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BIOHYDROGEN PRODUCTION OF A CO-CULTURE CONSISTING OF HALOPHYTIC CYANOBACTERIUM PHORMIDIUM KEUTZINGIANUM AND ACTIVATED SLUDGE BACTERIA USING DIFFERENT EXOGENOUS CARBON SUBSTRATES AND SALT CONCENTRATIONS

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Maitha Mohammed Sultan Omran AL Nuaimi

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Declaration of Original Work

I, Maitha Mohammed Al Nuaimi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Biohydrogen production of a co-culture consisting of halophytic cyanobacterium Phormidium keutzingianum and activated sludge bacteria using different exogenous carbon substrates and salt concentrations*", hereby, solemnly declare that this is the original research work done by me under the supervision of Dr. Ashraf Aly Hassan, in the College of Engineering at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma, or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Various studies have proved the ability of different types of cyanobacteria and algae to produce hydrogen (H_2) by splitting water molecules into H_2 and oxygen (O_2) using specialized enzymes (hydrogenase and nitrogenase enzymes) through the biophotolysis process. However, the production of O_2 acts as the main process inhibitor. Several researchers studied this O_2 sensitivity and proposed effective solutions to regulate O_2 concentration. By co-culturing algae with aerobic bacteria, the consumption of the resulting oxygen could be attained and thus reducing the sensitivity of the enzyme to the evolved O_2. In this study, a microbial consortium (co-culture) consisting of cyanobacteria *Phormidium keutzingianum* and activated sludge bacteria ASB was established to regulate O_2 concentration and enhance H_2 production. Different co-culturing ratios (algae: bacteria) such as 2:1,1:1, and 1:2 were tested to find the optimum ratio for H_2 production. The effects of different exogenous carbon substrates (simple sugars) such as glucose, sorbitol, and mannitol were analyzed by supplementing the co-cultures with 10 g/L of sugar. In addition to study the effect of salt (NaCl) on H_2 production, different salt concentrations of 0, 10, and 20 g/L were tested. Results indicated that the amount of cumulative H_2 produced changed significantly by varying the carbon substrate. Glucose-supplemented co-culture produced the lowest amount of H_2 (278 ml L^{-1}) as compared to sorbitol-supplemented co-culture which produced the maximum amount of H_2 (980 ml L^{-1}). On the other hand, mannitol-supplemented co-culture produced (562 ml L^{-1}) of H_2. The results also showed that the addition of salt (NaCl) negatively affected H_2 production. By increasing the salinity level from 0-2%, the amount of total gas produced by glucose-supplemented co-culture was reduced from 2275 ml L^{-1}l to 734 ml L^{-1}, whereas cumulative H_2 reduced from 980 ml L^{-1} to 176.8 ml $L^{-1}$and 562 ml L^{-1} to 333 ml L^{-1} in sorbitol and mannitol-supplemented co-culture, respectively. This study proved the possibility of biohydrogen production by utilizing simple sugars and it can cause significant variations in the amount of the produced H_2 due to the differences in the metabolic pathways of different sugars by the involved cyanobacteria and bacterial cells. This study also shows that physical factors (such as the effect of salt) affected the H_2 production process due to variations in the tolerances of the involved cyanobacterial and bacterial cells toward different salt concentrations.

Keywords: Algae; Chlorophyll; Hydrogenase Enzyme; Metabolism; Nitrogenase Enzyme; Salt Tolerance; Wastewater

Title and Abstract (in Arabic)

انتاج الهيدروجين الحيوي في مزرعة ميكروبية مشتركة تتكون من البكتيريا الملحية الخضراء المزرقة *Phormidium kuetzigianum* وبكتيريا الحمأة النشطة باستخدام ركائز كربون خارجية و تركيزات ملح مختلفة

الملخص

اثبتت دراسات عديدة قدرة أنواع مختلفة من الخضراء المزرقة على إنتاج الهيدروجين عن طريق تفكيك جزيئات الماء إلى الهيدروجين والاكسجين باستخدام إنزيمات متخصصة (hydrogenase و nitrogenase) من خلال عملية التحلل الحيوي الاكسجين الناتج يعمل كمثبط رئيسي للعملية وأبلغت العديد من الدراسات عن حساسية الانزيمات للأكسجين واقترحت حلاً فعالاً لتنظيم تركيز الاكسجين من خلال الاستزراع المشترك للطحالب مع البكتيريا الهوائية والتي تساهم في استهلاك الأكسجين الناتج عن عملية التحلل الحيوي وبالتالي يقلل من حساسية الإنزيمات تجاه الاكسجين. في هذه الدراسة تم إنشاء اتحاد ميكروبي (مزرعة مشتركة) يتكون من البكتيريا الخضراء المزرقة *Phormidium keutzingianum* وبكتيريا الحمأة النشطة بهدف تنظيم تركيز الاكسجين وتعزيز إنتاج الهيدروجين . تم اختبار نسب الاستزراع المشترك المختلفة (الطحالب: البكتيريا) مثل 1:2 و 1:1 و 1: 2 لإيجاد النسبة المثلى لإنتاج الهيدروجين. تم تحليل تأثيرات ركائز الكربون الخارجية المختلفة (سكريات بسيطة) كالجلوكوز و السوربيتول والمانيتول من خلال تكميل المزرعة المشتركة المحضرة بـ 10 جم / لتر من الركيزة الكربونية. بالإضافة إلى دراسة تأثير ملح كلوريد الصوديوم على إنتاج الهيدروجين تم اختبار تركيزات ملح مختلفة مثل 0 و 10 و 20 جم / لتر. أشارت النتائج إلى أن كمية الهيدروجين التراكمية المنتجة تغيرت بشكل كبير بتغيير الركيزة الكربونية. على سبيل المثال أنتج الاستزراع المشترك المضاف إليه الجلوكوز أقل كمية من الهيدروجين (138 مل) مقارنة بالمزرعة المشتركة المكملة بالسوربيتول والتي أنتجت أكبر كمية الهيدروجين (490 مل). من ناحية أخرى، أنتجت المزرعة المشتركة المضاف إليها مانيتول (280 مل) من الهيدروجين. كما أظهرت النتائج أن إضافة ملح أثرت سلباً على إنتاج الهيدروجين. فبزيادة مستوى الملوحة من 0-2%، انخفضت كمية الغاز الكلية الناتجة عن الزراعة المشتركة المكملة بالجلوكوز من 1270 مل إلى 367 مل ، بينما انخفضت كمية الهيدروجين الكلية الناتجة من 490 مل إلى 88 مل ومن 280 مل إلى 166مل في المزرعة المشتركة المكملة بالسوربيتول والمانيتول على التوالي. اثبتت هذه الدراسة إمكانية إنتاج الهيدروجين الحيوي من خلال استخدام السكريات البسيطة ويمكن أن تسبب اختلافات كبيرة في كمية الهيدروجين الناتجة بسبب الاختلافات في مسارات التمثيل الغذائي للسكريات المختلفة من قبل الخلايا الطحلبية والبكتيرية المعنية. تظهر هذه الدراسة أيضًا أن العوامل الفيزيائية (مثل تأثير الملح) أثرت على عملية إنتاج الهيدروجين بسبب الاختلافات في تحمل الخلايا الطحلبية والبكتيرية المعنية تجاه تركيزات الملح المختلفة.

مفاهيم البحث الرئيسية : الطحالب، الكلوروفيل، انزيم الهيدروجيناز، الاستقلاب، انزيم النيتروجيناز، تحمل الملح، مياه الصرف

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Dedication

To my beloved parents, husband, my children (Rashid and Mohammed), and all my family

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List of Abbreviations

ASB	Activated Sludge Bacteria
ATP	Adenosine Triphosphate
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
FNR	Ferredoxin $NADP^+$ Oxidoreductase
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
OD	Optical Density
TOC	Total Organic Carbon
VFA	Volatile Fatty Acids

Chapter 1: Introduction

1.1 Overview

With the worldwide concern about the use of fossil fuels to produce electricity which results in increasing carbon emissions, looking for alternative energy sources that are clean and sustainable is required. Hydrogen (H_2) emerged as one of the candidates which considered to be a key fuel of the future as it has a high energy content per unit of weight around 140 MJ/kg compared to the typical solid fuels which produce around 50 MJ per unit of weight. Moreover, the only waste generated from hydrogen combustion is water making it one of the best environmentally friendly energy carriers (Chi & Yu, 2018).

Steam reforming, coal gasification, and water electrolysis are the classical methods of H_2 production (G. Yang & Wang, 2017; Balachandar et al., 2020). However, these processes are highly energy intensive and highly dependent on fossil fuels, which increases greenhouse gas emissions (Rittmann & Herwig, 2012; Pugazhendhi et al., 2019). Therefore, they are considered non-renewable and unstainable H_2 production processes.

Producing H_2 through different biological routes such as biophotolysis using algae and cyanobacteria or by fermentation routes such as photo and dark fermentation by photosynthetic and anaerobic bacteria has attracted a lot of interest in recent years. The biological H_2 production processes are considered less energy intensive and more environmentally friendly as they can be operated at ambient temperature (Silva et al., 2018; Srivastava et al., 2019).

Cyanobacterial biomass can be used as a food source or feedstock for various products such as antioxidants, pharmaceuticals and coloring agents. It could also be used as a prospective precursor for future biofuels such as biomethane, biohydrogen, bioethanol, and biodiesel. The biomass can be converted to biogas (biomethane) by anaerobic digestion. The photosynthetic system in cyanobacteria is able to diverge the electrons generated from the two primary reactions to produce H_2. Previous studies have been conducted on different algal species capable of producing H_2. Algal species

such as green algae *Chlamydomonas sp.* Is classified as one of the top H_2 producers and other algal genera such as *Anabaena, Chlorella, Oscillatoria,* and *Scenedesmus* have been widely studied to determine their ability to produce H_2 (Liu et al., 2019; Ruiz-Marin et al., 2020; L. Li et al., 2021; Grechanik et al., 2021).

The Calvin cycle in cyanobacteria leads to the production of carbohydrates, lipid, fatty acids, and proteins. Bioethanol can be produced from carbohydrates by fermentation. Biodiesel can be produced from lipids. CH_4, H_2, and e^- can be obtained from fatty acids acetate, butyrate, and propionate by fermentation (Parmar et al., 2011). Furthermore, cyanobacterial biomass is considered as one of the most beneficial bio accumulators due to its low-cost cultivation and absorption potential (Opeolu et al., 2010). They can degrade various toxic organic pollutants in the environment and convert them to less toxic or non-toxic compounds and utilize them as a source of nutrients. *Spirulina* sp., *Oscillatoria* sp., and *Westiellopsis* sp. are some of the common examples of the most commonly cyanobacterial species that were cultivated and used to treat industrial wastewater (Touliabah et al., 2022).

Microalgae/cyanobacteria can be found in nature in a symbiotic relationship with bacteria forming microbial consortia in different environments (Subashchandrabose et al., 2011). Algal-bacterial symbiosis can be employed as an inexpensive biological treatment method to remove organic pollutants from wastewater. By providing the essential oxygen, a critical electron acceptor for the aerobic bacterial breakdown of organic contaminants. On the other hand, bacteria provide CO_2 needed for microalgal photosynthesis. Therefore, the symbiotic relationship between cyanobacteria/microalgae and bacteria in wastewater treatment systems could support low-cost aeration, lessens the environmental impact related to other mechanical and chemical treatment approaches, improves nutrient recovery, and reduce CO_2 emissions (Muñoz & Guieysse, 2006). Moreover, the microbial community within the coculture provides a beneficial association through exchanging basic metabolites such as amino acids and growth-promoting supplements, like thiamin and indole acetic acid (Cole et al., 2014; Higgins et al., 2016). Therefore, this will increase biomass productivity and will reduce the cost of supplying more nutrients for algal growth.

Wastewater produced from domestic, industrial, and agricultural activities comprises a variety of inorganic and organic substances, as well as pathogens, nutrients including nitrogen, phosphorous, carbon content, and sediments that have a significant impact on the pH, chemical oxygen demand (COD), biochemical oxygen demand (BOD), and dissolved oxygen (DO). The selection of suitable and effective microbial consortia depends on the properties of wastewaters influence the choice of suitable and effective bacteria-cyanobacteria/microalgae consortia for enhanced performance in wastewater treatment systems (Monfet & Unc, 2017).

For example, dairy wastewater has high nitrogen and phosphorus content whereas domestic wastewater has high organic carbon content (Posadas et al., 2014; Chastain, 2017). Therefore, the partners within the consortia should have the potential to reduce the amount of carbon, nitrogen or phosphorus content in the wastewater. A microbial consortium consists of phenol-resistant microalgae such as *Chlorella vulgaris* and *Scenedesmus quadricauda* and heterotrophic bacteria *Pantoea agglomerans* and *Raoultella terrigena* showed the capability to grow in coke and olive washing wastewater and the potential to remove 99% of phenol containing compounds in wastewater (Maza-Márquez et al., 2014; Ryu et al., 2017). The inoculum ratio of 5:1 for algae to activated sludge used to treat municipal wastewater led to the reduction in nitrogen and phosphorus content by 95.8% and 93.5% (Perera et al., 2019). Cultivating *Chlorella* sp in centrate wastewater that contains a high concentration of nutrients led to the reduction of COD, total nitrogen, and total phosphorus by 70%, 61%, and 61% respectively (Min et al., 2011).

Thus, the symbiotic relationship between cyanobacteria/microalgae and bacteria in sewage or wastewater treatment systems is expected to double benefit the environment by reducing the amount of nutrients or organic matter in wastewater which in turn will benefit the algal biomass that can be further converted to produce biofuels and energy.

1.1.1 Biological H_2 production routes

Biological routes resulting in H_2 production can be classified into two main categories Photosyhthtic or photolytic (direct and indirect biophotolysis) and fermentative (photo and dark fermentation) (Nikolaidis & Poullikkas, 2017).

1.1.1.1 Biophotolysis

Both cyanobacteria and green algae utilize photosynthetic pigments such as chlorophyll and other pigments to capture light energy to split water molecules into H_2, oxygen (O_2), and electrons via a process called biophotolysis by performing the following reactions (Das & Veziroglu, 2008). Direct bio photolysis is the direct conversion of water into H_2 and O_2 using light energy. The advantage of this route is the production of H_2 from water directly by simply using solar energy. The unicellular green algae *Chlamydomonas reinhardtii* produce H_2 via direct photolysis (Sakurai et al., 2013; Tamburic et al., 2012). Direct bio-photolysis overall reactions described as follows:

$$H2O \rightarrow \frac{1}{2} O_2 + 2H^+ + 2\,e^- \text{ (in the presence of light) Equation (1)}$$

$$2H^+ + 2e^- \rightarrow H_2 \text{ Equation (2)}$$

By utilizing solar energy, photosystem II (PSII) splits water molecules into electrons, protons (H^+), and O_2. While electrons are moving through the photosynthetic membrane that connects PSII and photosystem I (PSI), Adenosine triphosphate (ATP) is being produced. Then, PSI transfers electrons to ferredoxin (Fd). Either the reduced Fd can donate electrons to [FeFe]-hydrogenase, which catalyzes the synthesis of H_2 in green algae, or it can reduce Nicotinamide adenine dinucleotide (NADP) to Nicotinamide adenine dinucleotide phosphate (NADPH) via the ferredoxin $NADP^+$ oxidoreductase (FNR) reaction, which is then employed in the Calvin cycle for CO_2 fixation (Akhlaghi & Najafpour-Darzi, 2020).

Cyanobacteria are similar to green algae in their photosynthetic ability. They can produce H_2 using two photosystems PSI and PSII and an electron transport chain. However, [NiFe]-hydrogenase is the enzyme responsible for H_2 production in

cyanobacteria. [NiFe]-hydrogenase is less sensitive to O_2 evolution as [FeFe]-hydrogenase in green algae (Sakurai et al., 2013). Which is one of the main benefits of producing H_2 using cyanobacteria. The requirement of high light intensity and inhibition of hydrogenase enzyme by the presence of a certain amount of O_2 are the main disadvantages of direct photolysis (Ni et al., 2006).

