.70
120	6.10	16.35	2.88	1.05	0	0.69
144	6.10	14.57	3.71		0	0.90
168	6.10	9.70	3.84	0.86	0	0.77
192	6.49	7.21	6.54		0	0
216	6.29	2.88	2.69	0.89	5.76	0
240	6.50	1.58	3.33	0.11	4.33	0

Table A.1.14 Hydrogen production data for 20 g/L lactose with 6 g/L NaCl concentration at anaerobic sludge experiment

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	V_{total} gas (mL)	H₂-1 (%)	V_t x H₂-1	V_{cum.} (mL)	Cumulative H₂ (mmol)
0	100.00	210.00	0	0	0	0	0	0	0
24	93.00	217.00	0.04	8.25	20.00	0.04	0.76	9.01	0.35
48	87.00	223.00	0.07	7.14	10.00	0.07	0.69	16.84	0.66
72	82.00	228.00	0.12	11.47	10.00	0.12	1.18	29.49	1.16
96	77.00	233.00	0.04	-	10.00	0.04	0.44	29.92	1.18
120	72.00	238.00	0.15	26.64	11.00	0.15	1.70	58.26	2.29
144	67.00	243.00	0.18	7.48	10.00	0.18	1.82	67.56	2.66
168	62.00	248.00	0.18	1.31	10.00	0.18	1.84	70.71	2.78
192	57.00	253.00	0.18	0.49	10.00	0.18	1.82	73.02	2.87
216	55.00	255.00	0.19	2.17	16.00	0.19	3.03	78.22	3.08
240	50.00	260.00	0.19	0.69	10.00	0.19	1.88	80.79	3.18

Appendix-2: Raw Data for Lactose (5-100 g/L) Concentrations Experiments

Table A.2.1 Raw data for 5 g/L of lactose with anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.2	5.00	0.61	0	2.85	0.19
18	5.1	1.33	0.41	0.61	2.43	0.11
45	6.7	0.95	0.59	0.35	2.10	0.18
57	6.5	0.71	1.09	0.44	2.72	0.41
78	6.3	0.60	1.44	0.62	1.45	0.89
101	6.7	0.57	2.26	1.04	0	1.36
125	6.8	0.54	2.58	1.27	0	1.24
150	6.7	0.47	2.40	1.34	0	1.35
170	6.7	0.47	2.23	1.35	0	1.43
251	6.9	0.42	1.85	1.77	0	1.48
269	6.9	0.37	2.24	1.16	0	1.75

Table A.2.2 Hydrogen production data for 5 g/L of lactose with anaerobic sludge experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150	160	0	0	0	0	0	0	0
18	143	167	0.04	7.18	10.00	0.04	0.43	7.61	0.31
45	137	173	0.07	4.06	9.00	0.07	0.59	12.26	0.49
57	131	179	0.07	1.63	10.00	0.07	0.72	14.60	0.59
78	126	184	0.07	0.38	11.00	0.07	0.79	15.77	0.63
101	120	190	0.07	0	9.00	0.07	0.60	16.37	0.66
125	114	196	0.07	1.40	7.00	0.07	0.50	18.27	0.73
150	107	203	0.08	1.37	11.00	0.08	0.83	20.47	0.82
170	100	210	0.07	0	8.00	0.07	0.57	21.05	0.85
251	95	215	0.08	2.70	10.00	0.08	0.83	24.58	0.99
269	90	220	0.07	0	10.00	0.07	0.73	25.31	1.02

Table A.2.3 Raw data for 10 g/L of lactose with anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.2	10.00	0.28	0.38	2.60	0.13
18	4.6	7.98	0.43	0.23	5.57	0.12
45	4.7	5.83	0.77	1.07	4.39	0.16
57	6.17	2.68	1.05	0.67	4.11	0
78	6.7	2.70	1.39	0.48	4.62	0
101	6.6	1.86	2.36	1.19	5.65	0
125	6.6	1.82	2.01	1.16	4.51	0
150	6.6	1.70	2.82	1.21	3.43	0
170	6.7	1.58	1.91	1.42	3.57	0.73
251	6.8	1.38	1.85	1.79	0	0.72
269	6.8	1.20	1.95	1.92	0	0.73

Table A.2.4 Hydrogen production data for 10 g/L of lactose with anaerobic sludge experiment

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	Vtotal gas (mL)	H₂-1 (%)	Vt x H₂-1	Vcum. (mL)	Cumulative H₂ (mmol)
0	150	160	0	0	0	0	0	0	0
18	144	166	0.04	6.14	10.00	0.04	0.37	6.51	0.26
45	138	172	0.04	0.10	10.00	0.04	0.36	6.98	0.28
57	133	177	0.10	12.01	10.00	0.10	1.03	20.01	0.80
78	127	183	0.11	2.38	10.00	0.11	1.13	23.52	0.95
101	121	189	0.05	0	8.00	0.05	0.41	23.92	0.96
125	116	194	0.12	13.45	9.00	0.12	1.07	38.44	1.55
150	111	199	0.12	1.81	12.00	0.12	1.50	41.74	1.68
170	106	204	0.13	2.50	8.00	0.13	1.07	45.32	1.82
251	101	209	0.19	12.31	11.00	0.19	2.09	59.72	2.40
269	96	214	0.18	0	11.00	0.18	1.94	61.65	2.48

Table A.2.5 Raw data for 30 g/L of lactose with anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.2	30.90	0	0.09	0	0.18
18	5	30.00	0	0.12	5.53	0.17
45	4.7	28.66	0.69	0.18	10.29	0.24
57	5.1	26.20	1.12	1.55	11.17	0.34
78	5.6	26.01	1.19		13.18	0.35
101	5.4	21.77	4.16	3.80	9.61	0
125	5.4	14.27	1.95	1.15	8.23	0
150	6	11.32	3.44	1.25	7.90	0
170	6.26	7.50	3.32	1.23	8.44	0
251	5.84	7.12	2.36	1.00	7.03	0
269	6.67	5.38	2.72	1.25	6.72	0

Table A.2.6 Hydrogen production data for 30 g/L of lactose with anaerobic sludge experiment

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	Vtotal gas (mL)	H₂-1 (%)	Vt x H₂-1	Vcum. (mL)	Cumulative H₂ (mmol)
0	150	160	0	0	0	0	0	0	0
18	144	166	0.03	5.64	17.00	0.03	0.58	6.22	0.25
45	138	172	0.07	6.57	12.00	0.07	0.85	13.64	0.55
57	132	178	0.08	2.33	10.00	0.08	0.82	16.79	0.68
78	127	183	0.08	0	7.00	0.08	0.55	17.34	0.70
101	120	190	0.12	8.57	12.00	0.12	1.45	27.37	1.10
125	115	195	0.14	3.90	10.00	0.14	1.38	32.65	1.31
150	109	201	0.15	3.54	7.00	0.15	1.06	37.25	1.50
170	103	207	0.16	2.31	9.00	0.16	1.43	40.99	1.65
251	97	213	0.17	4.17	20.00	0.17	3.47	48.63	1.96
269	92	218	0.17	0.18	6.00	0.17	1.02	49.84	2.00

Table A.2.7 Raw data for 50 g/L of lactose with anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.2	50.00	0	0.29	0	0.25
18	5.2	49.98	0	0.20	4.74	0.21
45	4.7	49.92	0.40	0.21	7.67	0.29
57	5.13	49.85	1.00	0.77	12.20	0.48
78	5.43	49.47	1.19	1.03	15.40	0
101	5.49	38.30	3.06	1.18	10.04	0
125	5.5	38.05	2.87	1.44	12.75	0
150	5.53	18.05	3.43	1.27	6.53	0
170	5.64	5.81	3.17	1.56	2.32	0
251	5.23	5.49	2.05	1.07	0.76	0
269	6.5	5.49	1.72	1.17	0.78	0

Table A.2.8 Hydrogen production data for 50 g/L of lactose with anaerobic sludge experiment

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	Vtotal gas (mL)	H₂-1 (%)	Vt x H₂-1	Vcum. (mL)	Cumulative H₂ (mmol)
0	150	160	0	0	0	0	0	0	0
18	143	167	0.03	5.51	10.00	0.03	0.33	5.84	0.23
45	137	173	0.03	0.18	10.00	0.03	0.33	6.35	0.26
57	130	180	0.03	0.52	10.00	0.03	0.35	7.21	0.29
78	125	185	0.04	1.84	10.00	0.04	0.44	9.49	0.38
101	118	192	0.06	3.82	10.00	0.06	0.62	13.92	0.56
125	113	197	0.07	1.67	8.00	0.07	0.55	16.14	0.65
150	107	203	0.09	3.76	12.00	0.09	1.02	20.93	0.84
170	100	210	0.09	1.62	8.50	0.09	0.77	23.31	0.94
251	94	216	0.12	7.61	21.00	0.12	2.58	33.50	1.35
269	89	221	0.12	0	8.00	0.12	0.94	34.43	1.38

Table A.2.9 Raw data for 75 g/L of lactose with anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.2	75.00	0.31	0.16	0	0.32
18	5.45	73.45	0.23	0.23	3.58	0.29
45	5.12	70.61	0.39	0.24	5.53	0.33
57	5.6	55.09	0.91	0.58	8.87	0.49
78	5.45	52.72	1.57	0.56	10.97	0.48
101	5.5	51.40	2.46	1.00	12.93	0.77
125	5.55	45.34	2.71	1.38	10.63	0
150	5.5	44.11	2.41	1.72	19.06	0
170	6	43.92	1.74	1.24	17.53	0
251	5.3	40.32	3.75	1.21	14.99	0.78
269	6.31	38.62	4.70	0.99	12.41	0

Table A.2.10 Hydrogen production data for 75 g/L of lactose with anaerobic sludge experiment

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	Vtotal gas (mL)	H₂-1 (%)	Vt x H₂-1	Vcum. (mL)	Cumulative H₂ (mmol)
0	150	160	0	0	0	0	0	0	0
18	144	166	0.03	5.44	9.00	0.03	0.30	5.74	0.23
45	139	171	0.03	0.16	8.00	0.03	0.26	6.17	0.25
57	133	177	0.03	0.41	10.00	0.03	0.34	6.92	0.28
78	126	184	0.03	0.18	9.00	0.03	0.30	7.40	0.30
101	118	192	0.03	0.19	10.00	0.03	0.33	7.93	0.32
125	113	197	0.04	1.64	8.00	0.04	0.33	9.90	0.40
150	106	204	0	0	1.00	0	0	9.90	0.40
170	100	210	0.03	7.01	18.00	0.03	0.60	17.51	0.70
251	94	216	0.07	7.48	9.00	0.07	0.60	25.60	1.03
269	88	222	0.07	0.41	5.00	0.07	0.34	26.35	1.06

Table A.2.11 Raw data for 100 g/L of lactose with anaerobic sludge experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.2	100.00	0	0.16	0.00	0.36
18	5.97	99.33	0	0.44	3.32	0.29
45	5.29	87.21	0	0.31	5.97	0.34
57	5.7	75.47	0.89	0.58	6.80	0.47
78	5.9	75.09	0.94	0.61	10.80	0.53
101	5.7	72.82	2.32	0.91	11.66	0.70
125	5.9	67.77	4.69	0.92	14.11	0.79
150	6.3	51.87	3.05	0.97	14.86	0
170	6.16	40.13	2.83	0.98	14.62	0
251	5.37	39.63	2.90	1.00	16.55	0.70
269	6.17	13.13	2.11	0.96	4.58	0.69

Table A.2.12 Hydrogen production data for 100 g/L of lactose with anaerobic sludge experiment

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	Vtotal gas (mL)	H₂-1 (%)	V_t x H₂-1	Vcum. (mL)	Cumulative H₂ (mmol)
0	150	160	0	0	0	0	0	0	0
18	144	166	0.03	5.44	17.00	0.03	0.56	6.00	0.24
45	138	172	0.03	0.20	9.00	0.03	0.30	6.49	0.26
57	132	178	0.03	0.34	10.00	0.03	0.34	7.17	0.29
78	125	185	0.03	0.20	7.00	0.03	0.23	7.60	0.31
101	119	191	0.03	0.32	8.00	0.03	0.27	8.19	0.33
125	113	197	0.03	0.26	5.00	0.03	0.17	8.62	0.35
150	107	203	0.03	0.27	10.00	0.03	0.35	9.24	0.37
170	100	210	0.04	0.58	16.00	0.04	0.58	10.39	0.42
251	94	216	0.07	6.65	18.00	0.07	1.19	18.24	0.73
269	88	222	0.06	0	6.17	0.06	0.36	18.60	0.75

Appendix-3: Raw Data for NaCl (0.1-2.505-5 g/L), Na₂S₂O₃ (0-100-200 mg/L), and mineral concentrations (Ni, Fe, Cu, Zn) (0-0.4-0.8 mM) with using the Box-Behnken Design (BBD), 10 g/L lactose and *Clostridium acetobutylicum* Experiments.

Table A.3.1 Raw data for 0.01 g/L of NaCl, no Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (1st experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.29	6.94	0.66	0.53	0.11	0.21
41	5.30	1.34	1.02	1.10	0	0
65	6.28	1.05	1.76		0	0
87	6.60	0.85	2.63	1.69	0.49	0
110	6.60	0.80	4.80	2.36	0	0
134	6.60	0.56			0	0
156	6.52	0.56	5.45	2.27	0	1.09
180	6.60	0.56	3.89	2.61	0	1.11
205	6.60	0.56	4.38	2.70	0	1.14
233	6.60	0.56	3.63	2.14	0	0

Table A.3.2 Hydrogen production data for 0.01 g/L of NaCl, no Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (1st experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	146.00	164.00	0.08	12.56	9.00	0.08	0.69	13.25	0.53
41	140.00	170.00	0.40	55.08	12.00	0.40	4.77	73.11	2.94
65	135.00	175.00	0.46	13.24	22.00	0.46	10.17	96.52	3.88
87	129.00	181.00	0.44	0.00	15.00	0.44	6.61	103.12	4.15
110	123.00	187.00	0.40	0.00	15.00	0.40	6.07	109.19	4.39
134	117.00	193.00	0.36	0.00	9.00	0.36	3.26	112.44	4.52
156	111.00	199.00	0.31	0.00	9.00	0.31	2.81	115.26	4.64
180	106.00	204.00	0.31	1.77	27.00	0.31	8.47	125.49	5.05
205	101.00	209.00	0.27	0.00	19.00	0.27	5.15	130.64	5.25
233	96.00	214.00	0.24	0.00	20.50	0.24	4.93	135.57	5.45

Table A.3.3 Raw data for 0.01 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and no minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (2nd experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.80	7.52	0.44	0.27	5.11	0.08
41	5.35	1.12	0.58	1.30	6.09	0
65	6.26	0.68	0.88	1.70	4.38	0
87	6.52	0.62	2.33	2.10	0	1.41
110	6.60	0.58	3.82	2.62	0	1.93
134	6.60	0.56	3.75	2.31	0	1.75
156	6.60	0.56	3.90	2.49	0	1.84
180	6.60	0.56	3.94	2.81	0	1.70
205	6.38	0.56	3.68	2.32	0	1.62
233	6.50	0.56	3.77	2.32	0	1.72

Table A.3.4 Hydrogen production data for 0.01 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and no minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (2nd experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	144.00	166.00	0.15	24.15	22.00	0.15	3.20	27.35	1.10
41	138.00	172.00	0.42	48.53	112.00	0.42	47.33	123.22	4.96
65	133.00	177.00	0.46	9.19	22.00	0.46	10.18	142.59	5.73
87	128.00	182.00	0.47	4.26	22.00	0.47	10.41	157.26	6.33
110	122.00	188.00	0.42	0.00	12.00	0.42	5.02	162.28	6.53
134	117.00	193.00	0.38	0.00	7.00	0.38	2.68	164.96	6.63
156	111.00	199.00	0.32	0.00	7.00	0.32	2.22	167.19	6.72
180	106.00	204.00	0.33	4.00	26.00	0.33	8.57	179.75	7.23
205	100.00	210.00	0.26	0.00	19.00	0.26	5.01	184.76	7.43
233	95.00	215.00	0.26	0.82	18.00	0.26	4.70	190.29	0

Table A.3.5 Raw data for 0.01 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.8 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (3rd experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.80	8.53	0.34	0.16	5.26	0
41	5.24	1.54	0.54	0.75	7.30	0
65	6.30	1.09	0.75	1.19	6.22	0
87	6.70	0.93	1.95	1.86	3.18	0
110	6.60	0.93	2.87	2.76	0	0.79
134	6.60	0.87	3.20	3.12	0	0
156	6.60	0.62	3.15	3.27	0	0.85
180	6.60	0.56	3.21	3.18	0	0.80
205	6.60	0.56	3.19	3.48	0	0.82
233	6.60	0.56	3.26	3.10	0	0.88

Table A.3.6 Hydrogen production data for 0.01 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.8 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (3rd experimental vessel)

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	Vtotal gas (mL)	H₂-1 (%)	Vt x H₂-1	Vcum. (mL)	Cumulative H₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	145.00	165.00	0.07	10.79	20.00	0.07	1.31	12.10	0.49
41	140.00	170.00	0.27	34.99	62.00	0.27	16.70	63.79	2.57
65	134.00	176.00	0.35	15.63	27.00	0.35	9.42	88.83	3.57
87	128.00	182.00	0.33	0.00	17.00	0.33	5.54	94.37	3.80
110	123.00	187.00	0.28	0.00	16.00	0.28	4.40	98.77	3.97
134	118.00	192.00	0.26	0.00	12.00	0.26	3.06	101.83	4.10
156	112.00	198.00	0.22	0.00	9.00	0.22	1.95	103.78	4.17
180	107.00	203.00	0.23	3.80	28.00	0.23	6.43	114.01	4.59
205	102.00	208.00	0.19	0.00	16.50	0.19	3.08	117.09	4.71
233	97.00	213.00	0.17	0.00	19.00	0.17	3.25	120.34	4.84

Table A.3.7 Raw data for 0.01 g/L of NaCl, 200 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (4th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	6.90	8.51	0.36	0.16	5.21	0
41	6.50	1.65	0.51	0.46	5.73	0
65	6.50	1.53	0.58	0.92	5.47	0
87	6.50	0.76	1.58	1.36	3.88	0.66
110	6.60	0.56	3.56	1.66	0	1.73
134	6.57	0.56	4.35	2.16	0	2.17
156	6.45	0.56	4.42	2.48	0	2.03
180	6.60	0.56	3.88	2.45	0	1.89
205	6.48	0.56	3.83	2.29	0	1.18
233	6.60	0.56	3.49	2.90	0	1.98

Table A.3.8 Hydrogen production data for 0.01 g/L of NaCl, 200 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (4th experimental vessel)

Time (hour)	V _I (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	147.00	163.00	0.07	10.84	20.00	0.07	1.33	12.17	0.49
41	143.00	167.00	0.22	26.69	52.00	0.22	11.68	50.54	2.03
65	137.00	173.00	0.28	10.98	32.00	0.28	8.97	70.50	2.84
87	131.00	179.00	0.34	12.67	10.00	0.34	3.42	86.59	3.48
110	125.00	185.00	0.36	6.07	22.00	0.36	8.00	100.65	4.05
134	119.00	191.00	0.34	0.00	11.00	0.34	3.71	104.36	4.20
156	113.00	197.00	0.30	0.00	10.00	0.30	3.01	107.38	4.32
180	108.00	202.00	0.29	0.00	26.50	0.29	7.78	115.16	4.63
205	103.00	207.00	0.25	0.00	18.00	0.25	4.45	119.61	4.81
233	98.00	212.00	0.23	0.00	22.00	0.23	5.04	124.64	5.01

Table A.3.9 Raw data for 2.505 g/L of NaCl, no Na₂S₂O₃, and no minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (5th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.80	6.21	0.45	0.29	5.15	0
41	5.10	1.30	0.67	1.06	5.48	0
65	6.20	0.86	0.85	1.42	4.48	0
87	6.49	0.67	2.19	1.87	0.46	0.70
110	6.49	0.63	3.78	2.60	0	1.07
134	6.60	0.56	3.53	2.32	0	1.02
156	6.60	0.56	4.52	3.23	0	1.34
180	6.60	0.56	2.76	2.82	0	1.04
205	6.45	0.56	3.92	2.73	0	1.09
233	6.50	0.56	3.83	3.22	0	1.05