On the other hand, cyanobacteria and microalgae can produce H_2 by utilizing the stored glycogen or starch content via indirect bio photolysis (Bolatkhan et al., 2019). In direct bio photolysis reaction describe as follows:

$$6\,CO_2 + 6\,H_2O \rightarrow C_6H_{12}O_6 + 6\,CO_2 \quad \text{Equation (3)}$$

$$C_6H_{12}O_6 + 6\,H_2O \rightarrow 12\,H_2 + 6\,CO_2 \quad \text{Equation (4)}$$

It is a two-step process where in the first step water split into protons (H^+) and O_2 is formed in the presence of light. Whereas in the second step, CO_2 will be fixed and carbohydrate will be stored in the intracellular reserves and used for H_2 production (Ghirardi et al., 2000). The main advantage of this is that O_2 evolution or photosynthesis is separated from H_2 evolution producing a relatively higher H_2 yield.

The requirement of continuous light energy supply and the requirement of significant ATP for the sustained nitrogenase activity are the main limitations for large scale application (Sharma & Arya, 2017).

1.1.1.2 Dark fermentation

Dark fermentation DF is one of the most studied routes of biological H_2 production. A wide range of waste materials such as food and agricultural wastes and wastewater can be utilized to produce H_2 via dark fermentation (Turon et al., 2016). In dark fermentation, H_2 is produced via the acetate-mediated pathway. The overall reaction is summarized as follows:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4\,H_2 + 2CO_2 + 2CH_3COOH \quad \text{Equation (5)}$$

Facultative and obligate anaerobic bacteria act on a substrate such as carbohydrates, proteins or lipids to produce H_2, carbon dioxide (CO_2) and organic

acids. *Clostridium* sp., *Bacillus* sp., and *Enterobacter* sp. are examples of H_2-producing microorganisms involved in DF (Ferreira & Gouveia, 2020).

Bacterial species of the genus *Clostridium* were reported to have the highest H_2 production yield (Silva et al., 2018). Due to its versatile metabolic pathway, it can produce a variety of by-products such as VFAs along with H_2. Where the amount of the produced VFAs and the yield of H_2 production varies based on the type of bacterial species (Bao et al., 2012).

A mixed culture of *Bacillus* sp. and *Brevumdimonas* sp. produced H_2 higher than the amount of H_2 produced by pure cultures by two times. This has been attributed to the difference in the metabolic pathway of each bacterial species (Bao et al., 2012).

The possibility of using a wide range of substrates without requiring light energy source to produce H_2 is an advantage of DF, However DF produce relatively lower H_2 production yield compared to other biological routes (Sharma & Arya, 2017).

1.1.1.3 Photo fermentation

Photosynthetic bacteria such as PNS bacteria require light energy to convert organic substrates into H_2 and CO_2 (Manish & Banerjee, 2008). In the presence of nitrogen (N_2), nitrogenase enzymes produce H_2 as a by-product of N_2 fixation (Akhlaghi & Najafpour-Darzi, 2020).

H_2 production in the presence of N_2:

$$N_2 + 8\,e^- + 8H^+ + 16\,ATP \rightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i(inorganic\ phosphate)$$ Equation (6)

In the absence of nitrogenase enzymes change their catalytic metabolism from N_2 fixation producing only H_2. The reason for favoring nitrogen limited condition (Akhlaghi & Najafpour-Darzi, 2020).

H_2 production in the presence of N_2:

$$8\,e^- + 8\,H^+ + 16\,ATP \rightarrow 4H_2 + 16\,ADP + 16\,P_i$$ Equation (7)

Rhodobacter sphaeroides, Rhodobacter capsulatus, Rhodobacter sulfidophilus, Rhodopseudomonas palustris, and Rhodospirillum rubrum are some examples of PNS bacteria that showed the ability to produce H_2 via photo fermentation (T. Y. Wu et al., 2012).

Under anaerobic, nitrogen-limited, and light conditions, substrates such as carbohydrates and organic acids are oxidized in TCA cycle to provide electrons (Lazaro & Hallenbeck, 2019). The generated electrons are used by the nitrogenase enzyme which is the primary enzyme for catalyzing H_2 production in photosynthetic bacteria. Therefore, H_2 can be produced as a result of the utilization of light energy by a photosynthetic membrane which provides the nitrogenase enzyme with ATP required for proton reduction (Sagir & Alipour, 2021).

The use of solar energy and various carbon sources, a wide range of light wavelengths (400–1000 nm), and operation at low pressures and temperatures make photo fermentation a viable process (Koku et al., 2002). Besides that, photo fermentative bacteria are efficient in removing the produced industrial organic wastes which is considered one of the main advantages of using photo fermentative bacteria. Disadvantages of photo fermentation include the pretreatment of industrial effluent and nitrogen-limited condition (Sharma & Arya, 2017).

1.2 Statement of the Problem

Currently, about 95% of the produced H_2 around the world comes from fossil fuels Figure 1 which results in the emission of a huge amount of greenhouse gases which contributes to exacerbating the problem of global warming. Therefore, looking for other environmentally friendly, efficient, and sustainable alternatives is important to meet the global energy demand. Water electrolysis is another method for H_2 production although it is considered a clean and efficient process for producing H_2, but it requires a large energy input. “Green H_2” is a term used for the H_2 produced using renewables which emerged as one of the appealing methods to produce H_2 however generating H_2 using renewables requires also a huge amount of energy which in turn makes green H_2 more expensive to obtain.

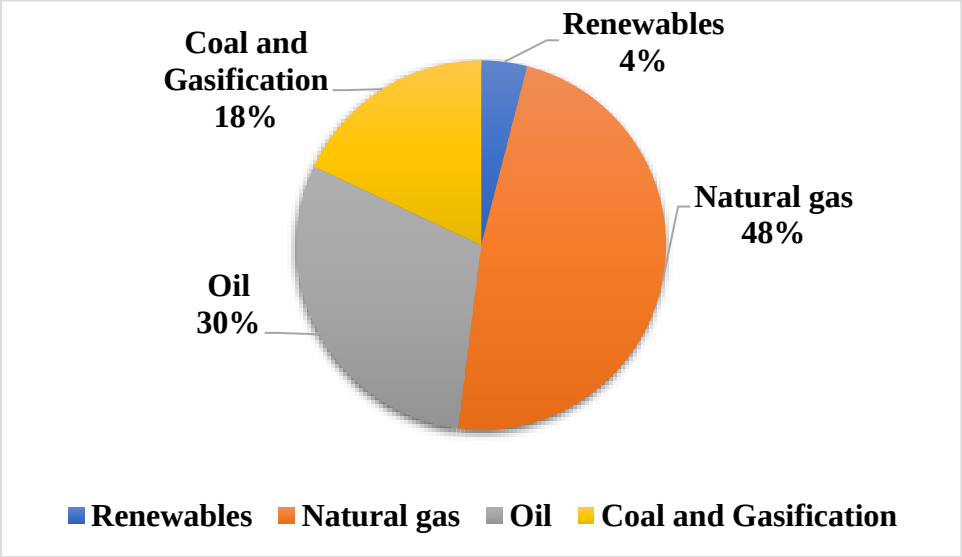

Figure 1: Global H_2 production by method as % of total metric tons (Balat, 2008)

Several microorganisms such as microalgae, cyanobacteria, and bacteria showed the potential to produce H_2 through different biological routes under specific conditions. The ability of these microorganisms to produce H_2 could offer a sustainable clean energy supply and a promising replacement for conventional fossil fuels. But so far, more studies are being conducted to overcome the barriers associated with biological H_2 production and that could affect its effectiveness. Low H_2 production yield is the main limitation of biological H_2 production which could be attributed to several reasons one of the main reasons is the inhibition of the hydrogenase enzyme activity due to the evolution of O_2 by photosynthesis.

Therefore, the anaerobic condition is a major requirement to maintain the enzymatic activity required for H_2 production. One of the proposed methods to overcome the inactivation of enzymatic activity as a result of O_2 evolution is to co-culture activated sludge bacteria (ASB) along with the algal cells as shown in Figure 2 The new microbial consortia interaction can boost H_2 production yield by the following:

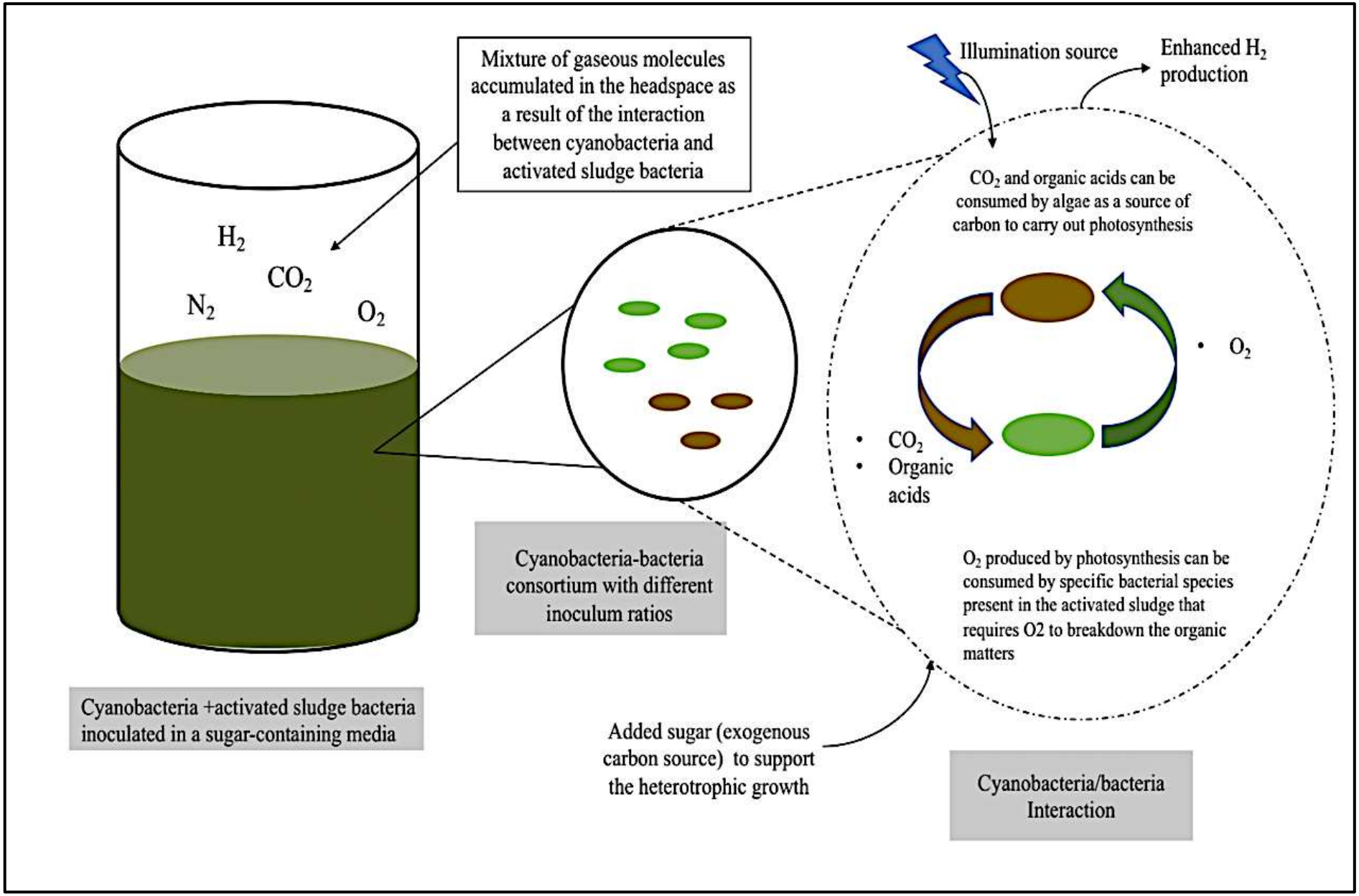

Figure 2: Proposed algal-bacterial interaction to boost H_2 production

presented in the medium to support carbon degradation, minimizing O_2 concentration in the medium.

(2) CO_2 produced as a result of bacterial fermentation can be consumed by algal cells to support its growth and to perform photosynthesis.

(3) Organic acids generated from bacterial fermentation can be consumed by photosynthetic bacteria to maintain energy for their growth and metabolism leading to an increase in H_2 production.

Although previous studies showed that co-culturing microalgae with ASB could lower O_2 concentration and significantly increase H_2 production yield (Fakhimi et al., 2020; Javed et al., 2022). However, to ensure sustainable H_2 production there are other factors are still needed to be evaluated to find the optimum conditions required for improved bio-H_2 production. In this study, *Phormidium keutzingium* a non-heterocystous filamentous halophytic cyanobacterium (no H_2 production data published yet) will be co-cultivated with ASB, and the possible factors that might be affecting H_2 production such as inoculum ratio (Cyanobacteria: ASB), type of the exogenous carbon substrates and other important growth parameters such as salinity will be investigated.

1.3 Research Objectives

This research aims to maximize the total produced H_2 by the filamentous cyanobacteria strain (*Phormidium Keutzingianum*) co-cultured with activated sludge bacteria (ASB) with different mixing ratios, different sugars (carbon substrate), and different salinities Figure 3 represents the main research objectives of this study.

Therefore, the following objectives have been set in order to fulfill this aim:

1. To study the impact of different exogenous carbon substrates such as glucose, sorbitol, and mannitol on the total produced hydrogen
2. To examine the effect of varying mixing ratios on the evolution of molecular O_2 and H_2 production. Mixing ratios of Cyanobacteria to ASB of 2:1, 1:1, and 1:2, respectively, were chosen to be tested in this study

3. To study the impact of different salt concentrations on the total produced H_2. Concentrations of 0, 10, and 20 g/L as salt (NaCl) concentrations will be tested in this study.

To address the research, we need to answer the following research questions:

- What is the effect of different inoculum ratios on the total volume of the produced hydrogen gas?
- How would different exogenous carbon sources improve the total produced hydrogen?
- How would different NaCl concentrations improve the total produced hydrogen?

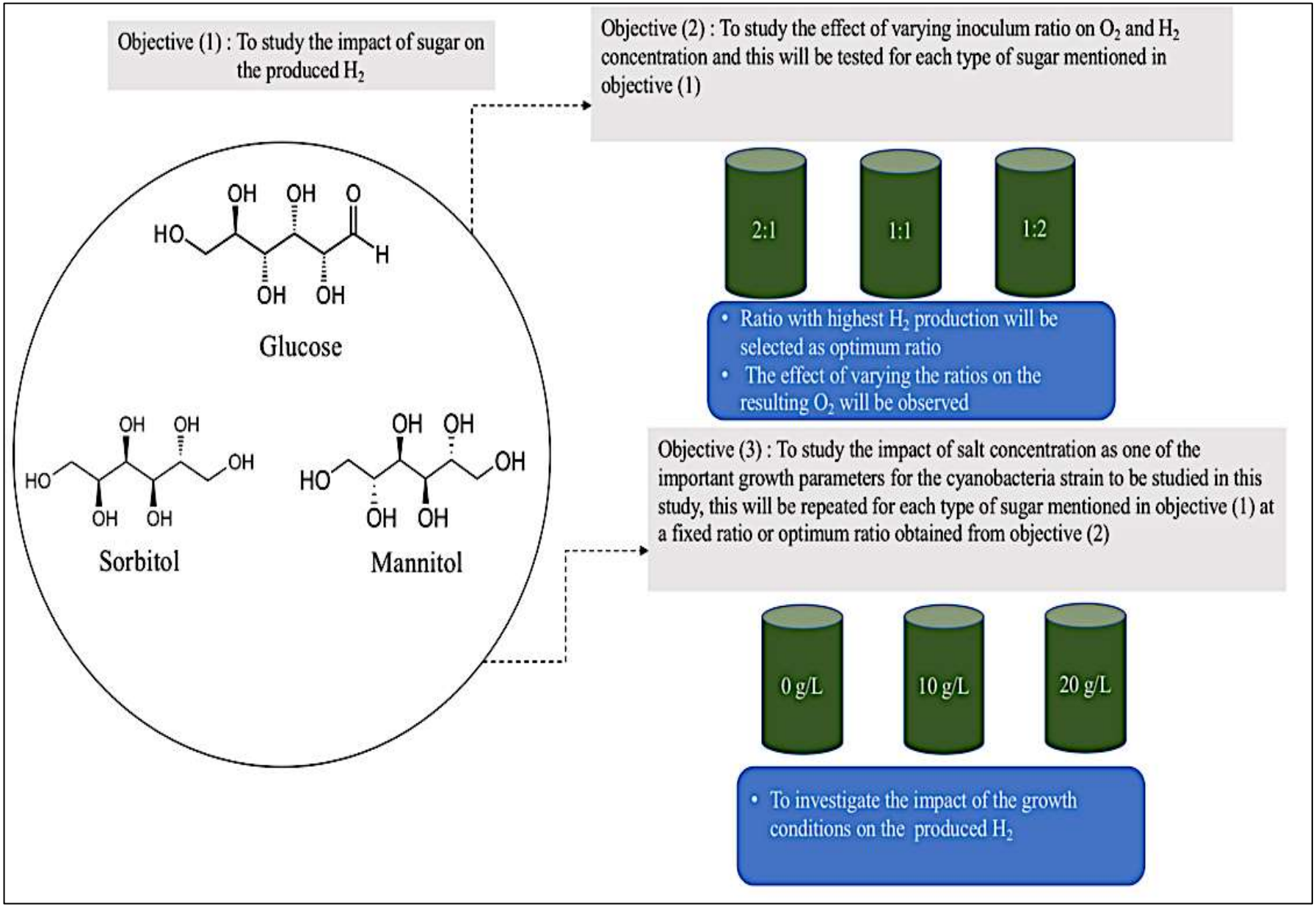

Figure 3: A schematic diagram for the research objectives

1.4 Relevant Literature

1.4.1 Co-culturing cyanobacteria with Activated sludge bacteria (ASB)

Previous studies mentioned proposed ideas to overcome the barriers of biological hydrogen production such as the low production yield, hydrogenase/ nitrogenase oxygen sensitivity, and the accumulation of Volatile fatty acids VFA by integrating biological systems (dark fermentation, photo-fermentation, and biophotolysis). Several studies have dealt with the advantages of integrating biological systems or mixed cultures in enhancing H_2 production (Melis & Melnicki, 2006; Fakhimi et al., 2020).

Chlorophyll and phycobilin molecules in algae and cyanobacteria have absorption maxima at 400-700 nm whereas bacteriochlorophylls in photosynthetic bacteria have absorption maxima at 400-600 nm and 800-1100 nm which could provide 25% additional energy of the solar spectrum to that of green algae or cyanobacteria, therefore co-cultivating those microorganisms could enhance H_2 production as a result of increasing the light conversion efficiency (Ghirardi et al., 2009). Moreover, the integration of more than one biological system could enhance the efficiency of the single system to produce H_2 at a higher rate (Hay et al., 2013). For example, H_2 production enhanced to 12 moles H_2 /mol hexose by combining dark and photo fermentation compared to 4 moles H_2/mole hexose by dark fermentation alone (Show et al., 2012). Co-culturing anaerobic bacteria (acidogenic bacteria) along with photosynthetic bacteria helped to alleviate the accumulation of volatile fatty acids by 40%, thus, enhancing H_2 production (Chandra & Venkata Mohan, 2014). Up to 2-fold improvement in H_2 production yield was observed by a co-culture of *Bacillus* sp. and *Enterobacter* sp. than by the individual strains (Patel et al., 2014). Based on previous studies done on the potential of bacterial monocultures to produce hydrogen in a co-culture, it has been concluded that pure monocultures produce low hydrogen yield compared to mixed-culture systems (Qian et al., 2011; Masset et al., 2012; Wu et al., 2012 X; Li et al., 2013; Patel et al., 2014; Pachapur et al., 2017; Xu et al., 2017).

The inactivation of hydrogenase activity is due to the presence of molecular oxygen which is considered as one of the critical problems associated with hydrogen production using algae and cyanobacteria. One of the recent strategies used to overcome this problem, therefore, increasing the biohydrogen production yield is to build a microbial consortium consisting of microalgae and aerobic bacterial species to increase O_2 consumption rate (Javed et al., 2022). It has been investigated that the co-culture facultative anaerobes with strict anaerobes solved the problem of O_2 sensitivity without the need of a reducing agent. A mixed culture of *Clostridium butyricum* and *Escherichia coli* showed their applicability for stable H_2 production due to the increase in substrate conversion efficiency (Seppälä et al., 2011). In a batch study using starch as a substrate, the capabilities of *C.butyricum* and *Enterobactrer aerogenes* to produce H_2 were tested. *E.aerogenes* was not able to produce H_2 by utilizing starch. However, *C.butyricum* was able to utilize starch to produce H_2 after a lag time of 12 h. The addition of a reducing agent such as 0.1% L-cysteine reduced the lag time to 5 h. Co-culturing *E.aerogenes* with *C.butyricum* led to a further reduction in lag time to 2 h and the amount of H_2 was much higher than the amount of H_2 produced when the reducing agent was added. *E.aerogenes* has the ability to consume O_2, therefore, the anaerobic condition was obtained which result in improving H_2 production without the need of a costly reducing agent (Yokoi et al., 1998).

Another advantage reported by Fakhimi et al. (2020) is that co-culturing these microorganisms allow various photosynthetic and fermentative metabolites to be exchanged which resulted in enhancing H_2 production. Examples include carbon, nitrogen, phosphorus, and sulfur, as well as growth stimulants like Vitamin B12 which are essential for the support of algae–bacteria interactions.(Fakhimi et al., 2020). Another study by Fakhimi et al. (2019) suggested that co-culturing bacterial strains *Pseudomonas putida, Escherichia coli, Rhizobium etli* with the green alga *Chlamydomonas reinhardti* resulted in an increase in H_2 production by 60%. This was more than the sum of respective monocultures as a result of the algal-bacterial interaction which allows algal cells to consume the produced acetic acid resulted from bacterial fermentation which in turn benefits both algal and bacterial H_2 production.

Ban et al. (2018) studied the effect of cultivating *Pseudomonas* sp. strain D with *Chlamydomonas reinhardtii* on H_2 production. The oxygen content in the reactor's headspace and the dissolved oxygen was consumed for bacterial growth which helped in creating anaerobic environment suitable for algal hydrogenase activity. Moreover, the algal-bacterial interaction proved to slow chlorophyll content reduction, maintain protein content and enhance starch accumulation, therefore, improving photolysis-mediated hydrogen production in green algae.

Studies showed the effect of co-cultivating algae with other bacteria such as *Azotobacter*, *Bradyrhizobium*, *Pseudomonas*, and *Escherichia* helps in improving hydrogen production by regulating the oxygen content (S. Wu et al., 2012; Xu et al., 2017). Another study reported that co-culturing *Chlamydomonas reinhardtii* with bacterial strains *pseudomonas* sp, *E.coli*, and *Rhizobium etli* could slower acetic acid uptake rate in the medium, therefore, lowering the oxygen accumulation rate and sustaining hypoxia which supports the hydrogen production (Fakhimi, Tavakoli, et al., 2019).

1.4.2 Effect of the culture conditions on H_2 production

Environmental conditions such as pH, temperature, light intensity, salinity, and nutrient availability are some of the main factors that could affect the hydrogen production rate and each microalgal species have different requirements for optimal hydrogen production. Essential elements in the growth medium such as carbon, sulfur, and nitrogen are important for cell growth and it has been reported previously that their presence or absence in the growth medium affects the H2 production yield in some cyanobacterial strains (Dutta et al., 2005).

1.4.2.1 pH

pH serves as one of the indirect indicators of algal growth (Zhou & Dunford, 2017). Also, it is an important factor to be considered in photobiological processes that produce H_2, as each microorganism has its optimal pH that regulates its metabolic pathway leading to H_2 production. Moreover, pH influences the substrate utilization efficiency, synthesis of proteins, and the release of metabolic

by-products. H_2 production usually occurs at the acidification stage of the metabolic pathway (Kothari et al., 2012). Generally, Acidic pH enhances biohydrogen production in bio photolytic and photo-fermentative processes (Mona et al., 2020). Table 1 includes the optimal pH for H_2 production by different bacterial and algal species (Melitos et al., 2021).

Table 1: Optimum pH for biological H_2 production in different studied algal and bacterial species

Species	**Optimum pH**
Chlamydomonas reinhardtii	7
Cyanobacterium Synechocystis and Cyanobacterium Gleocapsa	6.8-8.3
Oleaginous microalgae	6.8
Clostridium species	5-6
Rhodopseudomonas palustris	6.7-7.2
Rhodobacter sphaeroids	6.8-7.2
Rhodobacter Capsulatus	7

1.4.2.2 Effect of carbon sources on H_2 production yield

Organic carbon substrates could influence H_2 production by influencing nitrogenase activity (Neuer & Bothe, 1985). Sugar catabolism is important for maintaining the enzymatic activity and can lead to an increase in NADH which is an important substance for hydrogenase enzyme activity. For example, hexoses such as fructose and glucose produce 2 NADH/mol of hexose as a result of the conversion to pyruvate molecule. On the other hand, hexitol such as sorbitol and mannitol give 3 NADH/mol hexitol. Whereas hexonic and hexuronic acids (highly oxidized sugars) give less than 2 NADH/mol (Clark, 1989).

Previous studies showed that cyanobacteria are able to use a wide range of substrates to produce H_2. And it can cause a variable effect on the hydrogen

production yield of a specific microalgal strain (Yodsang et al., 2018). Various cyanobacterial strains grown under photoheterotrophic conditions showed the capability to use sugars as a reductant or source of carbon for H_2 production (Reddy PM et al., 1996). Substrate or carbon source is an important factor that influences H_2 production through dark fermentation (Silva et al., 2018).

Glucose was the optimum exogenous carbon source for H_2 production by a co-culture of Chlorella vulgaris and activated sludge bacteria compared to different sugars tested such as sorbitol and mannitol. Another study done on *A.variabilis* reported an increase in hydrogen production after adding fructose, which suggests that when fructose is metabolized, it serves as an additional source of reductant required by the nitrogenase enzyme responsible for H_2 production in this strain leading to an increase in the H_2 production rate (Reddy PM et al., 1996).

A study was done on a nitrogen-fixing unicellular cyanobacterium *Synechococcus sp.* strain Miami BG 043511that showed a capability to produce hydrogen using the intercellular glycogen which reduces with the reduction of glycogen content but restored by the addition of external organic substrates which worked effectively as electron donors enhancing H_2 production (Luo & Mitsui, 1994).

1.4.2.3 Effect of salinity on H_2 production yield

Guo et al. (2014) showed that fermentative hydrogen production is greatly influenced by several factors such as initial pH, temperature, substrate concentration, and salt concentration. It was reported that at different salt concentrations, the pH value decreased from 6.9 to 6.1 as a result of VFAs accumulation which could further affect the activity of H_2-producing microbes (Guo et al., 2014). Moreover, Zhang et al. (2017) reported that salinity can affect substrate utilization as the adaptation mechanism in a salty environment requires more energy. Therefore, the substrate utilization efficiency should be increased with increasing salt concentration. High salt concentration affects substrate metabolism, composition and the concentration of the VFA accompanied by hydrogen production. For example, mixed-acid fermentation was the fermentation type at a different salt concentration whereas at 0% salinity, it

was mainly ethanol-type fermentation and there was also an increase in acetic acid concentration with increasing salt concentration (Zhang et al., 2017). It has been reported by (Hao et al., 2006) that in anaerobic fermentation with sucrose, by increasing sodium ion concentration from 0-16,000 mg/L acetic acid concentration increased from 109.4 to 267.7 mg/L.

Taikhao et al. (2013) Reported the effect of salt on H_2 production, it was concluded that salt concentration can significantly affect cyanobacterial growth and therefore H_2 evolution. It was indicated that freshwater cyanobacterial species show different behavior than marine species in salt-containing medium. For example, higher NaCl concentration affected negatively the growth rate of freshwater cyanobacteria *Anabaena doliolum*. The freshwater cyanobacteria *N.muscorum* and *A.variabilis* SPU 003 produced more hydrogen in NaCl free medium than in NaCl containing medium (Shah et al., 2001, 2003).

Other H_2-producing microorganisms showed that their H_2 production yield was significantly affected when NaCl concentration increases. The H_2 production yield of *Clostridium butyricum* decreases when NaCl concentration exceeded 10.16 g/L. This was explained by the fact that the cell consumes more energy for maintenance than for a generation. This will shift the hydrogen production pathway by producing acetic acid instead of butyric acid, leading to decreasing hydrogen production yield. Several studies on bio-H_2 production concluded that higher salt concentration results in high energy consumption, low substrate conversion, and cell lysis affecting the ability of the cells to produce more H_2 (Lee et al., 2012). A study indicated the effect of the presence of salts on anaerobic fermentation using waste sludge. Salt ions could cause dehydration of the bacterial cells affecting the population and activity of the microbial communities due to the increase in osmotic pressure (Chen et al., 2008).

Chapter 2: Methods

2.1 Growth conditions of cyanobacterial strain

Cyanobacterial strain *Phormidium keutzingianum* (UTEX LB PS38) was obtained from the culture collection unit at the University of Texas, Austin, USA. The cyanobacterial strain was grown in a 1L Erlenmeyer flask at a temperature of 23°C and supplied by the light of continuous intensity of 2350 lux. 25 mL of stock culture was further transferred to an autoclaved 5L Schott bottle placed on a magnetic stirrer to provide a continuous stirring at 200 rpm for homogenous mixing of air in the culture. During the time of culturing, the purity of the strain was maintained and microscopically checked to avoid contamination.

The strain was suspended in BG-11 medium using the recommended growth medium recipe from UTEX which contained all of the necessary nutrients for cyanobacterial growth such as Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, $Co^{2+,}$ NO_3^{-} , MoO_4^{-2} , SO_4^{-2}, Cl^{-} , and HPO^{-}_4 ions. In addition, 1 ml of BG-11 trace metal solution was added to the growth medium.

2.2 Growth conditions of the ASB

The domestic activated sludge was obtained from the Al Saad wastewater treatment plant in Al Ain city, UAE. The activated sludge contained a diverse community of microorganisms, including bacteria, protozoa, filamentous bacteria, algae, and fungi. The activated sludge was kept in a refrigerator in a non-transparent container to prevent the microbial community, particularly algae, and fungi, from altering its composition prior to use. In addition, the shelf life of activated sludge was limited to one month to avoid the alteration of composition and characteristics of the microbial community in the activated sludge. In this study a mixture of bacterial strains present in activated sludge was used instead of pure bacterial strain.

2.3 Preparation of co-culture

All the experiments in this study were conducted in duplicate, and all the measurements such as gas compositions, organic carbon concentration, chlorophyll content, and optical densities were obtained following the methodologies of previously conducted studies that had a similar purpose of this study or dealt with similar microorganisms in this study (Javed et al., 2022; Zafar et al., 2022).

Different inoculum ratios (Cyanobacteria: ASB) ratios such as 2:1, 1:1, and 1:2 were tested to study the effect of co-culturing cyanobacteria with activated sludge bacteria on O_2 content and total H_2 production. Glucose, sorbitol, and mannitol were used as exogenous carbon substrates and added at a concentration of 10 g/L to each inoculum including the positive controls (containing only culture of *Phormidium* strain with the carbon substrate and growth medium). The harvested cyanobacteria and activated sludge were collected in a falcon tube of 50 ml and centrifuged at 8000 rpm for ten minutes.

The obtained supernatant was disposed of, and the wet biomass was washed with deionized water (DI), and then resuspended with an O_2-deficient TAP medium. A mixture of cyanobacteria and activated sludge were prepared at different mixing ratios in 500 ml Schott bottles (reactors). After finding the optimum ratio using different sugars. The effect of different salinities (0%,1% and 2%) 0, 10 and 20 g/L of NaCl was tested following the same preparation method discussed above.