Table A.3.10 Hydrogen production data for 2.505 g/L of NaCl, no Na₂S₂O₃, and no minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (5th experimental vessel)

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	Vtotal gas (mL)	H₂-1 (%)	Vt x H₂-1	Vcum. (mL)	Cumulative H₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	147.00	163.00	0.11	18.22	32.00	0.11	3.58	21.80	0.88
41	142.00	168.00	0.37	43.89	72.00	0.37	26.62	92.31	3.71
65	137.00	173.00	0.43	11.59	27.00	0.43	11.50	115.40	4.64
87	131.00	179.00	0.43	4.02	10.00	0.43	4.34	123.76	4.98
110	125.00	185.00	0.39	0.00	10.00	0.39	3.93	127.69	5.14
134	120.00	190.00	0.36	0.00	10.00	0.36	3.63	131.32	5.28
156	114.00	196.00	0.32	0.00	10.00	0.32	3.18	134.50	5.41
180	109.00	201.00	0.32	2.31	26.50	0.32	8.53	145.34	5.85
205	104.00	206.00	0.26	0.00	22.00	0.26	5.81	151.15	6.08
233	99.00	211.00	0.25	0.00	19.00	0.25	4.81	155.97	6.27

Table A.3.11 Raw data for 2.505 g/L of NaCl, no Na₂S₂O₃, and 0.8 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (6th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.30	7.61	0.34	0.15	5.69	0
41	4.80	1.75	0.48	0.43	5.84	0
65	6.16	0.82	0.86	0.77	4.58	0.24
87	6.55	0.81	2.70	1.77	0	1.14
110	6.60	0.77	3.60	2.18	0	1.47
134	6.60	0.56	3.99	2.30	0	1.56
156	6.60	0.56	3.60	2.34	0	1.36
180	6.60	0.56	3.85	3.28	0	1.39
205	6.60	0.56	3.72	2.31	0	1.38
233	6.60	0.56	3.78	2.76	0	1.39

Table A.3.12 Hydrogen production data for 2.505 g/L of NaCl, no Na₂S₂O₃, and 0.8 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (6th experimental vessel)

Time (hour)	V _i (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total gas} (mL)	H ₂ -1 (%)	V _{t x} H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	148.00	162.00	0.06	10.47	22.00	0.06	1.42	11.89	0.48
41	144.00	166.00	0.22	26.85	52.00	0.22	11.69	50.43	2.03
65	139.00	171.00	0.31	14.95	27.00	0.31	8.25	73.64	2.96
87	133.00	177.00	0.35	9.40	47.00	0.35	16.37	99.41	4.00
110	127.00	183.00	0.30	0.00	9.00	0.30	2.73	102.14	4.11
134	122.00	188.00	0.27	0.00	7.00	0.27	1.91	104.05	4.18
156	116.00	194.00	0.24	0.00	15.00	0.24	3.63	107.67	4.33
180	111.00	199.00	0.22	0.00	18.50	0.22	4.11	111.78	4.50
205	106.00	204.00	0.20	0.00	22.00	0.20	4.39	116.17	4.67
233	101.00	209.00	0.20	0.05	20.00	0.20	3.90	120.12	4.83

Table A.3.13 Raw data for 2.505 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (7th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.70	9.87	0.38	0.17	5.97	0
41	4.84	2.39	0.50	0.38	5.08	0
65	6.01	1.57	0.69	0.99	4.67	0.23
87	6.60	1.43	1.99	1.42	0.76	1.41
110	6.50	1.12	3.30	1.82	0	1.94
134	6.60	0.57	3.91	2.05	0	2.33
156	6.37	0.56	3.77	2.10	0	2.22
180	6.60	0.56	2.32	2.43	0	1.76
205	6.54	0.56	4.08	2.43	0	2.33
233	6.54	0.56	3.71	2.57	0	2.08

Table A.3.14 Hydrogen production data for 2.505 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (7th experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	147.00	163.00	0.08	13.10	17.00	0.08	1.37	14.47	0.58
41	141.00	169.00	0.22	24.06	42.00	0.22	9.24	47.77	1.92
65	136.00	174.00	0.31	17.49	42.00	0.31	13.19	78.45	3.16
87	130.00	180.00	0.36	9.82	42.00	0.36	15.04	103.31	4.16
110	125.00	185.00	0.30	0.00	17.00	0.30	5.11	108.42	4.36
134	120.00	190.00	0.30	1.53	8.00	0.30	2.40	112.36	4.52
156	114.00	196.00	0.26	0.00	25.00	0.26	6.62	118.98	4.79
180	109.00	201.00	0.23	0.00	17.50	0.23	4.04	123.02	4.95
205	104.00	206.00	0.20	0.00	20.00	0.20	4.01	127.03	5.11
233	99.00	211.00	0.20	0.00	19.00	0.20	3.71	130.74	5.26

Table A.3.15 Raw data for 2.505 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (8th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.46	6.95	0.36	0.14	5.72	0
41	4.82	1.39	0.47	0.31	5.69	0
65	6.29	1.01	0.61	0.61	4.52	0
87	6.53	0.86	2.06	1.00	3.71	1.18
110	6.50	0.82	4.00	1.48	0	2.67
134	6.50	0.73	4.53	1.56	0	2.93
156	6.50	0.56	4.14	1.67	0	2.53
180	6.50	0.56	3.44	2.28	0	2.53
205	6.50	0.56	4.63	1.83	0	2.88
233	6.50	0.56	4.07	1.98	0	2.68

Table A.3.16 Hydrogen production data for 2.505 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (8th experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	147.00	163.00	0.06	10.50	15.00	0.06	0.97	11.46	0.46
41	143.00	167.00	0.19	20.40	12.00	0.19	2.22	34.08	1.37
65	138.00	172.00	0.23	8.10	29.00	0.23	6.57	48.75	1.96
87	132.00	178.00	0.28	10.17	20.00	0.28	5.52	64.45	2.59
110	127.00	183.00	0.17	0.00	9.00	0.17	1.55	66.00	2.65
134	121.00	189.00	0.25	15.43	8.00	0.25	1.98	83.41	3.35
156	115.00	195.00	0.21	0.00	8.00	0.21	1.71	85.12	3.42
180	110.00	200.00	0.16	0.00	8.50	0.16	1.37	86.49	3.48
205	104.00	206.00	0.05	0.00	2.00	0.05	0.09	86.58	3.48
233	99.00	211.00	0.12	15.97	9.50	0.12	1.15	103.70	4.17

Table A.3.17 Raw data for 2.505 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (9th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.60	5.40	0.41	0.23	5.07	0
41	5.00	1.97	0.55	0.73	4.33	0
65	6.29	1.80	0.77	1.26	3.71	0
87	6.50	1.69	2.12	1.54	0.69	0.91
110	6.50	1.57	3.40	2.25	0	0.75
134	6.60	1.19	3.77	2.18	0	0.76
156	6.43	0.56	3.17	2.27	0	0.46
180	6.56	0.56	3.28	2.94	0	0.44
205	6.56	0.56	3.89	2.66	0	0.67
233	6.56	0.56	3.54	2.97	0	0.62

Table A.3.18 Hydrogen production data for 2.505 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (9th experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	146.00	164.00	0.11	18.34	17.00	0.11	1.90	20.24	0.81
41	141.00	169.00	0.31	33.72	62.00	0.31	19.10	73.05	2.94
65	135.00	175.00	0.36	11.70	39.00	0.36	14.21	98.96	3.98
87	129.00	181.00	0.40	8.27	27.00	0.40	10.74	117.97	4.74
110	124.00	186.00	0.36	0.00	22.00	0.36	7.81	125.78	5.06
134	119.00	191.00	0.32	0.00	9.00	0.32	2.91	128.68	5.18
156	113.00	197.00	0.25	0.00	8.00	0.25	1.99	130.68	5.26
180	107.00	203.00	0.26	4.54	6.00	0.26	1.58	136.80	5.50
205	101.00	209.00	0.22	0.00	20.00	0.22	4.49	141.30	5.68
233	96.00	214.00	0.21	0.00	17.00	0.21	3.57	144.87	5.83

Table A.3.19 Raw data for 2.505 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (10th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.70	7.42	0.38	0.16	5.93	0.07
41	4.93	1.69	0.50	0.45	5.04	0
65	6.24	1.00	0.75	0.87	5.85	0
87	6.57	0.77	2.51	1.21	1.25	0.27
110	6.60	0.53	4.12	1.68	0	0.67
134	6.60	0.56	4.22	1.64	0	0.81
156	6.57	0.56	4.38	1.94	0	0.85
180	6.60	0.56	2.80	2.47	0	0.75
205	6.60	0.56	3.76	2.35	0	0.96
233	6.58	0.56	3.40	2.64	0	0.96

Table A.3.20 Hydrogen production data for 2.505 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (10th experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	145.00	165.00	0.08	12.62	52.00	0.08	3.98	16.60	0.67
41	141.00	169.00	0.23	26.30	47.00	0.23	10.82	53.72	2.16
65	136.00	174.00	0.32	16.27	32.00	0.32	10.15	80.15	3.22
87	130.00	180.00	0.37	11.71	32.00	0.37	11.89	103.75	4.17
110	125.00	185.00	0.33	0.00	17.00	0.33	5.55	109.30	4.40
134	120.00	190.00	0.29	0.00	10.00	0.29	2.88	112.18	4.51
156	114.00	196.00	0.27	0.00	8.00	0.27	2.18	114.36	4.60
180	109.00	201.00	0.28	3.27	29.00	0.28	8.17	125.79	5.06
205	103.00	207.00	0.20	0.00	22.00	0.20	4.31	130.10	5.23
233	98.00	212.00	0.23	8.12	19.00	0.23	4.36	142.58	5.73

Table A.3.21 Raw data for 2.505 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (11th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.68	7.27	0.38	0.16	5.10	0
41	5.17	0.68	0.52	0.44	5.95	0
65	6.39	0.64	1.00	0.73	4.43	0.50
87	6.60	0.60	2.68	1.06	0.15	1.93
110	6.70	0.58	4.36	1.53	0	0.63
134	6.60	0.58	4.57	1.48	0	0.66
156	6.60	0.56	5.30	1.74	0	0.99
180	6.60	0.56	3.27	2.32	0	0.36
205	6.36	0.56	4.47	1.82	0	0.72
233	6.60	0.56	4.39	2.34	0	0.53

Table A.3.22 Hydrogen production data for 2.505 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (11th experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	146.00	164.00	0.09	14.55	52.00	0.09	4.61	19.16	0.77
41	141.00	169.00	0.23	24.04	47.00	0.23	10.73	53.93	2.17
65	135.00	175.00	0.32	16.89	27.00	0.32	8.56	79.38	3.19
87	129.00	181.00	0.36	10.58	32.00	0.36	11.68	101.64	4.09
110	124.00	186.00	0.30	0.00	7.00	0.30	2.13	103.77	4.17
134	119.00	191.00	0.27	0.00	9.00	0.27	2.46	106.24	4.27
156	113.00	197.00	0.16	0.00	7.00	0.16	1.12	107.36	4.32
180	108.00	202.00	0.24	16.68	31.50	0.24	7.53	131.57	5.29
205	102.00	208.00	0.20	0.00	20.00	0.20	4.03	135.59	5.45
233	97.00	213.00	0.19	0.00	20.00	0.19	3.87	139.46	5.61

Table A.3.23 Raw data for 2.505 g/L of NaCl, 200 mg/L of Na₂S₂O₃, and no minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (12th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.50	3.85	0.69	0.36	5.84	0
41	5.80	0.86	0.60	1.59	4.34	0.11
65	6.49	0.85	1.37	1.58	0.33	1.61
87	6.50	0.81	2.16	1.92	0	1.92
110	6.50	0.68	3.15	2.49	0	0.31
134	6.60	0.56	3.03	2.43	0	0.17
156	6.58	0.56	3.28	2.58	0	0.12
180	6.60	0.56	2.42	3.55	0	0.97
205	6.56	0.56	3.09	3.31	0	0.07
233	6.52	0.56	2.17	4.17	0	0.85

Table A.3.24 Hydrogen production data for 2.505 g/L of NaCl, 200 mg/L of Na₂S₂O₃, and no minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (12th experimental vessel)

Time (hour)	V _i (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	147.00	163.00	0.24	38.39	114.00	0.24	26.85	65.23	2.62
41	142.00	168.00	0.42	31.77	74.00	0.42	30.90	127.91	5.14
65	136.00	174.00	0.39	0.00	27.00	0.39	10.51	138.41	5.57
87	130.00	180.00	0.38	0.28	12.00	0.38	4.53	143.23	5.76
110	124.00	186.00	0.38	2.58	12.00	0.38	4.55	150.37	6.05
134	118.00	192.00	0.36	0.00	9.00	0.36	3.23	153.60	6.18
156	112.00	198.00	0.35	0.00	7.00	0.35	2.43	156.03	6.28
180	107.00	203.00	0.34	1.00	33.00	0.34	11.33	168.36	6.77
205	101.00	209.00	0.28	0.00	20.00	0.28	5.63	173.99	7.00
233	96.00	214.00	0.27	0.00	18.00	0.27	4.85	178.84	7.19

Table A.3.25 Raw data for 2.505 g/L of NaCl, 200 mg/L of Na₂S₂O₃, and 0.8 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (13th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.65	7.96	0.41	0.21	5.80	0
41	5.00	1.62	0.49	0.70	5.33	0
65	6.32	1.49	0.78	1.02	4.69	0.30
87	6.30	1.22	2.60	1.59	0	2.21
110	6.50	0.68	3.30	1.80	0	1.36
134	6.50	0.56	3.58	1.89	0	1.51
156	6.39	0.56	3.67	2.60	0	0.62
180	6.60	0.56	3.13	2.24	0	0.41
205	6.60	0.56	4.19	2.87	0	0.90
233	6.60	0.56	3.85	2.93	0	0.43

Table A.3.26 Hydrogen production data for 2.505 g/L of NaCl, 200 mg/L of Na₂S₂O₃, and 0.8 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (13th experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	147.00	163.00	0.11	17.80	29.00	0.11	3.17	20.97	0.84
41	142.00	168.00	0.31	34.82	72.00	0.31	22.55	78.33	3.15
65	136.00	174.00	0.35	8.21	27.00	0.35	9.44	95.99	3.86
87	130.00	180.00	0.31	0.00	17.00	0.31	5.21	101.20	4.07
110	125.00	185.00	0.26	0.00	7.00	0.26	1.81	103.01	4.14
134	119.00	191.00	0.23	0.00	6.00	0.23	1.38	104.39	4.20
156	113.00	197.00	0.20	0.00	18.00	0.20	3.60	107.98	4.34
180	108.00	202.00	0.16	0.00	26.00	0.16	4.12	112.10	4.51
205	102.00	208.00	0.13	0.00	22.00	0.13	2.91	115.01	4.63
233	97.00	213.00	0.11	0.00	19.00	0.11	2.18	117.18	4.71

Table A.3.27 Raw data for 5 g/L of NaCl, no Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (14th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.68	7.50	0.39	0.18	5.02	0.07
41	5.10	1.70	0.50	0.58	6.39	
65	6.40	0.97	0.85	0.95	5.17	0.12
87	6.50	0.72	2.57	1.20	1.71	1.09
110	6.50	0.58	3.92	1.52	0	0.66
134	6.43	0.56	4.26	1.54	0	0.79
156	6.47	0.56	4.26	1.87	0	0.81
180	6.52	0.56	3.62	1.81	0	0.87
205	6.49	0.56	4.24	2.28	0	0.70
233	6.43	0.56	1.67	4.64	0	0.84

Table A.3.28 Hydrogen production data for 5 g/L of NaCl, no Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (14th experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	146.00	164.00	0.08	13.84	42.00	0.08	3.54	17.39	0.70
41	141.00	169.00	0.27	32.55	62.00	0.27	17.02	66.95	2.69
65	136.00	174.00	0.34	13.17	24.50	0.34	8.39	88.51	3.56
87	131.00	179.00	0.36	4.77	24.00	0.36	8.63	101.91	4.10
110	126.00	184.00	0.30	0.00	15.00	0.30	4.56	106.47	4.28
134	120.00	190.00	0.26	0.00	9.00	0.26	2.37	108.83	4.38
156	114.00	196.00	0.24	0.00	18.00	0.24	4.28	113.11	4.55
180	109.00	201.00	0.15	0.00	18.00	0.15	2.72	115.83	4.66
205	103.00	207.00	0.19	9.09	17.50	0.19	3.33	128.25	5.16
233	98.00	212.00	0.18	0.00	17.00	0.18	3.13	131.39	5.28

Table A.3.29 Raw data for 5 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and no minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (15th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.72	7.89	0.49	0.30	5.28	0
41	5.69	0.90	0.60	1.49	4.65	0
65	6.46	0.88	1.08	1.60	4.58	0
87	6.42	0.76	1.89	1.79	1.66	0.79
110	6.30	0.71	2.89	1.91	0	1.48
134	6.60	0.56	3.72	2.26	0	0.96
156	6.50	0.56	3.51	2.28	0	0.83
180	6.50	0.56	2.38	2.30	0	1.52
205	6.50	0.56	4.07	2.97	0	0.23
233	6.50	0.56	3.45	2.81	0	0.83

Table A.3.30 Hydrogen production data for 5 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and no minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (15th experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	148.00	162.00	0.12	18.92	10.00	0.12	1.17	20.09	0.81
41	145.00	165.00	0.04	0.00	12.00	0.04	0.45	20.54	0.83
65	139.00	171.00	0.06	4.31	10.00	0.06	0.62	25.47	1.02
87	133.00	177.00	0.07	2.12	9.00	0.07	0.64	28.23	1.14
110	127.00	183.00	0.08	1.87	8.00	0.08	0.63	30.74	1.24
134	121.00	189.00	0.06	0.00	5.00	0.06	0.28	31.02	1.25
156	115.00	195.00	0.04	0.00	12.00	0.04	0.44	31.46	1.27
180	110.00	200.00	0.04	0.26	12.00	0.04	0.45	32.17	1.29
205	104.00	206.00	0.04	0.22	12.00	0.04	0.45	32.85	1.32
233	99.00	211.00	0.04	0.19	10.00	0.04	0.37	33.41	1.34

Table A.3.31 Raw data for 5 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.8 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (16th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.69	5.79	0.38	0.18	5.94	0
41	5.40	2.35	0.48	0.82	6.05	0
65	6.48	1.02	0.82	1.27	6.08	0
87	6.44	0.97	2.23	1.33	1.25	0
110	6.46	0.83	4.05	1.83	0	0.64
134	6.48	0.81	4.69	2.13	0	0.89
156	6.50	0.56	3.60	1.98	0	0.64
180	6.50	0.56	2.49	2.25	0	0.50
205	6.50	0.56	2.46	3.05	0	0.79
233	6.50	0.56	3.88	1.61	0	0.50

Table A.3.32 Hydrogen production data for 5 g/L of NaCl, 100 mg/L of Na₂S₂O₃, and 0.8 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (16th experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	147.00	163.00	0.07	11.88	9.00	0.07	0.66	12.54	0.50
41	144.00	166.00	0.32	41.92	12.00	0.32	3.89	58.35	2.35
65	138.00	172.00	0.35	6.54	22.00	0.35	7.72	72.60	2.92
87	132.00	178.00	0.33	0.00	12.00	0.33	3.96	76.56	3.08
110	126.00	184.00	0.27	0.00	8.00	0.27	2.12	78.68	3.16
134	120.00	190.00	0.21	0.00	9.50	0.21	2.03	80.71	3.25
156	114.00	196.00	0.11	0.00	7.00	0.11	0.78	81.49	3.28
180	109.00	201.00	0.13	3.99	9.50	0.13	1.22	86.70	3.49
205	103.00	207.00	0.12	0.00	10.50	0.12	1.30	88.00	3.54
233	98.00	212.00	0.11	0.00	11.00	0.11	1.26	89.25	3.59