2.4 Experimental setup

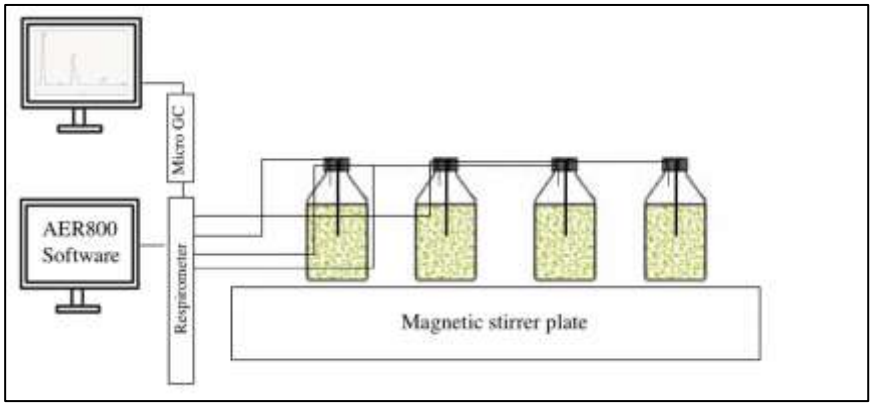

Figure 4: Experimental setup

As shown in Figure 4, the experiment was conducted using a set of eight glass 500 ml bottles (reactors) placed on a magnetic stirrer plate. Each reactor had two ports, one for the gas measurement connected to the respirometer (AER-800) and the second port for liquid sampling. The liquid samples were taken on daily basis for the measurement of optical density, chlorophyll content, and total organic carbon (TOC) concentration. Gases from respirometer were injected into Micro GC (490 Micro GC system) for measuring the generated gas composition, separately from each reactor through an automatic 16 valve actuator. The effect of each carbon substrate was studied using three distinct cyanobacteria to ASB inoculum ratios – 2:1 v/v (300:200 mL), 1:1 v/v (250:250 mL), and 1:2 v/v (200:300 mL). Each ratio, including the control, was performed in duplicates.

Nitrogen (N_2) gas was purged in each reactor for ten minutes after the addition of the inoculum and substrate to the reactor in order to create an anaerobic condition essential for hydrogen production. The rubber stopper caps were used to seal the reactors. The respirometer was used to measure the total volume (mL) of gases produced in each reactor. Each reactor was subjected to continuous light for a photoperiod of 24:0 h. In order to avoid the external mixing of atmospheric air, the gases were then passed through a portable actuator with 16 valves to a Micro gas

chromatography (GC) thermal conductivity detector (TCD) to determine their composition.

2.5 Analytical method

AER-800, a Challenge Technology, Arkansas, 8-channel respirometer with eight bottles and a magnetic stirrer plate, was used to measure the amount of gases produced. A 16-port valve actuator (VICI) (1/16″, Valco Instruments, Switzerland) is used to switch between the reactors and prevent ambient air from mixing. Gas chromatography was performed by a 490 Micro GC equipped with a thermal conductivity detector (TCD) using argon as an inert gas carrier. The column temperature and injector temperature was set at 80°C and 50°C, respectively. A standard GC method was developed for the detection of methane (CH_4), nitrogen (N_2), oxygen (O_2), and hydrogen (H_2), at retention times of 1.5, 1.8, 2.3, and 3.7 minutes, respectively, using 20 m Molsieve 5A column. In a second column (10 m PoraPLOT Q column), carbon dioxide (CO_2) was detected at a retention time of 1.0 minutes. TOC analyzer (Analytik Jena multi N/C 2100) was used to measure the concentrations of reducing sugar and organic carbon. Optical densities (OD) of the pure and co-culture were measured at wavelengths of 620 and 400 nm for cyanobacteria and bacterial strain, respectively.

To measure the chlorophyll content a sample of cyanobacteria and activated sludge coculture (200 µL) was collected from the reactors every day and then centrifuged at 8,000 rpm to get the pure co-culture pallet at the bottom of the Eppendorf tube (2.5 mL). The pallets were resuspended in 800 µL of 95% (v/v) ethanol and stored in the refrigerator at 4°C for 24 h for the algal cells to rupture and release the chlorophyll pigment. Then, the supernatant was collected from the Eppendorf tube after centrifugation at 8,000 rpm for 10 min and used to determine the optical density at 665 nm (Chl a absorption), 649 nm (Chl b absorption), and 750 nm (turbidity correction). Chlorophyll concentration calculated based on the equation given as

$$\text{Chl} = 5\frac{6}{25} \times (A_{664} - A_{750}) + 22\frac{6}{25} \times (A_{649} - A_{750})$$

Chapter 3: Results and Discussions

This section will present the main findings of this study and will answer the main research question. For example, the effects of different exogenous carbon substrates on H_2 production will be discussed in the following sections: 3.1 for glucose, 3.2 for sorbitol and 3.3 for mannitol. Under these sections other important findings will be presented such as the of impact of different mixing ratios (cyanobacteria: ASB) on the cumulative gas production and other important experimental variables such as the utilization of organic carbon content and the impact of exogenous carbon sources on the growth of cyanobacteria or ASB that are involved in the mixture.

Other important objectives in this study is to study the impact of salinity and pH change on H_2 production. In section 3.4 the impact of the salinity on H_2 production as an important factor that could influence H_2 production will be discussed. Then, section 3.5 will present the pH change throughout the experimentation period and how H_2 production will be affected accordingly.

3.1 Glucose

Carbon sources are mostly utilized to support cell growth and product formation (S. T. Yang, 2007). Most organisms utilize glucose as a carbon source for metabolites, energy sources, and for biopolymers synthesis (Jeckelmann & Erni, 2020). Glucose has been used in various water quality applications. Glucose treatment resulted in reducing harmful algal blooms by controlling the growth of Proteobacteria and cyanobacteria. Introducing glucose to the water samples resulted in the inhibition of cyanobacterial nutrient uptake and results in inhibiting their growth. Consequently, the carbon source was readily available for proteobacteria which utilized the available glucose to promote their growth (Vesper et al., 2022). Previous studies showed that glucose addition enhanced the biological treatment of the wastewater by the reduction of TOC, COD, and UV_{254} (N. Li et al., 2021).

In terms of biogas production, glucose is one of the preferred carbon substrates for H_2 production through fermentation. Several wastewater bacterial species such as *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Clostridium butyricum* produced

H_2 by utilizing glucose as a carbon substrate (Rashid et al., 2013). Pairs of co-culture *C.butyricum* and *R sphaeriods , Klebsiella pneumoniae* and *Rhodospirillum rubrum* have been studied for H_2 production using glucose as a carbon source (Fang et al., 2006).

Previous studies on H_2 production using heterocystous cyanobacteria depended on the internal carbohydrate reserve (glycogen) as a source of energy or by providing CO_2 to support H_2 production. However, many cyanobacterial strains couldn't show the optimum activity under these conditions due to limited amount of glycogen. Therefore, the addition of an external carbon source (glucose) has been used to improve the H_2 production during anaerobic fermentation and to regulate the oxygen concentration during photosynthetic growth activity. The study showed that the O_2 level was consistent over the incubation period. Glucose is consumed, and CO_2 evolved as a result of glucose metabolism however CO_2 was subsequently fixed by photosynthesis and O_2 was generated. Whereas, a specific level of O_2 is required to maintain nitrogenase activity which is supported by the oxidative metabolism of carbohydrates in vegetative cells (Yeager et al., 2011).

3.1.1 Impact of mixing ratios

3.1.1.1 Cumulative gas production

The cumulative gas production was evaluated using co-cultures of cyanobacteria and activated sludge supplemented with 10 g L^{-1} glucose at three different inoculum ratios 2:1, 1:1, and 1:2. The gas production lasted for 3 days in all co-cultures. Cumulative gas production presented in Figure 5 for each mixing ratio 2:1, 1:1 and 1:2 was 2236, 2275, and 2232 ml L^{-1}, respectively. The evolved gas mixture in all inoculum ratios was composed of H_2, O_2, N_2, and CO_2. The maximum H_2 was produced in 1:1 ratio as 278 ml L^{-1} followed by 1:2 and 2:1 ratio as 230 ml L^{-1} and 212 ml L^{-1}, respectively. On the other hand, the cumulative O_2 produced by 2:1, 1:1, and 1:2 ratios were 394, 451 and 351 ml L^{-1} respectively. The amount of N_2 produced in 2:1 ratio was 1592.8 ml L^{-1}, 1:1 ratio was1737 ml L^{-1}), and 1:2 ratio

(1162 ml L^{-1}). CO_2 generated in 2:1 ratio was 81.3 ml L^{-1}, whereas 1:1 ratio produced the highest amount of CO_2 101 ml $L^{-1}$and 1:2 ratio produced 86.5 ml L^{-1}.

The results indicated that there was no significant difference in the amount of the total produced gas by varying the inoculum ratio. In all glucose-supplemented co-cultures, as shown in Table 2, N_2 occupied 60% of the gas mixture accumulated in the reactor's headspace followed by O_2 and H_2 which make up to 15 and 8% of the total cumulative gas. Whereas the least amount of 3% of CO_2 was generated in total cumulative gas. In addition, there was a significant correlation between the daily H_2 and O_2 concentrations as shown in Figure 6. On day 1, O_2 concentration was 20% in 2:1 and 16% in both 1:1 and 1:2 ratio. O_2 concentration started to decline to reach 13% on day 3 in 2:1, 14% in 1:1, and 15% in 1:2 ratio. In contrast, on the same days (day 1 and day3), it was observed that there was a significant improvement in H_2 concentration. H_2 concentration was 1.5% in 2:1 and 9% in 1:1 and 9% in 1:2 ratio on day 1. With the reduction in O_2 concentration, H_2 concentration increased to reach a maximum concentration on day 3 of 12% in 2:1, 14% in 1:1, and 12% in 1:2 ratio. This indicates that the reduction in O_2 concentration contributed to enhancing H_2 concentration.

Table 2: Cumulative gas production for different mixing ratios in glucose-supplemented co-cultures

	2:1	**1:1**	**1:2**
H_2	212 (10%)	278 (10%)	230 (8%)
O_2	394 (15%)	451 (17%)	351 (15%)
N_2	1593 (62%)	1737 (65%)	1162 (52%)
CO_2	81.3 (3%)	101 (3.8%)	86.5 (3.8%)

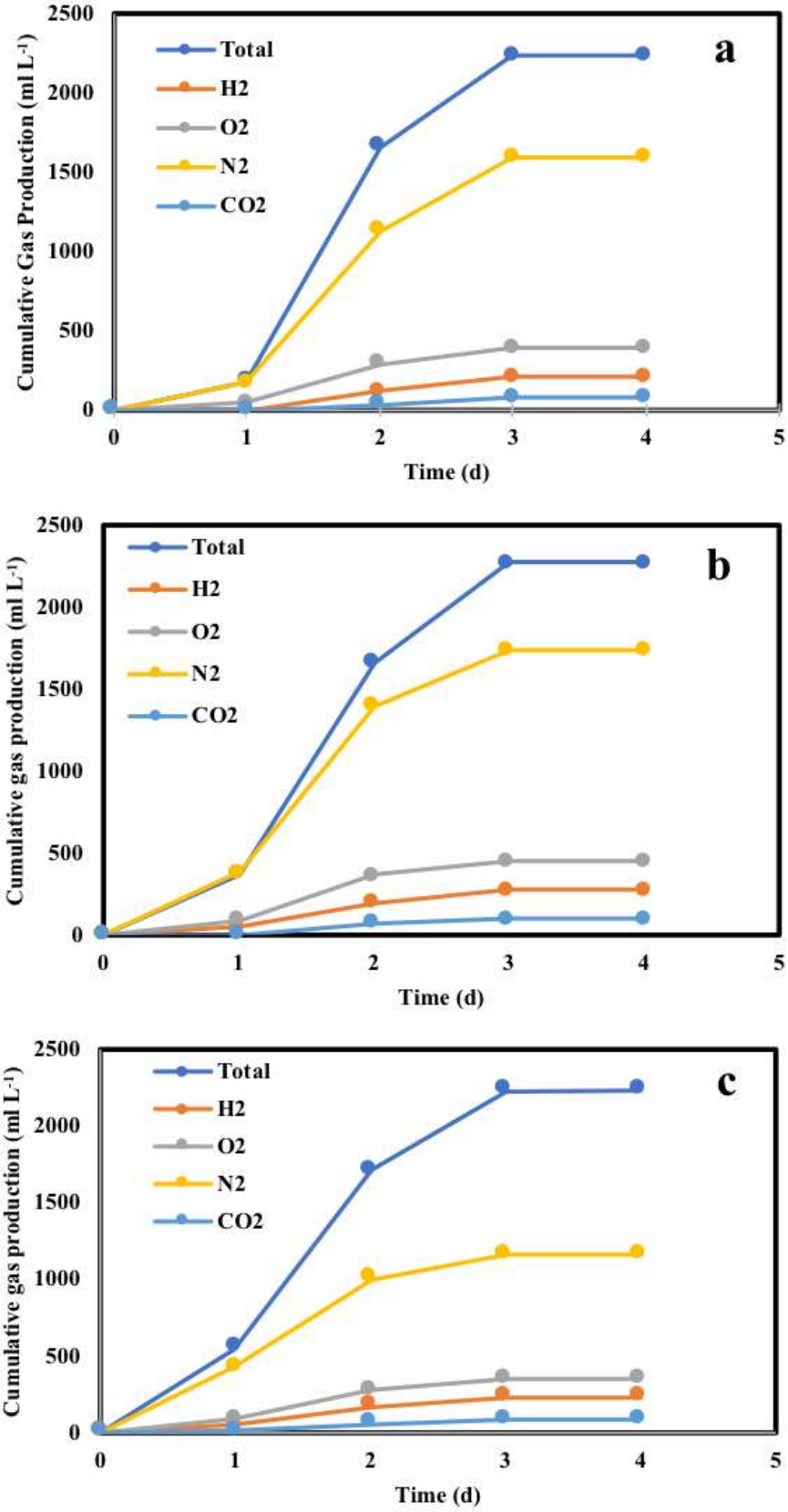

Figure 5: Cumulative gas production using different mixing ratios a)2:1 b)1:1 c)1:2

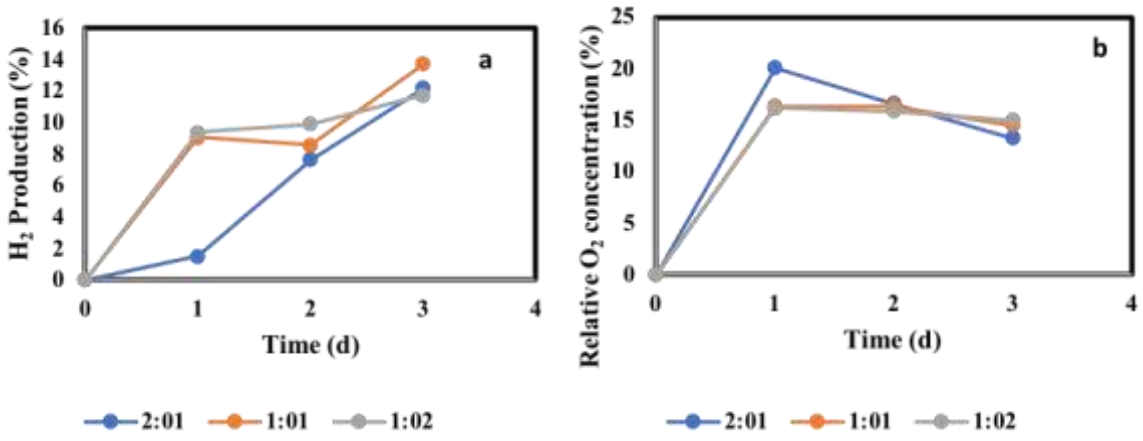

Figure 6: The relationship between the daily H_2 and O_2 production, a) H_2 production and b) Relative O_2 concentration

Although previous studies showed that glucose has been used for fermentative H_2 production using various wastewater bacterial species or microalgal species. However, most of the conducted studies used pure bacterial cultures than mixed cultures that combined activated sludge bacteria and cyanobacteria. One of the limited studies showed that the microalgae *Chlorella vulgaris* when co-cultivated with activated sludge bacteria produced 37% H_2 out of 3341 mL L^{-1} of the total produced gas using glucose as a substrate (Javed et al., 2022). In the current study, the concentration of N_2 (very high) and CO_2 (very low) suggested that microbial competition between the involved microorganisms for the same substrate (glucose) might be one of the reasons that led to the obvious differences in the proportions of the total produced gas (Rafrafi et al., 2013). For example, glucose is one of the preferred substrates for different wastewater bacterial species including denitrifying bacteria responsible for producing N_2. In this study, it is observed that glucose was mainly utilized to catalyze denitrification. It has been reported that glucose is one of the preferred carbon sources for several denitrifying bacteria (Rajta et al., 2020).