Table A.3.33 Raw data for 5 g/L of NaCl, 200 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (17th experimental vessel)

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	10.00	0	0	0	0
16	4.66	7.13	0.38	0.17	6.10	0
41	4.80	1.22	0.53	0.51	5.56	0
65	6.36	1.14	0.75	1.02	5.14	0
87	6.60	1.03	2.11	1.18	1.19	1.34
110	6.50	0.71	4.02	1.77	0	0.88
134	6.60	0.56	4.14	1.70	0	0.69
156	6.60	0.56	3.80	1.78	0	1.46
180	6.60	0.56	2.70	1.76	0	1.35
205	6.60	0.56	4.15	1.77	0	0.93
233	6.60	0.56	4.06	1.54	0	0.68

Table A.3.34 Hydrogen production data for 5 g/L of NaCl, 200 mg/L of Na₂S₂O₃, and 0.4 mM of minerals (Ni, Fe, Cu, Zn) with *Clostridium acetobutylicum* experiment (17th experimental vessel)

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total gas} (mL)	H ₂ -1 (%)	V _{t x} H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
16	146.00	164.00	0.08	13.30	12.00	0.08	0.97	14.27	0.57
41	141.00	169.00	0.27	31.87	72.00	0.27	19.25	65.39	2.63
65	135.00	175.00	0.31	9.01	18.00	0.31	5.57	79.97	3.22
87	129.00	181.00	0.36	10.27	22.00	0.36	7.83	98.08	3.94
110	124.00	186.00	0.33	0.00	10.00	0.33	3.29	101.37	4.08
134	118.00	192.00	0.29	0.00	6.00	0.29	1.75	103.12	4.15
156	112.00	198.00	0.24	0.00	25.00	0.24	6.06	109.18	4.39
180	107.00	203.00	0.21	0.00	8.50	0.21	1.78	110.96	4.46
205	102.00	208.00	0.19	0.00	18.00	0.19	3.41	114.37	4.60
233	97.00	213.00	0.17	0.00	9.50	0.17	1.63	116.00	4.67



Appendix-4: Raw data for CaCO₃ (0-4 g/L) Concentrations at 20 g/L Cheese Whey Powder (CWP) with *Clostridium acetobutylicum* Experiment.

Table A.4.1 Raw data for no CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	14.75	0	0	0	0
17	4.87	7.08	0.05	1.38	0.62	0
41	5.00	1.46	0.06	0.30	0.81	0
67	6.30	1.23	0.12	0.78	0.47	0
89	6.75	0.97	0.17	0.25	0	0.14
113	6.49	0.87	0.31	0.47	0	0.21
136	6.50	0.86	0.30	0.25	0	0
162	6.63	0.79	0.32	0.27	0	0.20

184	6.73	0.74	0.36	0.34	0	0.23
208	6.79	0.71	0.29	0.29	0	0.17
233	6.79	0.68	0.24	0	0	0.16

Table A.4.2 Hydrogen production data for no CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
17	142.00	168.00	0.05	9.04	35.00	0.05	1.88	10.92	0.44
41	138.00	172.00	0.18	21.49	32.50	0.18	5.77	38.18	1.54
67	132.00	178.00	0.27	16.92	32.50	0.27	8.66	63.77	2.56
89	126.00	184.00	0.28	3.62	32.50	0.28	9.02	76.42	3.07
113	120.00	190.00	0.24	0.00	17.50	0.24	4.20	80.62	3.24
136	113.00	197.00	0.22	0.00	6.00	0.22	1.31	81.93	3.30
162	107.00	203.00	0.18	0.00	7.00	0.18	1.25	83.18	3.35
184	101.00	209.00	0.19	2.74	7.00	0.19	1.30	87.22	3.51
208	94.00	216.00	0.17	0.00	8.00	0.17	1.37	88.59	3.56
233	87.00	223.00	0.12	0.00	12.50	0.12	1.44	90.03	3.62



Table A.4.3 Raw data for 0.1 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	14.75	0	0	0	0
17	4.86	6.15	0.05	0.02	0.63	0
41	5.00	2.04	0.07	0.07	0.76	0
67	5.60	1.75	0.15	0.22	0.22	0.08
89	6.59	1.51	0.20	0.23	0	0.13
113	6.60	1.33	0.31	0.33	0	0.18
136	6.70	1.12	0.31	0.29	0	0.16
162	6.93	1.05	0.31	2.04	0	0.16
184	5.93	1.04	0.31	2.42	0	0.15
208	6.90	0.99	0.31	0.45	0	0.17
233	7.80	0.66	0.30	0.36	0	0.17

Table A.4.4 Hydrogen production data for 0.1 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total} gas (mL)	H ₂ -1 (%)	V _t x H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
17	143.00	167.00	0.06	9.75	42.50	0.06	2.48	12.23	0.49
41	139.00	171.00	0.24	30.55	42.50	0.24	10.02	52.80	2.12
67	133.00	177.00	0.29	11.89	42.50	0.29	12.53	77.23	3.11
89	127.00	183.00	0.31	5.19	22.50	0.31	7.06	89.48	3.60
113	121.00	189.00	0.27	0.00	12.50	0.27	3.42	92.90	3.74
136	115.00	195.00	0.05	0.00	7.00	0.05	0.36	93.26	3.75
162	109.00	201.00	0.23	36.39	8.00	0.23	1.85	131.49	5.29
184	103.00	207.00	0.21	0.00	12.00	0.21	2.51	134.00	5.39
208	95.00	215.00	0.20	0.77	8.00	0.20	1.64	136.41	5.49
233	89.00	221.00	0.20	0.23	8.00	0.20	1.60	138.25	5.56

Table A.4.5 Raw data for 0.5 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	14.75	0	0	0	0
17	5.10	2.99	0.06	0.03	0.79	0
41	5.70	1.64	0.06	0.08	0.84	0
67	6.30	1.00	0.18	0.20	0.34	0.11
89	6.50	0.76	0.22	0.18	0.10	0.17
113	6.6	0.65	0.40	0.30	0	0.26
136	7.13	0.57	0.41	0.29	0	0.25
162	6.63	0.65	0.39	0.49	0	0.24
184	6.53	0.79	0.42	0.45	0	0.24
208	6.89	0.53	0.40	0.44	0	0.24
233	4.70	0.53	0.35	0.17	0	3.32

Table A.4.6 Hydrogen production data for 0.5 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total} gas (mL)	H ₂ -1 (%)	V _t x H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
17	143.00	167.00	0.11	18.27	9.00	0.11	0.98	19.25	0.77
41	138.00	172.00	0.22	19.72	12.50	0.22	2.76	41.74	1.68
67	132.00	178.00	0.23	2.61	10.00	0.23	2.28	46.63	1.88
89	126.00	184.00	0.24	3.69	12.50	0.24	3.01	53.33	2.14
113	120.00	190.00	0.24	1.90	12.50	0.24	3.04	58.26	2.34
136	114.00	196.00	0.04	0.00	7.00	0.04	0.26	58.52	2.35
162	108.00	202.00	0.16	25.23	8.00	0.16	1.28	85.03	3.42
184	102.00	208.00	0.15	0.00	10.00	0.15	1.45	86.48	3.48
208	96.00	214.00	0.05	0.00	7.00	0.05	0.34	86.82	3.49
233	91.00	219.00	0.10	12.53	12.50	0.10	1.30	100.65	4.05

Table A.4.7 Raw data for 1 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	14.75	0	0	0	0
17	5.90	3.42	0.07	0.02	0.95	0
41	6.40	1.95	0.12	0.09	0.57	0.09
67	6.10	1.70	0.27	0.18	0	0.22
89	6.90	1.52	0.30	0.19	0	0.23
113	6.60	1.38	0.42	0.30	0	0.28
136	7.93	1.18	0.40	0.39	0	0.26
162	7.13	1.02	0.40	0.42	0	0.25
184	7.83	0.91	0.41	0.42	0	0.26
208	6.69	0.83	0.40	0.38	0	0.25
233	7.19	0.65	0.40	0.39	0	0.24

Table A.4.8 Hydrogen production data for 1 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total gas} (mL)	H ₂ -1 (%)	V _{t x} H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
17	144.00	166.00	0.05	7.47	6.00	0.05	0.27	7.74	0.31
41	139.00	171.00	0.17	20.78	12.50	0.17	2.07	30.58	1.23
67	133.00	177.00	0.18	2.99	10.00	0.18	1.77	35.34	1.42
89	127.00	183.00	0.16	-2.27	12.50	0.16	1.98	35.05	1.41
113	121.00	189.00	0.12	0.00	12.50	0.12	1.56	36.61	1.47
136	115.00	195.00	0.11	0.00	9.00	0.11	0.96	37.57	1.51
162	109.00	201.00	0.08	0.00	8.00	0.08	0.67	38.24	1.54
184	102.00	208.00	0.05	0.00	15.00	0.05	0.75	38.99	1.57
208	95.50	214.50	0.05	0.00	7.00	0.05	0.34	39.33	1.58
233	90.50	219.50	0.04	0.00	10.00	0.04	0.40	39.73	1.60

Table A.4.9 Raw data for 2 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	14.75	0	0	0	0
17	6.00	2.04	0.06	0.02	0.81	0
41	6.50	1.95	0.12	0.11	0.53	0.08
67	6.70	1.90	0.27	0.19	0	0.20
89	6.80	1.46	0.30	0.19	0	0.21
113	6.70	1.78	0.43	0.32	0	0.27
136	8.03	1.64	0.41	0.40	0	0.24
162	7.13	0.97	0.43	0.43	0	0.25
184	6.93	0.66	0.41	0.39	0	0.23
208	6.69	0.63	0.42	0.39	0	0.24
233	7.19	0.70	0.40	0.40	0	0.23