Thus, insufficient glucose was left for fermentative H_2 production as both H_2 and CO_2 concentrations were relatively low compared to N_2 concentration. Another reason for the low H_2 concentration could be the presence of H_2-consuming microorganisms in the mixed culture that could utilize glucose to promote their

growth and increase their abundance in the culture. In addition, the production of other metabolites such as volatile fatty acids (VFAs) mainly acetic acid and butyric acid might be the reason for low H_2 production (Kraemer & Bagley, 2007).

3.1.1.2 TOC concentration

The measurement of organic carbon content might be an indication of the capability of microorganisms to metabolize organic content to support their growth or to enhance different biological routes such as H_2 production and denitrification. In this study , a reduction in total organic carbon content in 2:1, 1:1, and 1:2 ratio was observed. The initial TOC concentration in 2:1 and 1:1 ratio was 4.77 and 3.45 g/L reaching a final concentration in both ratios of 1.98 g/L as shown in Figure 7. However, the initial TOC concentration in 1:2 ratio was 3.57 g/L and the final concentration was 3.32 g/L. Therefore, almost 58% and 42% of the TOC was consumed in ratios 2:1 and 1:1 ratio, respectively. On the other hand, only 7% of the TOC was utilized by 1:2 ratio at the end of the experimentation period.

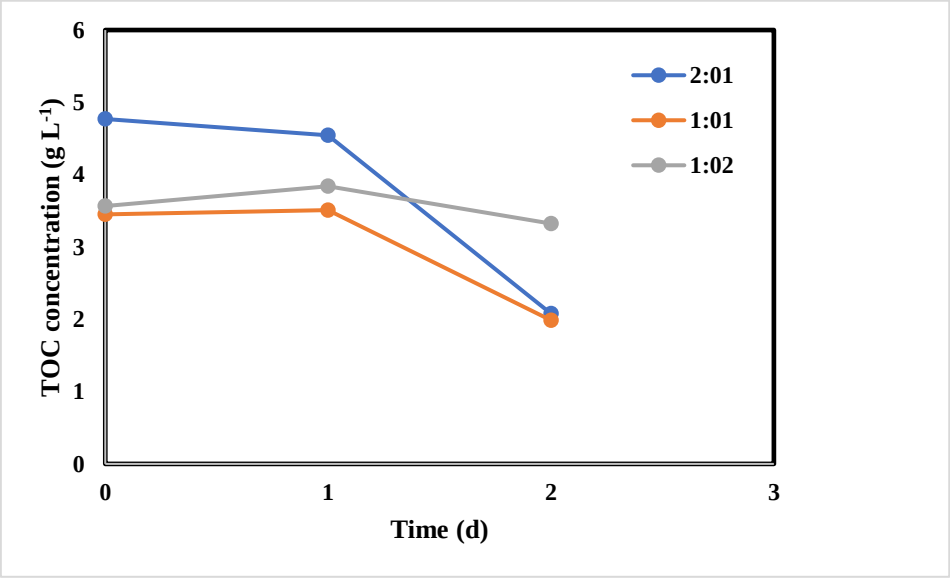

Figure 7: TOC concentration at different mixing ratios in glucose-supplemented co-cultures

It can be observed from the results that 2:1 and 1:1 were the best-performing ratios in carbon content utilization are 2:1 and 1:1 ratio. From those, 1:1 ratio

produced the largest amount of H_2 which could be related to the ability of both cyanobacterial and bacterial cells to utilize the organic carbon for the production of H_2. Although 2:1 and 1:1 ratios consumed large amounts of carbon content; however, the consumed amount was supporting other biological routes than H_2 production which could be the conversion of nitrate and nitrite into N_2 gas based on the large amount of N_2 compared to the amount of the produced H_2 and CO_2. A previous study showed that 60% of the external carbon was consumed as an electron donor for denitrification while 40% was consumed as a substrate for other metabolic activities (Kraemer & Bagley, 2007).

3.1.1.3 Optical density and chlorophyll measurement

Optical density at a wavelength of λ 620 nm (OD_{620}) measurement showed that glucose supplementation can support the growth of the algal cells in all the tested ratios. However, it can be observed from the algal growth behavior shown in Figure 8 (a) that the growth rate was higher on the first day of the experiment compared to the second day. For example, the initial absorbances value in 2:1 , 1:1 and 1:2 were 3.35 , 6.1 and 6.85 respectively. On day 2 the absorbance values reached 8.1 , 9 and 11.8. after that the absorbance value on day 3 didn't change and became almost stable.

Similarly, it can be observed based on the optical density at a wavelength of λ 400 nm (OD_{400}) that the bacterial growth rate on the first day was higher than the day after based on the bacterial growth behavior shown in Figure 8 (b) the initial absorbances were 8.95, 6.1 and 6.85 2:1 , 1:1 and 1:2 respectively. On the second day of the experiment the absorbance value reached 13.25, 15.9 and 19.6 and after that the growth behavior became constant. This can be attributed to the higher amount of O_2 that was available to support bacterial growth on the first day compared to the days after. When the amount of O_2 started to decrease it affected the growth behavior.

Several studies showed that exogenous glucose can be used to enhance the growth behavior of many algal and cyanobacterial species. Glucose is known to support the heterotrophic growth of several chlorella species (Perez-Garcia et al., 2010). The filamentous cyanobacteria *Spirulina Plantensis* showed the ability to

metabolize glucose to enhance the production of biomass and photosynthetic pigments (Marquez et al., 1993). *Chlorella homosphaera* microalgae and *Arthrospira platensis* cyanobacteria were cultivated in a glucose-containing medium under mixotrophic conditions which resulted in a biomass increase up to 2.8 and 3.5-fold respectively (Margarites et al., 2017). Different monosaccharides and disaccharides such as glucose, fructose, lactose and sucrose have been used for mixotrophic cultivation of *Spirulina Plantensis* and showed different effective transport and assimilation mechanisms (Chojnacka et al., 2003). Although glucose supported the growth of algal and bacterial cells in this study; however, biomass productivity showed less support for H_2 production as the microbial consortia consist of various microorganisms that could cause a shift in the metabolic pathways affecting the overall H_2 production.

Similarly, Chlorophyll measurements showed that there is a reduction in chlorophyll content in 2:1 ratio from Day 0 to Day 2 from 2.8 μg/mL to 1.5 μg/mL as shown in Figure 8 (c). After Day 2, chlorophyll content started to slightly increase again till the end of the experimentation days reaching a final concentration of 2 μg/mL. On the other hand, the chlorophyll concentration in 1:1 ratio was almost stable throughout the experimentation period where the initial concentration was 1.96 μg/mL reaching a final concentration of 2 μg/mL. In 1:2 the chlorophyll content slightly decreased from 1.1 μg/mL reaching a final concentration of 1.04 μg/mL at the end of incubation period. It can be observed from the chlorophyll content in all the tested ratios that one of the growth factors such as the non-saline environment was not supporting the cyanobacterial cell's growth and could be responsible for the reduction in chlorophyll concentration as NaCl is one of the important salts for the growth of *Phormidium* sp38.

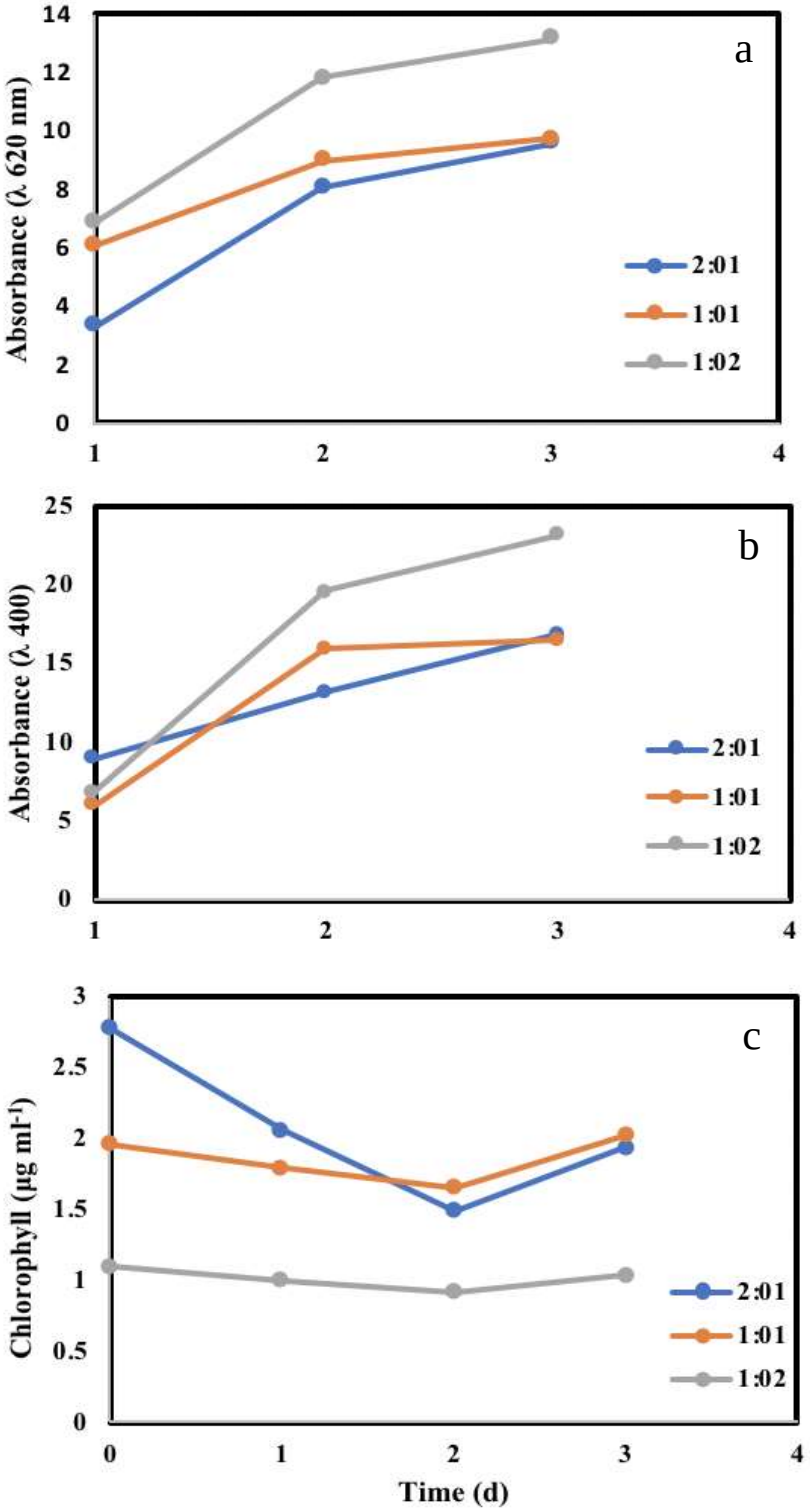

Figure 8: The growth behavior of cyanobacteria and bacteria at different mixing ratios in glucose-supplemented co-cultures a) Absorbance at 620 nm b) Absorbance at 400 nm c) Chlorophyll content

3.2 Sorbitol

Sorbitol is a naturally occurring sugar alcohol in some fruits such as apples, apricots, and cherries and can be industrially produced by the hydrogenation of glucose (Tiefenbacher, 2017). It is widely used in the food industry as a low-calorie sweetener, humectant, texturizer, or softener and it has also been used as a starting material for the production of many pharmaceuticals such as sorbose or ascorbic acid (Gérard & Rabot, 2010). Furthermore, sorbitol can be used as a probiotic due to its poor absorption in the small intestine, bacteria in the colon utilize it as a fermentation substrate (Zarour et al., 2017). Limited studies showed that sorbitol has been used as a carbon substrate for H_2 production. Therefore, the current study analyzed the impact of utilizing sorbitol on H_2 production.

3.2.1 Impact of mixing ratios

3.2.1.1 Cumulative gas production

When incubated under anaerobic conditions, sorbitol-supplemented co-cultures produced a mixture of gases H_2, O_2, N_2, and CO_2. Figure 9 represents the total volume of the gas mixture and individual total volumes of gases accumulated in the reactor headspace by the optimum ratio 2:1 throughout the experimentation period. The maximum volume of the cumulative gas produced using 2:1 ratio is 2950 ml L^{-1} consisting mainly of H_2 (980 ml L^{-1}), CO_2 (1014 ml L^{-1}), N_2 (663 ml L^{-1}), and fractions of O_2 (166 ml L^{-1}). It can be observed that H_2 has the highest concentration (33%) of the total accumulated gas. Followed by CO_2 and N_2 which are 34%% and 22% respectively of the total accumulated gases. Moreover, few amounts of O_2 evolved in sorbitol-supplemented co-culture (5%) of the total cumulative gases.

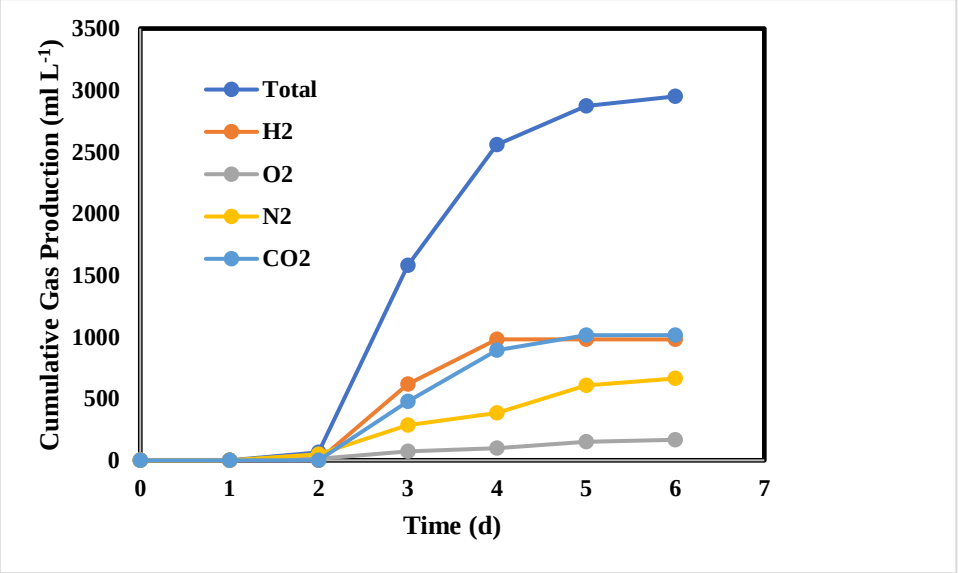

Figure 9: Cumulative gas production at optimum co-culturing ratio in sorbitol supplemented co-culture

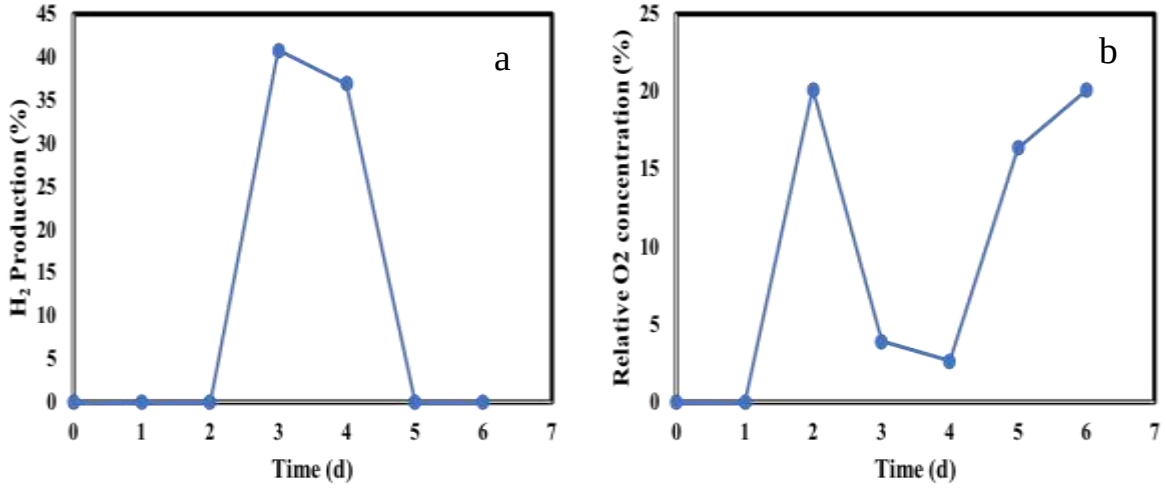

Figure 10: The relationship between the daily H_2 and O_2 production, a) H_2 production and b) Relative O_2 concentration

There is an obvious correlation between the amount of produced H_2 and O_2. The maximum H_2 production was obtained on the same days (Day 3 and Day 4) when O_2 concentration was at a minimum level as shown in Figure 10. This can be explained by the symbiotic relationship between cyanobacteria and ASB which worked significantly to uptake the produced O_2 which may enhance the potential of

the co-culture to produce more H_2 by maintaining the anaerobic condition required for fermentative H_2 production.