Table A.4.10 Hydrogen production data for 2 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total} gas (mL)	H ₂ -1 (%)	V _t x H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
17	144.00	166.00	0.09	14.77	12.50	0.09	1.11	15.89	0.64
41	139.00	171.00	0.16	13.00	12.50	0.16	2.03	30.91	1.24
67	134.00	176.00	0.10	0.00	10.00	0.10	0.95	31.87	1.28
89	128.00	182.00	0.04	0.00	11.00	0.04	0.41	32.28	1.30
113	121.00	189.00	0.04	0.02	12.50	0.04	0.45	32.75	1.32
136	116.00	194.00	0.04	0.20	8.00	0.04	0.29	33.24	1.34
162	109.00	201.00	0.04	0.44	8.00	0.04	0.30	33.97	1.37
184	102.00	208.00	0.04	0.00	12.00	0.04	0.42	34.39	1.38
208	95.00	215.00	0.03	0.03	9.00	0.03	0.31	34.73	1.40
233	90.00	220.00	0.03	0.35	10.00	0.03	0.35	35.42	1.42

Table A.4.11 Raw data for 3 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	14.75	0	0	0	0
17	6.00	3.10	0.07	0.02	0.93	0
41	6.50	1.91	0.10	0.12	0.54	0.07
67	6.60	1.86	0.25	0.28	0	0.19
89	6.60	1.77	0.28	0.18	0	0.19
113	6.60	1.41	0.40	0.29	0	0.23
136	7.43	1.25	0.37	0.47	0	0.22
162	7.13	1.17	0.35	0.21	0	0.22
184	6.53	0.87	0.37	0.41	0	0.21
208	8.09	0.70	0.38	0.47	0	0.23
233	8.09	0.63	0.39	0.50	0	0.22

Table A.4.12 Hydrogen production data for 3 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
17	143.00	167.00	0.09	14.86	92.50	0.09	8.23	23.10	0.93
41	138.00	172.00	0.13	7.88	52.50	0.13	6.94	37.91	1.52
67	133.00	177.00	0.17	6.70	12.50	0.17	2.08	46.69	1.88
89	127.00	183.00	0.13	0.00	12.50	0.13	1.66	48.34	1.94
113	120.00	190.00	0.10	0.00	12.50	0.10	1.24	49.58	1.99
136	114.00	196.00	0.08	0.00	6.00	0.08	0.49	50.07	2.01
162	108.00	202.00	0.07	0.00	8.00	0.07	0.58	50.66	2.04
184	102.00	208.00	0.07	0.00	12.50	0.07	0.84	51.50	2.07
208	96.00	214.00	0.03	0.00	10.00	0.03	0.34	51.84	2.08
233	91.00	219.00	0.06	4.92	26.00	0.06	1.45	58.21	2.34

Table A.4.13 Raw data for 4 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	14.75	0	0	0	0
17	6.10	2.21	0.07	0.02	0.91	0
41	6.50	1.88		0.11	0.24	0
67	6.60	1.75	0.22	0.18	0	0
89	6.70	1.30	0.28	0.19	0	0.21
113	6.70	1.22	0.39	0.30	0	0.24
136	6.73	1.22	0.38	0.42	0	0.23
162	6.73	1.02		0.20	0	0.23
184	6.53	0.81	0.39	0.38	0	0.25
208	7.19	0.76	0.40	0.41	0	0.24
233	8.09	0.70	0.39	0.42	0	0.23

Table A.4.14 Hydrogen production data for 4 g/L of CaCO₃ concentrations at 20 g/L CWP with *Clostridium acetobutylicum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
17	143.00	167.00	0.05	8.70	112.50	0.05	5.86	14.56	0.59
41	138.00	172.00	0.14	16.22	52.50	0.14	7.61	38.39	1.54
67	133.00	177.00	0.15	2.21	22.50	0.15	3.45	44.05	1.77
89	128.00	182.00	0.12	0.00	12.50	0.12	1.55	45.60	1.83
113	123.00	187.00	0.10	0.00	9.00	0.10	0.87	46.47	1.87
136	118.00	192.00	0.06	0.00	6.00	0.06	0.38	46.84	1.88
162	111.50	198.50	0.08	3.05	8.00	0.08	0.61	50.50	2.03
184	105.00	205.00	0.07	0.00	10.00	0.07	0.68	51.17	2.06
208	98.50	211.50	0.04	0.00	8.00	0.04	0.33	51.51	2.07
233	92.00	218.00	0.06	3.24	7.00	0.06	0.39	55.13	2.22

Appendix-5: Raw data for four different microorganisms (anaerobic sludge, *Clostridium acetobutylicum*, *C. beijerinckii*, *C. pasteurianum*) at 25 g/L Cheese Whey Powder (CWP) Experiments.

Table A.5.1 Raw data for anaerobic sludge at 25 g/L CWP experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	20.00	0	0	0	0
19	4.97	7.25	0.77	1.82	8.23	0
47	5.60	3.14	0.36	1.31	3.76	0
67	6.15	1.93	0.37	1.20	3.43	0
91	6.48	1.88	1.45	2.47	6.89	0
116	6.47	1.47	2.30	2.52	4.54	0
140	6.50	1.21	2.74	2.63	4.97	0
188	6.40	0.97	4.27	2.94	0	2.66

216	6.30	0.91	4.61	3.19	0	2.80
239	6.40	0.78	4.47	3.22	0	2.82
263	6.40	0.65	4.54	3.12	0	2.83

Table A.5.2 Hydrogen production data for anaerobic sludge at 25 g/L CWP experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
19	143.00	167.00	0.17	29.02	60.00	0.17	10.43	39.45	1.59
47	136.00	174.00	0.37	34.71	112.50	0.37	41.21	115.37	4.64
67	129.00	181.00	0.38	4.21	12.50	0.38	4.69	124.28	5.00
91	122.00	188.00	0.38	3.64	5.00	0.38	1.90	129.82	5.22
116	116.00	194.00	0.35	0.00	11.50	0.35	4.02	133.84	5.38
140	109.00	201.00	0.35	3.27	12.50	0.35	4.42	141.54	5.69
188	99.00	211.00	0.34	0.12	22.00	0.34	7.43	149.09	6.00
216	93.00	217.00	0.35	4.48	32.50	0.35	11.34	164.91	6.63
239	87.00	223.00	0.32	0.00	11.00	0.32	3.53	168.44	6.77
263	80.00	230.00	0.29	0.00	6.50	0.29	1.86	170.30	6.85

Table A.5.3 Raw data for *C. acetobutylicum* at 25 g/L CWP experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	20.00	0	0	0	0
19	4.80	9.74	0.33	0.63	11.91	0
47	5.20	3.71	0	0.36	6.14	0
67	6.37	3.09	0.37	0.89	6.18	0
91	6.60	1.65	1.35	2.07	9.00	0
116	6.60	1.17	2.69	2.13	8.72	0
140	6.60	1.11	2.40	1.99	9.30	0
188	6.60	1.08	3.04	2.42	9.13	0
216	6.60	0.82	3.29	2.51	8.43	0
239	6.70	0.79	3.46	2.40	7.89	0
263	6.70	0.75	2.49	2.52	8.86	0

Table A.5.4 Hydrogen production data for *C. acetobutylicum* at 25 g/L CWP experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total} gas (mL)	H ₂ -1 (%)	V _t x H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
19	143.00	167.00	0.06	10.24	30.00	0.06	1.84	12.08	0.49
47	136.00	174.00	0.12	10.82	32.50	0.12	3.93	26.83	1.08
67	129.50	180.50	0.19	13.17	17.50	0.19	3.32	43.31	1.74
91	122.50	187.50	0.21	5.20	10.00	0.21	2.10	50.61	2.04
116	115.50	194.50	0.19	0.00	10.00	0.19	1.86	52.47	2.11
140	109.00	201.00	0.18	0.00	9.50	0.18	1.69	54.16	2.18
188	99.00	211.00	0.06	0.00	8.00	0.06	0.45	54.61	2.20
216	94.00	216.00	0.13	16.34	8.00	0.13	1.04	71.98	2.90
239	88.00	222.00	0.13	0.51	5.00	0.13	0.65	73.14	2.94
263	81.00	229.00	0.12	0.00	8.00	0.12	0.95	74.09	2.98

Table A.5.5 Raw data for *C. beijerinckii* at 25 g/L CWP experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	20.00	0	0	0	0
19	5.20	8.78	0.95	1.15	5.88	0
47	5.50	6.49	0.66	0.74	3.32	0
67	6.40	1.84	0.79	1.02	3.96	0
91	6.70	1.76	2.10	2.29	7.00	0
116	6.70	1.60	2.89	2.79	5.79	0
140	6.70	1.47	2.87	2.72	5.72	0
188	6.70	1.30	3.35	1.62	5.91	0
216	6.70	1.17	3.19	1.17	4.15	0
239	6.70	1.02	3.34	1.32	5.45	0
263	6.70	0.56	3.26	1.42	4.35	0

Table A.5.6 Hydrogen production data for *C. beijerinckii* at 25 g/L CWP experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total gas} (mL)	H ₂ -1 (%)	V _{t x} H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
19	143.00	167.00	0.27	45.04	112.50	0.27	30.34	75.38	3.03
47	136.00	174.00	0.18	0.00	112.50	0.18	20.60	95.98	3.86
67	130.00	180.00	0.35	31.34	10.00	0.35	3.51	130.83	5.26
91	123.50	186.50	0.35	2.51	8.00	0.35	2.82	136.15	5.48
116	116.50	193.50	0.34	0.07	6.00	0.34	2.04	138.26	5.56
140	109.50	200.50	0.32	0.00	9.00	0.32	2.86	141.12	5.68
188	99.50	210.50	0.28	0.00	8.00	0.28	2.21	143.33	5.76
216	93.50	216.50	0.28	3.52	10.00	0.28	2.85	149.70	6.02
239	87.50	222.50	0.24	0.00	9.50	0.24	2.26	151.96	6.11
263	80.00	230.00	0.25	4.94	12.50	0.25	3.15	160.05	6.44



Table A.5.7 Raw data for *C. pasteurianum* at 25 g/L CWP experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	20.00	0	0	0	0
19	5.00	10.03	1.56	3.23	1.02	0.91
47	5.30	3.22	0.98	2.13	0.36	0.48
67	5.70	2.45	1.01	2.79	0.17	0
91	6.48	2.13	2.41	5.96	0	0
116	6.46	1.95	3.29	6.80	0	0
140	6.44	1.61	3.72	6.42	0	0
188	6.40	1.47	3.75	6.05	0	0
216	6.40	1.09	3.68	5.69	0	0
239	6.42	1.09	3.57	5.42	0	0
263	6.49	0.67	3.57	5.71	0	0



Table A.5.8 Hydrogen production data for *C. pasteurianum* at 25 g/L CWP experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	Vtotal gas (mL)	H ₂ -1 (%)	Vt x H ₂ -1	Vcum. (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
19	144.00	166.00	0.44	72.77	175.00	0.44	76.72	149.49	6.01
47	137.00	173.00	0.53	19.16	198.00	0.53	105.22	273.87	11.01
67	131.00	179.00	0.49	0.00	32.50	0.49	15.92	289.79	11.66
91	124.00	186.00	0.51	7.13	10.00	0.51	5.10	302.02	12.15
116	117.00	193.00	0.41	0.00	12.50	0.41	5.15	307.17	12.35
140	110.00	200.00	0.40	0.20	12.00	0.40	4.78	312.15	12.55
188	100.00	210.00	0.39	1.93	10.00	0.39	3.89	317.96	12.79
216	94.00	216.00	0.41	7.62	5.00	0.41	2.07	327.65	13.18
239	87.50	222.50	0.39	0.00	10.00	0.39	3.86	331.52	13.33
263	78.50	231.50	0.32	0.00	8.00	0.32	2.56	334.08	13.44

Appendix-6: Raw data for Organism (biomass) Concentrations (0.5-3 g/L) with *Clostridium pasterianum* at 45 g/L Cheese Whey Powder (CWP) Experiments.