The O_2 concentration started to again rise which might be due to photosynthetic activity in the presence of light however H_2 started to decline again after the increase in O_2 concentration. It can be concluded from the results that sorbitol supplementation could support fermentation more than photosynthesis which can be observed from the amount of evolved H_2 which was produced simultaneously with a high amount of CO_2. On the other hand, the minor amount of O_2 indicates either low photosynthetic activity or the ability of the ASB to uptake O_2 in the presence of sorbitol.

The metabolically engineered strain of *E. coli* (DJT135) showed the ability to ferment different sugars and sugar derivatives including sorbitol to produce H_2. The maximum H_2 yield was 1.5 mol H_2 mol^{-1} (Ghosh & Hallenbeck, 2009). Another study analyzed the potential of *Enterobacter aerogenes* strain (E.82005) to produce H_2 in batch cultures using different carbon substrates including sorbitol, the maximum yield obtained was 1.6 mol mol^{-1} sorbitol (Nakashimada et al., 2002).

3.2.1.2 TOC concentration

Figure 11 represents the changes in the concentration of TOC in sorbitol-supplemented co-culture. It can be observed that there was an accumulation in the TOC during the first days of the experiment. The accumulation of the TOC was least observed in ratio 2:1 ratio; however, in 1:1 and 1:2 ratio it was increased reaching a maximum TOC concentration of 6.73 g/L and 7.73 g/L, respectively. However, in 2:1 ratio, the initial TOC concentration was 4.4 g/L reaching a final concentration of 3.6 g/L at the end of the experimentation period. It can be concluded that the utilization of the carbon content using the 2:1 ratio was better than the 1:1 and 1:2 ratios.

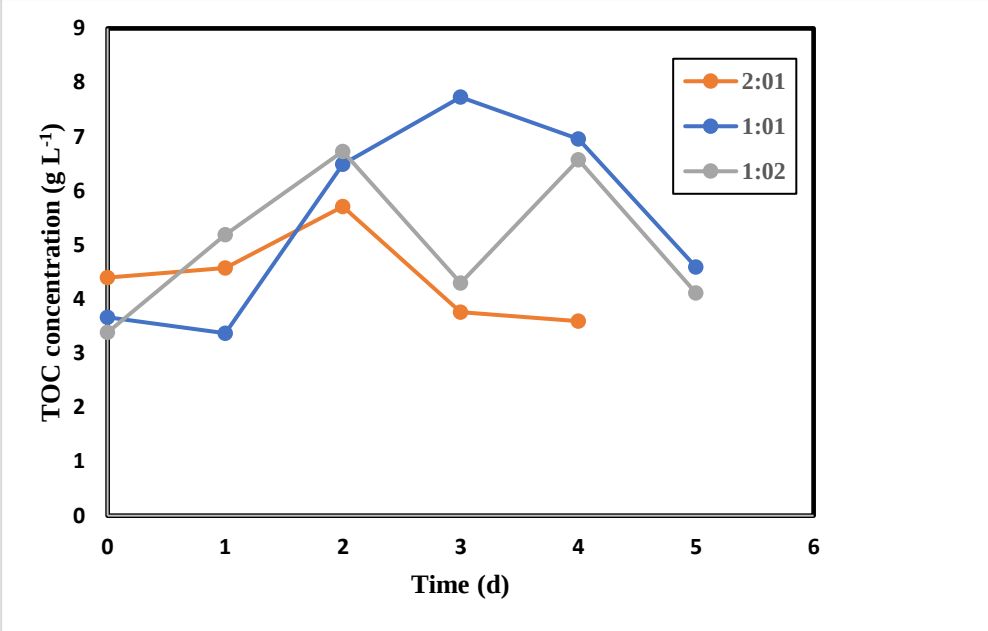

Figure 11: TOC concentration at different mixing ratios in sorbitol-supplemented co-cultures

3.2.1.3 Optical density and chlorophyll measurement

Absorbance at 620 nm and 400 nm and chlorophyll measurements were obtained to examine the effect of the carbon source (sorbitol) on growth behavior. OD_{600} measurements shown in Figure 12 (a) indicated that sorbitol supplementation did not show a significant increase in algal growth in all the tested ratios. For example, in 2:1 ratio there was an increase in OD value on the first day of the experiment then it started to decrease gradually from day 2 till the end of the experiment. Also, the growth in 1:2 ratio there was a significant increase in growth only from day 1 to day 2 then it became stable. However, ratio 1:1 showed better growth compared to the other ratios which increased from day 1 till the last day of the experiment. Based on the OD_{400} Figure 12 (b), it can be observed that sorbitol supplementation started to support bacterial growth in 2:1 and 1:2 ratio after day 2; however, there was a decline in bacterial growth in 1:1 ratio indicating no bacterial growth. In terms of chlorophyll content Figure 12 (c), there was an increase in 2:1 and 1:2 ratio from 0.6 µg/mL and 0.4 µg/mL reaching a maximum chlorophyll concentration of 1.2 and 0.8 µg/mL, respectively. However, there was no significant

change in chlorophyll content in ratio 1:1. Therefore, it can be concluded from the growth behavior of cyanobacteria and bacteria in sorbitol supplemented medium that sorbitol might not be one of the preferred carbon substrates that could support the heterotrophic growth of the currently studied cyanobacteria or the microbial community in the ASB.

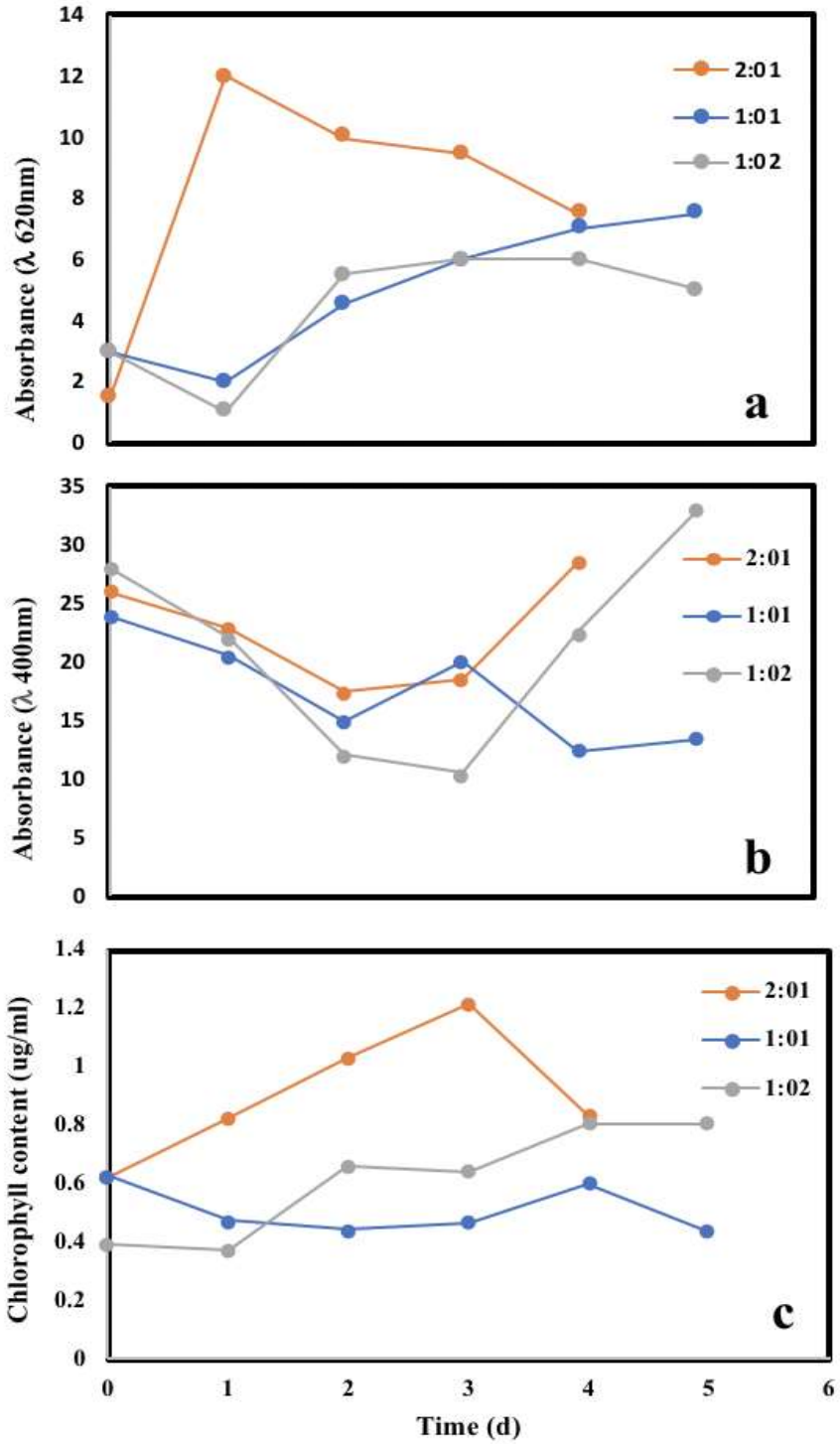

Figure 12: The growth behavior of cyanobacteria and bacteria at different mixing ratios in glucose-supplemented co-cultures a) Absorbance at 620 nm b) Absorbance at 400 nm c) Chlorophyll content

3.3 Mannitol

Mannitol is a polyol (sugar alcohol) and an isomer of the sugar alcohol "sorbitol"(Hiremath et al., 2018). It is considered as one of the most commonly used hyperosmotic agents. Many plants, algae, fungi, and bacteria naturally contain mannitol, which is used for energy storage, fermentation, osmoregulation, scavenging of reactive oxygen species, and pathogen defense. Commercial mannitol used in food and medical industries is produced through either the chemical hydrogenation of fructose or by extracting it from seaweed. Mannitol has many medical applications. For intracranial hypertension treatment, and as a scavenging agent for free radicals and reactive oxygen species.

Mannitol applications in water or wastewater quality or energy production are rare in the literature. However, seaweed's mannitol has been used in one of the previously conducted studies as a substrate for H_2 production giving an optimal specific H_2 yield of 224.2 ml H_2/g mannitol (Xia et al., 2015). Another study showed the introduction of mannitol to a nitrogen-rich wastewater sample increases the microbial abundance of the bacteria encoding nitrous oxide reductase genes, enhanced dehydrogenase activity, and antioxidant enzymes (X. Zhang et al., 2016).

3.3.1 Impact of mixing ratios

3.3.1.1 Cumulative gas production

Co-cultures with three different mixing ratios 2:1,1:1 and 1:2 were supplemented with mannitol as an exogenous carbon source. The gas production lasted for 3 days prior to process inhibition. The total cumulative gas produced using 2:1, 1:1, and 1:2 ratio was 3297 mL L^{-1}, 3243 mL L^{-1}, and 3063 mL L^{-1}, respectively, as shown in Figure 13. The maximum amount of H_2 produced using 2:1 ratio as 561.7 mL L^{-1} which was higher than the amount produced using 1:1 and 1:2 ratio as 310 mL L^{-1} and 278 mL L^{-1}, respectively. H_2 occupied 17% of the total accumulated gas in the reactor's headspace of 2:1 co-culture. Whereas 1:1 and 2:1 co-culture produced H_2 to be 9% of the total accumulated gas using both ratios.

Mannitol is more reduced sugar than glucose therefore it can provide more reducing equivalents that might enhance H_2 production. It can be concluded that mannitol was not fermented efficiently by the microorganisms in the culture resulting in low CO_2 concentration and consequently low fermentative H_2 production. Although mannitol should give a higher H_2 yield however the higher yield is attributed to the use of heat-pre-treated sludge. In contrast, the activated sludge in this study was not treated therefore there might be a probability of the presence of H_2-consuming microorganisms that could shift the metabolic pathway obtaining a lower H_2 yield.

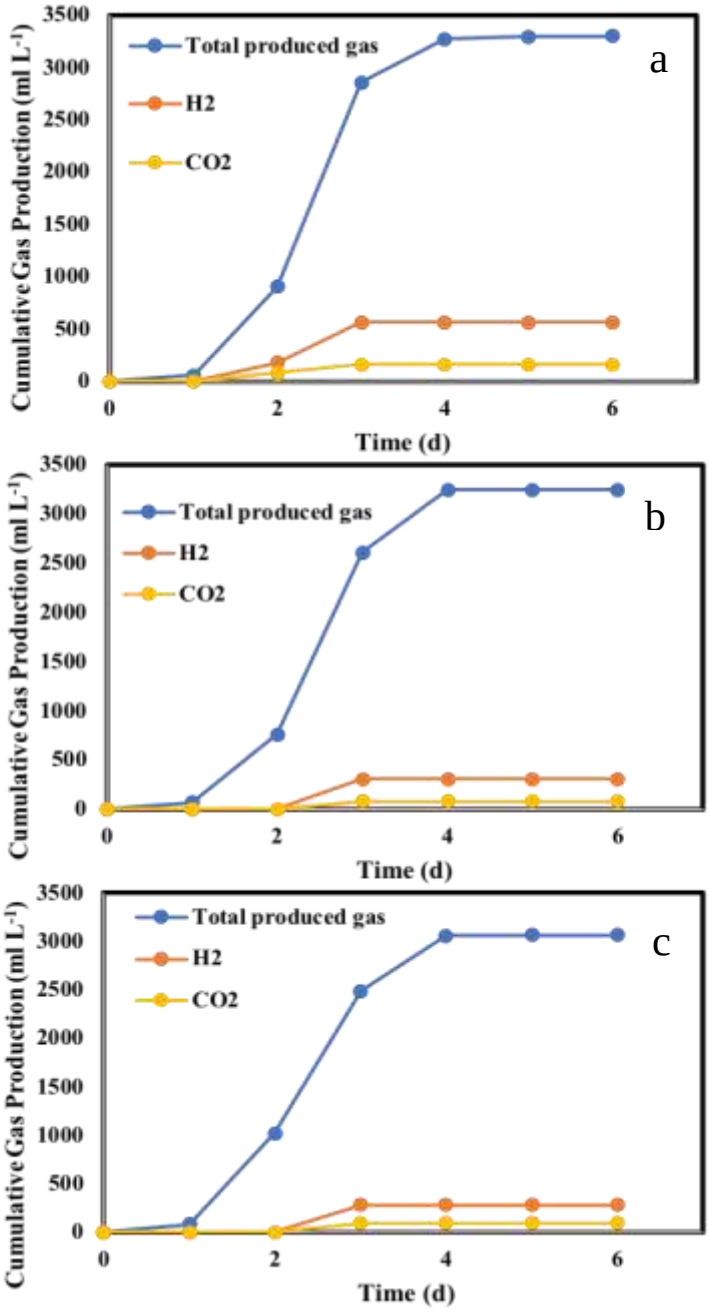

Figure 13: Cumulative gas production using different mixing ratios in mannitol supplemented co-cultures a) 2:1 b) 1:1 c) 1:2

3.3.1.2 TOC concentration

The concentration of the TOC of the mannitol-supplemented co-culture was quantified throughout the experimentation period to evaluate the capability of the involved microorganisms to utilize the TOC content in the culture medium for different microbial activities. In all of the co-culture ratios, there was a reduction in the TOC concentration and the microorganisms were able to degrade the carbon content in 5 days period. The initial TOC concentrations in 2:1, 1:1, and 1:2 was 3.3,3.8, and 4.16 g/L respectively, as shown in Figure 14. Where the final TOC concentration for all the tested ratios was 1.5 g/L. Thus, 2:1, 1:1 and 1:2 consumed 54.5%,60.5% and 64% respectively of the TOC content by the end of the experimentation period. It can be concluded that the microorganisms in all co-cultures were able to use mannitol as an exogenous carbon substrate to support their growth or other microbial activities such as nutrient removal processes or in H_2 production. However, 2:1 ratio consumed more carbon content than 1:1 and 1:2 ratio. Therefore, the co-culture with co-culturing ratio 1:2 consumed the highest amount of TOC based on the higher number of heterotrophic bacteria present in the culture which relies on the carbon content to support their growth. Moreover, based on the amount of produced H_2 it can be assumed that the microbes in the culture might degrade part of the carbon content to enhance H_2 production.