Table A.6.1 Raw data for 0.5 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	43.38	0	0	0	0
20	5.00	20.51	0.03	0.09	0.67	0
44	5.30	15.77	0.08	0.19	0.79	0
68	5.70	13.44	0.22	0.31	0.94	0
118	6.48	8.31	0.21	0.48	1.49	0
139	6.46	7.76	0.43	0.97	3.58	0

Table A.6.2 Hydrogen production data for 0.5 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	Vtotal gas (mL)	H₂-1 (%)	Vt x H₂-1	Vcum. (mL)	Cumulative H₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
19	142.50	167.50	0.17	28.56	52.50	0.17	8.95	37.52	1.51
47	136.00	174.00	0.45	49.37	112.50	0.45	50.39	137.28	5.52
67	129.50	180.50	0.56	22.35	122.50	0.56	68.06	227.69	9.16
91	122.50	187.50	0.52	0.00	125.50	0.52	64.98	292.67	11.77
116	116.00	194.00	0.54	7.01	42.50	0.54	22.81	322.49	12.97
140	109.00	201.00	0.57	10.17	32.50	0.57	18.48	351.14	14.12
188	102.00	208.00	0.60	10.26	10.00	0.60	5.99	367.38	14.78
216	95.00	215.00	0.49	0.00	17.50	0.49	8.65	376.03	15.12
239	88.00	222.00	0.52	8.74	10.00	0.52	5.18	389.95	15.68
263	81.50	228.50	0.45	0.00	12.50	0.45	5.66	395.60	15.91

Table A.6.3 Raw data for 1 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	43.38	0	0	0	0
20	5.50	22.14	0.03	0.10	0.59	0
44	4.97	18.14	0.09	0.22	0.73	0
68	5.11	15.37	0.18	0.32	0.81	0
118	5.26	6.73	0.20	0.45	1.38	0
139	6.105	4.50	0.38	0.99	3.09	0

Table A.6.4 Hydrogen production data for 1 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	V_l(mL)	V_g (mL)	H₂-1 (%)	VH₂-head space (mL)	Vtotal gas (mL)	H₂-1 (%)	Vt x H₂-1	Vcum. (mL)	Cumulative H₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
19	142.00	168.00	0.20	33.60	115.50	0.20	23.10	56.70	2.28
47	135.50	174.50	0.47	47.66	112.50	0.47	52.39	156.76	6.30
67	128.50	181.50	0.55	19.34	152.50	0.55	84.53	260.63	10.48
91	121.00	189.00	0.52	0.00	52.50	0.52	27.04	287.66	11.57

116	114.50	195.50	0.52	3.80	21.50	0.52	11.12	302.58	12.17
140	108.00	202.00	0.56	11.14	22.50	0.56	12.51	326.23	13.12
188	101.50	208.50	0.55	1.59	12.50	0.55	6.83	334.65	13.46
216	94.00	216.00	0.51	0.00	11.00	0.51	5.58	340.23	13.68
239	87.50	222.50	0.50	2.07	12.50	0.50	6.28	348.58	14.02
263	80.50	229.50	0.46	0.00	10.00	0.46	4.64	353.22	14.21

Table A.6.5 Raw data for 1.5 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	43.38	0	0	0	0
20	4.93	26.78	0.04	0.12	0.58	0
44	4.94	25.20	0.11	0.29	0.65	0
68	5.14	24.71	0.21	0.45	0.93	0
118	5.22	8.11	0.23	0.48	1.24	0
139	5.95	1.78	0.57	1.52	3.85	0

Table A.6.6 Hydrogen production data for 1.5 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	V _I (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total} gas (mL)	H ₂ -1 (%)	V _t x H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
19	143.50	166.50	0.28	46.19	92.50	0.28	25.66	71.85	2.89
47	137.00	173.00	0.52	43.83	95.00	0.52	49.43	165.11	6.64
67	130.50	179.50	0.56	10.68	130.00	0.56	72.93	248.72	10.00
91	124.00	186.00	0.55	1.34	135.00	0.55	74.06	324.12	13.04

116	117.00	193.00	0.06	0.00	20.50	0.06	1.26	325.38	13.09
140	110.00	200.00	0.54	96.39	22.50	0.54	12.18	433.95	17.45
188	103.50	206.50	0.51	0.00	12.50	0.51	6.33	440.28	17.71
216	96.50	213.50	0.50	2.74	6.00	0.50	3.01	446.03	17.94
239	90.00	220.00	0.49	1.33	12.50	0.49	6.17	453.53	18.24
263	83.00	227.00	0.46	0.00	10.50	0.46	4.80	458.33	18.43

Table A.6.7 Raw data for 2 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	43.38	0	0	0	0
20	4.96	34.98	0.03	0.06	0.59	0
44	4.97	17.94	0.10	0.22	0.64	0
68	5.17	12.70	0.19	0.29	0.72	0
118	5.28	11.81	0.23	0.44	1.26	0
139	5.98	3.17	0.41	0.98	2.50	0

Table A.6.8 Hydrogen production data for 2 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total} gas (mL)	H ₂ -1 (%)	V _t x H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
19	143.00	167.00	0.17	29.02	52.50	0.17	9.12	38.15	1.53
47	135.50	174.50	0.47	53.43	147.50	0.47	69.69	161.27	6.49
67	128.00	182.00	0.55	18.39	155.50	0.55	86.16	265.83	10.69
91	121.00	189.00	0.54	1.91	112.50	0.54	61.17	328.91	13.23

116	115.00	195.00	0.54	3.34	52.50	0.54	28.57	360.81	14.51
140	109.00	201.00	0.56	6.10	22.50	0.56	12.56	379.47	15.26
188	102.50	207.50	0.51	0.00	12.50	0.51	6.43	385.90	15.52
216	95.50	214.50	0.46	0.00	9.50	0.46	4.38	390.28	15.70
239	89.00	221.00	0.41	0.00	8.00	0.41	3.26	393.54	15.83
263	82.00	228.00	0.32	0.00	12.50	0.32	4.00	397.54	15.99

Table A.6.9 Raw data for 2.5 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	43.38	0	0	0	0
20	4.78	32.86	0.02	0.04	0.79	0
44	4.91	15.47	0.06	0.13	0.88	0
68	5.07	14.33	0.16	0.23	1.05	0
118	5.16	12.85	0.19	0.31	1.42	0
139	5.49	1.88	0.45	0.94	3.54	0

Table A.6.10 Hydrogen production data for 2.5 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	V _i (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total} gas (mL)	H ₂ -1 (%)	V _t x H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
19	143.50	166.50	0.42	69.18	52.50	0.42	21.81	90.99	3.66
47	136.50	173.50	0.32	0.00	99.00	0.32	32.16	123.15	4.95
67	130.00	180.00	0.45	25.28	92.50	0.45	41.95	190.38	7.66
91	122.50	187.50	0.49	10.70	72.50	0.49	35.70	236.77	9.52

116	116.00	194.00	0.50	4.36	30.00	0.50	14.95	256.09	10.30
140	109.50	200.50	0.50	3.40	12.50	0.50	6.24	265.73	10.69
188	103.00	207.00	0.45	0.00	10.50	0.45	4.74	270.46	10.88
216	96.00	214.00	0.44	1.45	8.00	0.44	3.55	275.46	11.08
239	88.50	221.50	0.42	0.00	32.50	0.42	13.65	289.10	11.63
263	81.50	228.50	0.39	0.00	12.50	0.39	4.87	293.97	11.82

Table A.6.11 Raw data for 3 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	43.38	0	0	0	0
20	4.54	34.98	0	0.04	0.92	0
44	4.91	17.94	0.06	0.08	1.04	0
68	5.01	16.16	0.13	0.17	1.35	0
118	5.05	15.17	0.13	0.25	1.80	0
139	5.43	14.43	0.27	0	4.25	0

Table A.6.12 Hydrogen production data for 3 g/L organism concentration at 45 g/L CWP experiment

Time (hour)	V _i (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total} gas (mL)	H ₂ -1 (%)	V _t x H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	150.00	160.00	0.00	0.00	0.00	0.00	0.00	0.00	0
19	143.00	167.00	0.04	6.38	52.50	0.04	2.01	8.38	0.34
47	135.50	174.50	0.27	40.67	72.50	0.27	19.55	68.60	2.76
67	129.00	181.00	0.40	24.85	72.50	0.40	28.80	122.24	4.92
91	121.50	188.50	0.36	0.00	30.00	0.36	10.86	133.10	5.35

116	114.50	195.50	0.34	0.00	20.00	0.34	6.85	139.95	5.63
140	108.00	202.00	0.40	14.57	32.50	0.40	13.11	167.63	6.74
188	101.00	209.00	0.44	11.46	12.50	0.44	5.56	184.65	7.43
216	94.00	216.00	0.39	0.00	10.50	0.39	4.10	188.74	7.59
239	88.00	222.00	0.35	0.00	12.50	0.35	4.36	193.11	7.77
263	81.00	229.00	0.30	0.00	12.50	0.30	3.79	196.90	7.92

Appendix-7: Raw data for Substrate (CWP) Concentrations with *Clostridium pasterianum* Experiments.