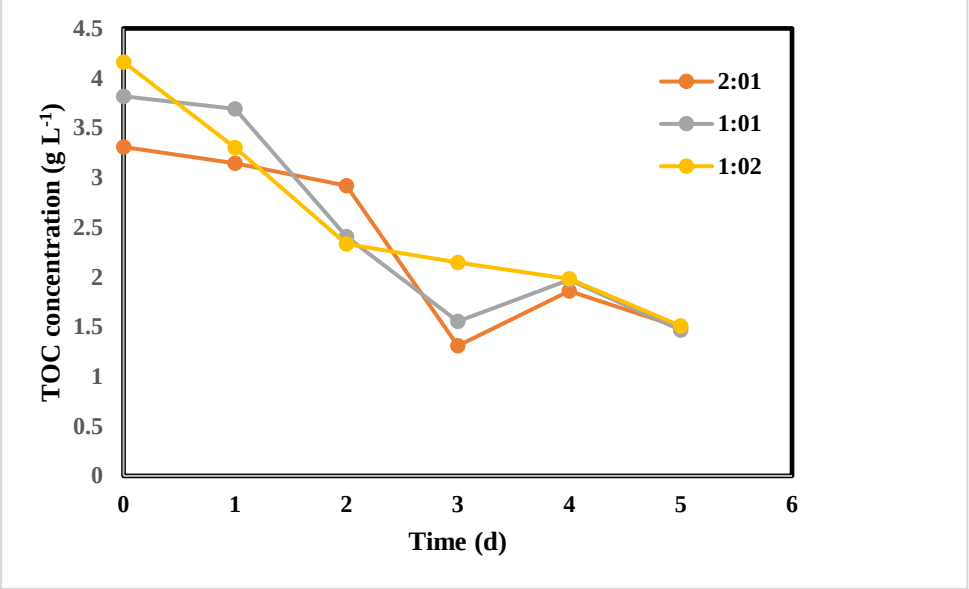

Figure 14: TOC concentration at different mixing ratios in mannitol-supplemented co-cultures

3.3.1.3 Optical density and chlorophyll measurement

Optical density at absorbances 620 nm and 400 nm and chlorophyll measurements were obtained to study the effect of mannitol on microbial growth. Based on the OD_{600} and chlorophyll content of all the tested ratios, it was observed that the cyanobacterial cells can survive for a short period of time maximum of 2-3 days in all the tested ratios under heterotrophic conditions in the presence of mannitol. The initial absorbance values at 620 nm in 2:1,1:1, and 1:2 was 1.5, 5, and 2, respectively, as shown in Figure 15 a. The growth pattern was increased reaching a maximum value on day 2 of 7.5,12.5, and 9.5. After day 2, a decline in absorbance value was observed in all tested ratios reaching a final absorbance value equal to the initial absorbance values.

Similarly, the OD_{400} (absorbances at 400 nm) suggests that mannitol supplementation could support the heterotrophic growth of the ASB to some extent. As shown in Figure 15 b, there was an increase in growth behavior in all the tested ratios. The initial absorbance values in 2:1 , 1:1 and 1:2 were 1, 11, and 2.5 respectively. The increase in growth was observed reaching maximum absorbances of 16, 23.5 and 17.5 on day 2. After day 2, the growth behavior started to decline again

to reach 9, 18 and 9.5. Based on the observations in OD400 measurements for both cyanobacteria and ASB it can be concluded that mannitol led to the increase in their biomass within a short period of time. However, a sudden decline that happened after the second day might be due to several reasons such as the depletion of essential nutrients or the reduction in pH value.

Similarly, In all the inoculum ratios, chlorophyll concentration started to rise on the first day of the experiment in ratios 2:1 and 1:1 from 2.4 and 2.56 μg/mL reaching a maximum concentration of 4.16 and 5.12 μg/mL then it started to decline again till the end of the experiment reaching a final concentration of 1.96 and 2.22 μg/mL, respectively, as shown in Figure 15 c. However, in 1:2 ratio, the chlorophyll concentration continues to increase after Day 2 till the last day of the experiment from 1.26 μg/mL to 3.36 μg/mL. The observed trend suggests that mannitol could support the algal growth to some extent in all the tested ratios.

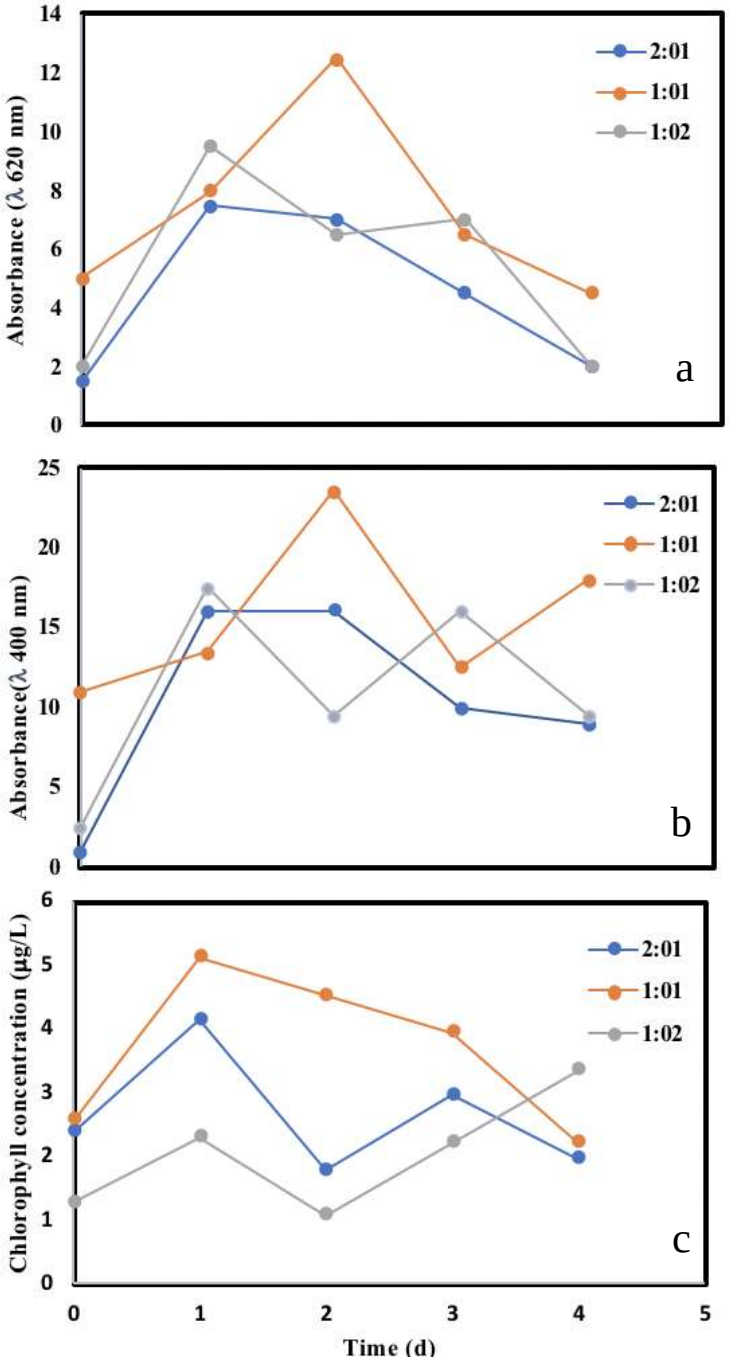

Figure 15: The growth behavior of cyanobacteria and bacteria at different mixing ratios in mannitol supplemented co-cultures a) Absorbance at 620 nm b) Absorbance at 400 nm c) Chlorophyll content

3.4 Impact of salinity on H_2 production

Although salts are necessary to support life on earth, excessive salt concentrations have a number of negative impacts, including the inhibition of important biochemical machinery (Al-Saari et al., 2019). Ionic strength is one the of key factors affecting H_2 generation and can either stimulate or hinder H_2 production. Low ionic strength may not affect H_2 production or perhaps be stimulatory. In contrast, high ionic strength may cause cell lysis. Therefore, an optimum ionic strength is necessary for optimized H_2 production (Alshiyab et al., 2008). Sodium (Na^+), magnesium (Mg^{2+}), zinc (Zn^{2+}), and iron (Fe^{3+}) are the most essential metal ions affecting H_2 production (Lin & Lay, 2005) .Na^+ is one of the key elements for bacterial growth (Van Niel et al., 2003), substrate uptake, and the yield of fermentative metabolites (Casey et al., 2013). Osmotic stress caused by high salinity (> 1.2% w/v NaCl) inhibits the growth of gram-negative, non-halophilic bacteria, which ultimately impacts their fermentative metabolism (Nakamura, 1977) . When these microbes are exposed to high salt concentrations, they can experience both osmotic stress and ion toxicity (Garcia et al., 1997). Although Na^+ ions are frequently found in wastewater in low concentrations. However high Na^+ concentrations could have an inhibitory effect on sewage and activated sludge treatment (Hao et al., 2006).

The optimum salt concentration for growing the cyanobacterial strain used in the current study *Phormidium keutzingianum* is 1%. However, the behavior of this cyanobacteria might differ during the anaerobic fermentation. Therefore, this section will discuss the effect of different salt concentrations (0,10, and 20 g/L) on the total gas and H_2 production.

The effect of salinity on H_2 production was evaluated using different salt concentrations 0,10 and 20 g_{Nacl} L^{-1}. Results shown Figure 16**Error! Reference s ource not found.** indicated that the maximum gas production obtained by glucose-supplemented co-cultures was 2275 ml L^{-1} at 0 g_{Nacl} L^{-1}. Further increase in salt concentration led to the reduction of the total produced gas. The total produced gas using salt concentrations of 10 and 20 g L^{-1} was 908 and 734 ml L^{-1}, respectively. Therefore, the increase in salt concentration caused 64% and 71% reduction, in 10

g_{NaCl} L^{-1} and 20 g_{NaCl} L^{-1} respectively, in total produced gas. On the other hand, the maximum cumulative gas produced by sorbitol-supplemented co-culture was 2950 mL L^{-1} and 2856 mL L^{-1} in non-saline conditions and at a salt concentration of 10 g_{NaCl} L^{-1} respectively. Further increase in salt concentration caused a reduction in cumulative gas production as a salt concentration of 20 g_{NaCl} L^{-1} produced a total gas of 1900 ml L^{-1}. The reduction in cumulative gas production was also observed in mannitol-supplemented co-cultures. The total amount of gas produced in non-saline conditions was 3297 ml L^{-1} a further increase in salt concentrations to 10 and 20 g_{NaCl} L^{-1} led to a reduction in the cumulative gas production 1664 ml $L^{-1}$and 1610 ml L^{-1}.

In terms of H_2 production, NaCl inhibits H_2 production by the glucose-supplemented co-culture. However, sorbitol and mannitol-supplemented co-cultures tolerated H_2 production in NaCl containing medium. Sorbitol-supplemented co-culture produced the maximum amount of H_2 produced by NaCl-free co-culture was 980 ml L^{-1} followed by 480 ml L^{-1} of H_2 by the co-culture grown in 10 g_{Nacl} L^{-1}. Further increase in NaCl concentration led to a reduction in the cumulative produced H_2 as 176.8 ml L^{-1} at 20 g_{Nacl} L^{-1}. The addition of salt 10 g_{Nacl} $L^{-1}$and 20 g_{Nacl} L^{-1} to the sorbitol supplemented co-culture resulted in a 50% and 82% reduction in the amount of the cumulative H_2, respectively. Mannitol supplemented co-culture produced 562 ml L^{-1} in NaCl-free co-culture whereas the coculture with a salt concentration of 10 g_{Nacl} L^{-1} produced 470 ml L^{-1}. Further increase in salt concentration led to a reduction in the cumulative H_2 as 333 ml L^{-1} produced by co-culture using salt concentration of 20 g_{Nacl} L^{-1}.

The results suggest that the optimum salinity level for H_2 production by the currently studied co-cultures is 0% salinity whatever the type of substrate used (glucose, sorbitol, or mannitol). However, the resistance of the microbes to the change in the salinity level in sorbitol and mannitol-containing mediums was better than in the glucose-containing medium according to the percent of the reduction in gas production. A similar reduction in H_2 production as a result of salt addition was observed in previous investigations where it was studied the impact of salt added to a fermentation medium for H_2 production (Alshiyab et al., 2008). The results found that

the productivity of *C.acetobutylicum* decreased by 18% from 0 to 5 g_{Nacl} L^{-1} as a result of the negative impact of salt addition on glucose degradation. Similarly, it was found that the H_2 production in heat-treated anaerobic mixed cultures decreased by 64% when the salt concentration was increased from 0 to 8.4 g_{Nacl} L^{-1} (Zheng et al., 2005). Another study showed that the abundance of H_2-producing species belonging to genera *Clostridium*, *Escherichia*, and *Enterobacter* was significantly lowered at a salt concentration above 9 g_{Nacl} L^{-1}(Pierra et al., 2014). A reduction in H_2 production of mixed anaerobic culture consists of sewage sludge *C.butyricum* and *C.acetobutylicum* as a result of increasing the concentration of NaCl from 0-30 g/L (Zheng et al., 2005). Another study investigated the ability of the *Clostridium butyricum* TM-9A to produce H_2 in the presence of various NaCl (2.0 – 20 g/L) concentrations (Pierra et al., 2014). The optimum H_2 production of 61 mmol L^{-1} was attained in the absence of NaCl. With the increase in salt content up to 20 g/L (2%) there was a gradual reduction in H_2 production. Niel et al. (2003) inferred that the increase in the concentration of NaCl in the fermentation medium led to the reduction in H_2 production and proposed that this reduction is due to the increase in ionic strength in the fermentation medium that could have an impact on the metabolic pathway of the bacteria.