Table A.7.1 Raw data for 40 g/L of CWP with *Clostridium pasterianum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	45.36	0	0	0	0
21	4.90	24.12	0.12	0.23	0.20	0
46	5.09	9.69	0.21	0.44	0.24	0
67	5.74	6.48	0.29	0.53	0.06	0.15
91	5.78	5.19	0.37	0.73	0.07	0.21
115	6.13	5.10	0.46	0.76	0	0
139	6.50	3.46	0.43	0.78	0	0
163	6.37	2.82	0.45	0.79	0	0
211	6.90	2.48	0.53	0.79	0	0
235	6.50	2.08	0.54	0.79	0	0
259	6.37	1.29	0.50	0.79	0	0

Table A.7.2 Hydrogen production data for 40 g/L of CWP with *Clostridium pasterianum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total gas} (mL)	H ₂ -1 (%)	V _{t x} H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	200.00	110.00	0.00	0.00	0.00	0.00	0.00	0.00	0
21	193.00	117.00	0.33	38.29	206.00	0.33	67.42	105.72	4.25
46	186.00	124.00	0.59	34.39	192.50	0.59	112.84	252.96	10.17
67	179.00	131.00	0.61	6.64	132.50	0.61	80.24	339.84	13.67
91	172.00	138.00	0.61	4.24	157.50	0.61	95.38	439.46	17.68
115	165.00	145.00	0.59	1.80	51.50	0.59	30.32	471.59	18.97
139	160.00	150.00	0.57	0.00	22.50	0.57	12.82	484.41	19.48
163	153.00	157.00	0.56	2.51	12.50	0.56	7.00	493.93	19.87
211	145.00	165.00	0.48	0.00	7.00	0.48	3.36	497.29	20.00
235	139.00	171.00	0.43	0.00	28.00	0.43	11.91	509.20	20.48
259	133.00	177.00	0.42	2.16	10.00	0.42	4.23	515.60	20.74

Table A.7.3 Raw data for 80 g/L of CWP with *Clostridium pasterianum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	82.12	0.06	0	0	0
21	4.58	77.38	0.11	0.14	0.45	0.040
46	5.12	45.86	0.32	0.34	0.32	0
67	5.20	41.91	0.38	0.55	0.21	0
91	5.52	30.84	0.45	0.72	0.27	0
115	5.58	22.84	0.52	0.85	0.07	0
139	5.89	20.37	0.54	0.87	0.13	0
163	6.08	9.20	0.52	0.95	0.18	0
211	5.80	4.16	0.53	1.09	0.49	0
235	6.14	3.57	0.60	1.08	0.56	0
259	7.00	3.37	0.06	1.10	0.76	0

Table A.7.4 Hydrogen production data for 80 g/L of CWP with *Clostridium pasterianum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total gas} (mL)	H ₂ -1 (%)	V _{t x} H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	200.00	110.00	0.00	0.00	0.00	0.00	0.00	0.00	0
21	193.00	117.00	0.03	3.86	92.50	0.03	3.05	6.91	0.28
46	185.00	125.00	0.46	53.98	215.00	0.46	99.48	160.37	6.45
67	177.00	133.00	0.59	21.14	220.00	0.59	130.64	312.14	12.55
91	169.00	141.00	0.59	4.14	125.00	0.59	73.69	389.98	15.68
115	161.50	148.50	0.56	0.00	102.50	0.56	57.83	447.81	18.01
139	154.50	155.50	0.16	0.00	62.50	0.16	9.80	457.61	18.40
163	147.00	163.00	0.57	68.12	42.50	0.57	24.12	549.85	22.11
211	139.50	170.50	0.56	3.56	102.50	0.56	57.75	611.15	24.58
235	132.00	178.00	0.55	2.41	42.50	0.55	23.51	637.07	25.62
259	125.00	185.00	0.58	9.37	8.00	0.58	4.66	651.10	26.19

Table A.7.5 Raw data for 100 g/L of CWP with *Clostridium pasteurianum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	104.75	0	0	0	0
21	5.10	74.49	0.09	0.26	0.57	0.02
46	5.14	53.62	0.16	0.42	0.48	0
67	5.26	47.81	0.29	0.64	0.50	0
91	5.32	36.20	0.37	0.85	0.76	0
115	5.44	28.92	0.45	0.89	0.80	0
139	5.62	22.49	0.56	0.77	0.98	0
163	5.80	22.07	0.55	0.81	1.11	0
211	5.55	14.10	0.64	0.92	1.55	0
235	5.86	13.36	0.62	0.94	1.84	0
259	5.66	8.91	0.50	0.91	1.91	0

Table A.7.6 Hydrogen production data for 100 g/L of CWP with *Clostridium pasterianum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total gas} (mL)	H ₂ -1 (%)	V _{t x} H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	200.00	110.00	0.00	0.00	0.00	0.00	0.00	0.00	0
21	194.00	116.00	0.34	39.17	192.50	0.34	65.01	104.18	4.19
46	186.00	124.00	0.58	32.36	215.00	0.58	124.03	260.58	10.48
67	179.00	131.00	0.58	4.50	150.00	0.58	87.07	352.15	14.16
91	172.00	138.00	0.54	0.00	92.50	0.54	50.38	402.53	16.19
115	164.00	146.00	0.57	7.60	112.50	0.57	63.77	473.89	19.06
139	157.50	152.50	0.55	1.38	92.50	0.55	51.03	526.30	21.17
163	150.50	159.50	0.55	3.35	52.50	0.55	28.80	558.45	22.46
211	143.00	167.00	0.54	3.16	72.50	0.54	39.35	600.96	24.17
235	135.00	175.00	0.49	0.00	10.00	0.49	4.94	605.90	24.37
259	127.50	182.50	0.45	0.00	12.50	0.45	5.60	611.50	24.59

Table A.7.7 Raw data for 150 g/L of CWP with *Clostridium pasteurianum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	151.02	0	0	0	0
21	5.48	113.96	0.07	0.17	0.88	0.06
46	5.05	100.44	0.15	0.34	0.97	0
67	5.24	85.99	0.21	0.52	1.04	0
91	5.32	67.46	0.34	0.76	1.46	0
115	5.53	63.76	0.33	0.96	1.71	0
139	5.51	60.05	0.37	1.00	1.82	0
163	5.87	51.89	0.40	1.09	2.12	0
211	5.49	49.12	0.39	1.13	2.60	0
235	5.71	40.97	0.38	1.11	2.92	0
259	5.73	37.26	0.45	1.17	3.41	0

Table A.7.8 Hydrogen production data for 150 g/L of CWP with *Clostridium pasterianum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total gas} (mL)	H ₂ -1 (%)	V _{t x} H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	200.00	110.00	0.00	0.00	0.00	0.00	0.00	0.00	0
21	193.00	117.00	0.07	8.28	172.50	0.07	12.21	20.50	0.82
46	186.00	124.00	0.49	52.92	192.50	0.49	95.02	168.44	6.77
67	179.00	131.00	0.51	5.45	72.00	0.51	36.63	210.52	8.47
91	171.00	139.00	0.59	14.77	177.50	0.59	103.98	329.27	13.24
115	163.50	146.50	0.53	0.00	52.50	0.53	27.80	357.07	14.36
139	156.50	153.50	0.56	8.49	135.00	0.56	75.71	441.28	17.75
163	149.00	161.00	0.55	2.20	52.50	0.55	28.79	472.26	18.99
211	142.50	167.50	0.50	0.00	42.50	0.50	21.46	493.71	19.86
235	134.00	176.00	0.47	0.00	10.00	0.47	4.70	498.41	20.05
259	126.50	183.50	0.44	0.00	10.00	0.44	4.42	502.83	20.22

Table A.7.9 Raw data for 200 g/L of CWP with *Clostridium pasteurianum* experiment

Time (hour)	pH	Total Sugar Concentration (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Lactic Acid (g/L)	Propionic Acid (g/L)
0	7.20	202.09	0	0	0	0
21	5.20	142.56	0.06	0.16	1.06	0.1
46	5.20	138.86	0.10	0.23	1.21	0.1
67	5.14	131.94	0.18	0.41	1.36	0
91	5.25	113.17	0.26	0.14	1.87	0
115	5.30	109.71	0	0.66	2.07	0
139	5.38	109.46	0	0.69	2.41	0
163	5.42	101.06	0	0.75	2.81	0
211	5.26	98.10	0	0.80	3.38	0
235	5.51	92.42	0	0.79	3.43	0
259	5.29	88.47	0	0.76	3.69	0

Table A.7.10 Hydrogen production data for 200 g/L of CWP with *Clostridium pasterianum* experiment

Time (hour)	V _l (mL)	V _g (mL)	H ₂ -1 (%)	VH ₂ -head space (mL)	V _{total gas} (mL)	H ₂ -1 (%)	V _{t x} H ₂ -1	V _{cum.} (mL)	Cumulative H ₂ (mmol)
0	200.00	110.00	0.00	0.00	0.00	0.00	0.00	0.00	0
21	193.00	117.00	0.03	3.86	42.50	0.03	1.40	5.26	0.21
46	186.00	124.00	0.25	27.11	52.50	0.25	13.11	45.49	1.83
67	179.50	130.50	0.42	24.06	75.00	0.42	31.63	101.18	4.07
91	172.50	137.50	0.44	5.36	23.50	0.44	10.32	116.86	4.70
115	165.50	144.50	0.39	0.00	9.00	0.39	3.53	120.39	4.84
139	158.00	152.00	0.39	2.90	8.00	0.39	3.14	126.43	5.08
163	151.00	159.00	0.38	0.00	6.00	0.38	2.27	128.70	5.18
211	144.50	165.50	0.36	0.00	8.50	0.36	3.02	131.72	5.30
235	136.00	174.00	0.34	0.00	6.50	0.34	2.18	133.91	5.39
259	128.50	181.50	0.33	0.81	6.00	0.33	1.96	136.68	5.50