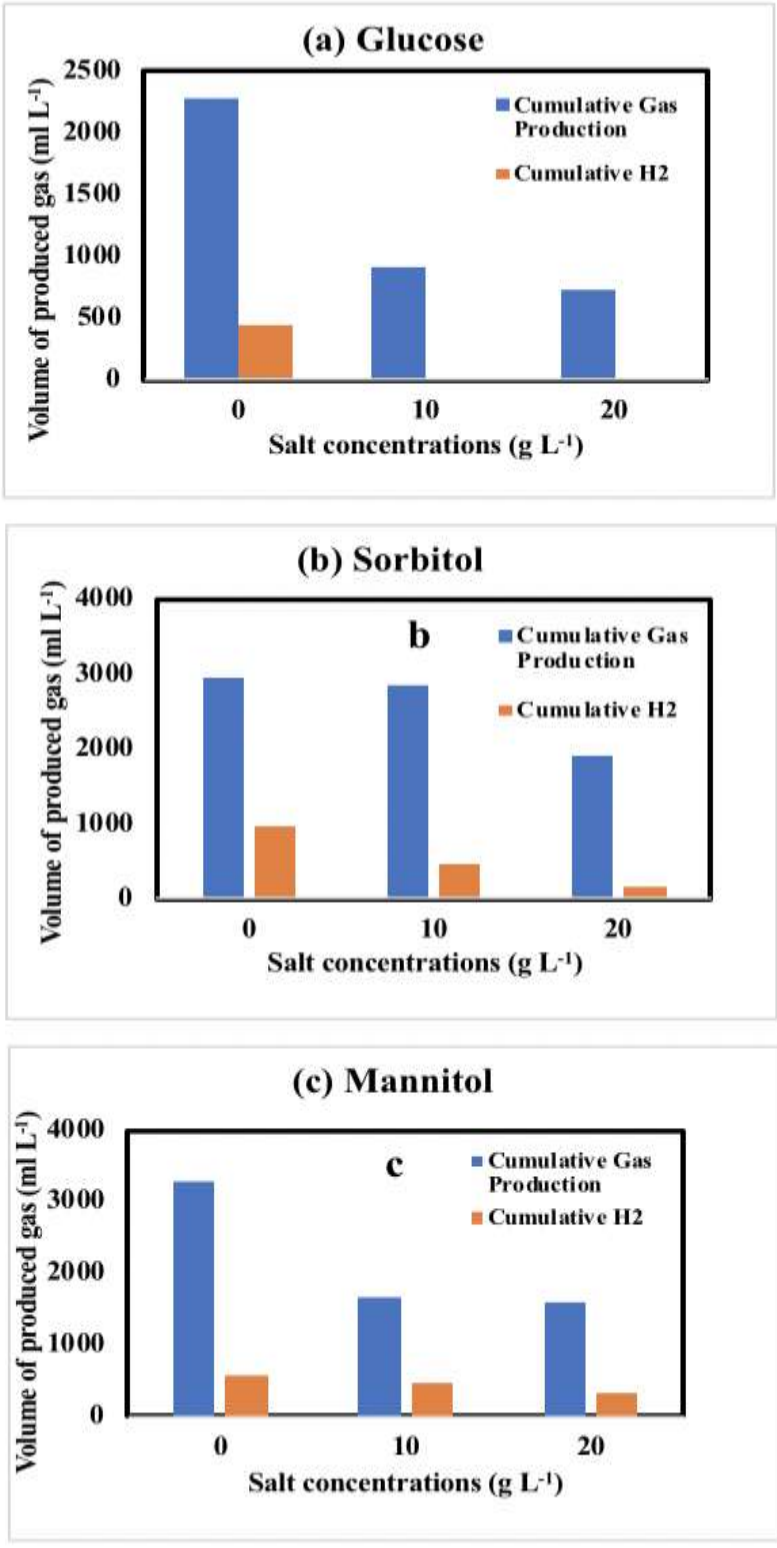

Figure 16: The effect of different salt concentrations on gas production

3.5 The effect of pH changes on H_2 production

The degree of substrate hydrolysis, hydrogenase activity, and metabolic pathways are all generally impacted by pH, which is a crucial factor in fermentation (Kim et al., 2011). Hence, variations in substrate and energy usage, protein synthesis, the creation of different storage products, and metabolite levels all respond differently to pH changes (Mu et al., 2006). The operational pH also influences the relative amounts of microbial species in mixed anaerobic cultures (Cappai et al., 2014). Extremely acidic or basic pH levels can have a negative impact on the activity of H_2-producing bacteria. As ATP is needed to sustain cell neutrality rather than producing H_2. Hydrogenase activity can be inhibited by low pH (Mohd Yasin et al., 2011). In fermentation metabolism, the pH drop is an indication of the VFA generation and the buffering capacity of the system. The production of VFA affects the buffering capacity of the system which further results in a decline in the pH of the system. A drop in the pH related to the accumulation of organic acids could lead to the inhibition of H_2 production (Poggi-Varaldo & Oleszkiewicz, 1992).

It can be observed from the change in pH values in all co-cultures supplemented with glucose, sorbitol or mannitol that the acidity of the system increased throughout the experimentation period. For example, the initial pH in glucose-supplemented co-cultures as shown in Figure 17 (a) was around 8 – 9 reaching a final pH value of 5. Similarly, the initial pH value in sorbitol-supplemented co-cultures Figure 17 (b) was 7 and decreased gradually to reach a final pH value of 5 – 6. In addition, there was a gradual reduction in the pH values in all mannitol-supplemented co-cultures Figure 17 (c) starting with a pH value of 8 – 9 and ending up with pH value of 5.5.

The reduction in the pH value was also observed in saline conditions Figure 17 (d),(e) and (f). For example, the initial pH values of the co-cultures grown in glucose, sorbitol and mannitol-containing medium and in the presence of 10 g/L salt were 9, 7, and 8 then pH values were reduced gradually to reach 7, 6, and 5.7. similarly, the initial pH values in the similar co-cultures but grown in the presence of 20 g/L NaCl were 8, 6.48 and 7.7. the final pH 6.1, 5.5, and 5.5. In contrast, it can be observed that

the final pH value in saline conditions was higher than in non-saline conditions which could be related to the inability of microbes to further degrade the carbon substrate and produce more protons that lower the pH value.

The maximum H_2 production was obtained from day 1 to day 3 at a pH range of 7.3 – 5.1 in glucose-supplemented co-cultures. Whereas in sorbitol-supplemented the maximum H_2 production was obtained from day 3 to day 4 at a pH range of 7.3 – 5.1 Similarly the maximum H_2 production in mannitol-supplemented coculture was obtained from day2 -day3 at a pH range 7-5. Therefore, it can be concluded from the results that a pH value less than 6 could be considered as the inhibitive pH level of H_2 production. The acidification of the system is attributed to the accumulation of volatile fatty acids in the system throughout the experimentation period. Acid accumulation resulted in a pH drop and therefore inhibited H_2 production. At a pH level below 5, bacteria cannot maintain their metabolic activity (Mohan et al., 2007). The average optimum pH for fermentative H_2 production reported in previous studies varies from 5 – 6 (Jun et al., 2008). It has been demonstrated by a previous study that a pH less than 6 could reduce the chlorophyll content and inhibit microbial growth preventing the microbial community from generating more electrons and protons necessary for H_2 production. Another reason for stopping H_2 production at low pH is the incapability of

the bacterial partner in the co-culture to degrade the endogenous or exogenous carbon required to provide more protons in acidic conditions.

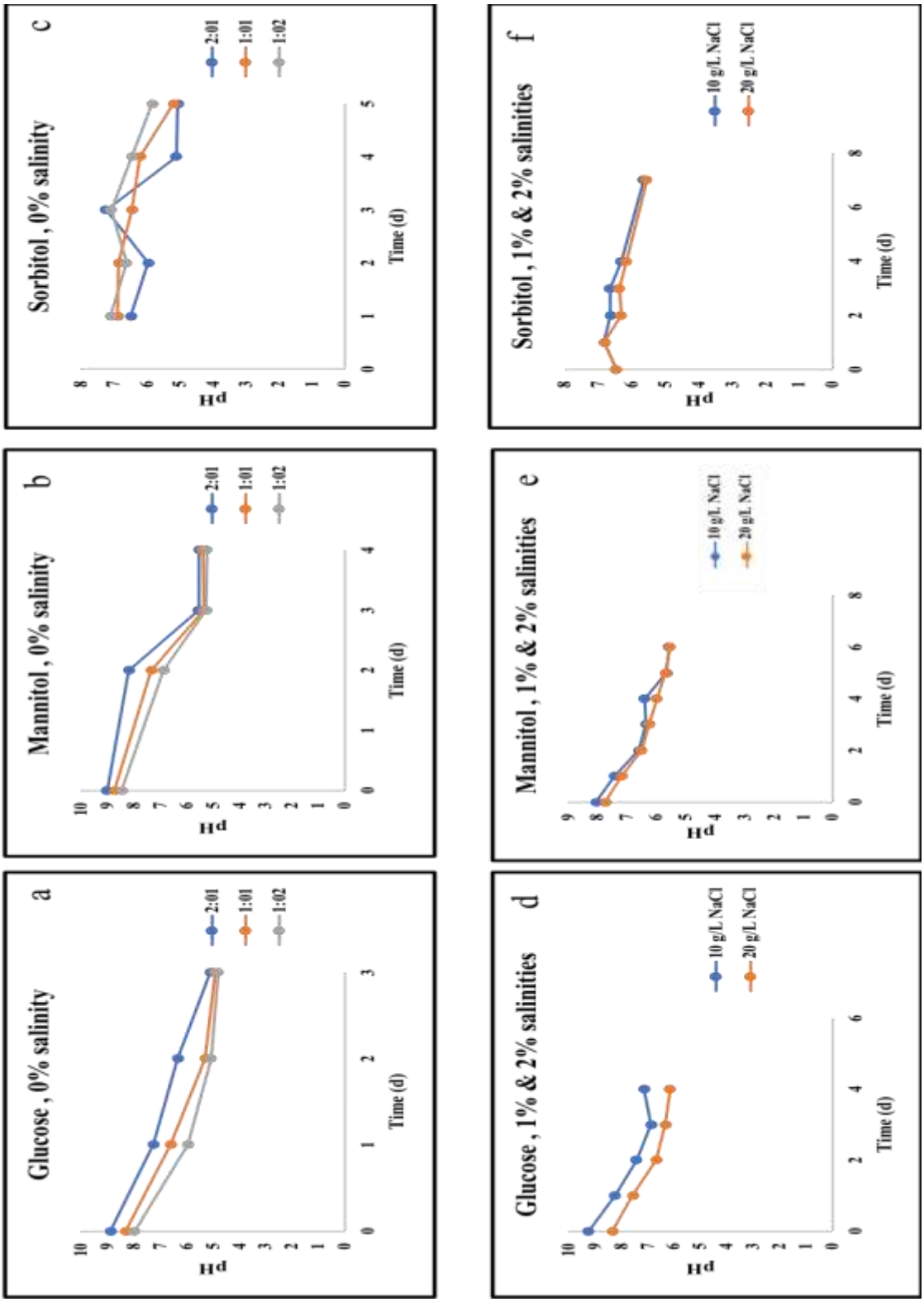

Figure 17: pH Changes in non-saline and saline conditions

Chapter 4: Conclusion

This study aimed to study the impact of different coculturing ratios, exogenous carbon substrates (glucose sorbitol and mannitol), and salinity on the biological H_2 production by a co-culture consisting of the halophytic cyanobacterium *Phormidium keutzingianum* and activated sludge bacteria.

It was found that there was a significant difference in the amount of cumulative H_2 produced by different carbon supplementation. Sorbitol supplementation resulted in the maximum amount of H_2 (980 ml L^{-1}) compared to other supplementations. The results also indicated that the addition of salt (NaCl) negatively affected H_2 production. By increasing the salinity level from 0-2%, the amount of H_2 produced by sorbitol-supplemented co-culture was reduced from (980 ml L^{-1} ml) to (176 ml L^{-1}). Therefore, based on the obtained results, it can be concluded that sorbitol could be one of the optimum sugar substrates for biological H_2 production by the studied algal/bacterial co-cultures. On the other hand, the addition of salt causes a negative effect on the ability of the microorganisms to produce H_2. Therefore, a non-saline environment is a suitable environment for H_2 production for the currently studied microorganisms.

Establishing a microbial consortium consisting of cyanobacteria and activated sludge bacteria could improve biological H_2 production via biophotolysis. Moreover, as a result of the symbiosis between microalgae and activated sludge bacteria, the developed microbial consortia can produce H_2 as a valuable by-product from wastewater. Therefore, this study can contribute to making H_2 production and wastewater treatment processes more sustainable and environmentally friendly. Although different carbon sources showed a significant difference in the amount of the produced H_2. However, further studies are required on the metabolic pathways of different H_2-producing algal or bacterial species by utilizing different organic substrates. In addition, studying the effect of stress conditions such as salinity on H_2 production and finding possible ways to maximize H_2 production in such conditions could contribute to alleviating the cost of biological H_2 production.

References

Akhlaghi, N., & Najafpour-Darzi, G. (2020). A comprehensive review on biological hydrogen production. *International Journal of Hydrogen Energy*, *45*(43), 22492–22512. https://doi.org/10.1016/j.ijhydene.2020.06.182

Al-Saari, N., Amada, E., Matsumura, Y., Tanaka, M., Mino, S., & Sawabe, T. (2019). Understanding the nacl-dependent behavior of hydrogen production of a marine bacterium, vibrio tritonius. *PeerJ*, *2019*(4), 2–16. https://doi.org/10.7717/peerj.6769

Alshiyab, H., Kalil, M. S., Hamid, A. A., & Wan Yusoff, W. M. (2008). Effect of salts addition on hydrogen production by C. acetobutylicum. *Pakistan Journal of Biological Sciences*, *11*(18), 2193–2200. https://doi.org/10.3923/pjbs.2008.2193.2200

Balachandar, G., Varanasi, J. L., Singh, V., Singh, H., & Das, D. (2020). Biological hydrogen production via dark fermentation: A holistic approach from lab-scale to pilot-scale. *International Journal of Hydrogen Energy*, *45*(8), 5202–5215. https://doi.org/10.1016/j.ijhydene.2019.09.006

Balat, M. (2008). Potential importance of hydrogen as a future solution to environmental and transportation problems. *International Journal of Hydrogen Energy*, *33*(15), 4013–4029. https://doi.org/10.1016/j.ijhydene.2008.05.047

Ban, S., Lin, W., Wu, F., & Luo, J. (2018). Algal-bacterial cooperation improves algal photolysis-mediated hydrogen production. *Bioresource Technology*, *251*, 350–357. https://doi.org/10.1016/j.biortech.2017.12.072

Bao, M., Su, H., & Tan, T. (2012). Biohydrogen production by dark fermentation of starch using mixed bacterial cultures of Bacillus sp and Brevumdimonas sp. *Energy and Fuels*, *26*(9), 5872–5878. https://doi.org/10.1021/ef300666m

Bolatkhan, K., Kossalbayev, B. D., Zayadan, B. K., Tomo, T., Veziroglu, T. N., & Allakhverdiev, S. I. (2019). Hydrogen production from phototrophic microorganisms: Reality and perspectives. *International Journal of Hydrogen Energy*, *44*(12), 5799–5811. https://doi.org/10.1016/j.ijhydene.2019.01.092

Cappai, G., De Gioannis, G., Friargiu, M., Massi, E., Muntoni, A., Polettini, A., Pomi, R., & Spiga, D. (2014). An experimental study on fermentative H2 production from food waste as affected by pH. *Waste Management*, *34*(8), 1510–1519. https://doi.org/10.1016/j.wasman.2014.04.014

Casey, E., Mosier, N. S., Adamec, J., Stockdale, Z., Ho, N., & Sedlak, M. (2013). Effect of salts on the Co-fermentation of glucose and xylose by a genetically engineered strain of Saccharomyces cerevisiae. *Biotechnology for Biofuels*, *6*(1), 83–92. https://doi.org/10.1186/1754-6834-6-83

Chandra, R., & Venkata Mohan, S. (2014). Enhanced bio-hydrogenesis by co-culturing photosynthetic bacteria with acidogenic process: Augmented dark-photo fermentative hybrid system to regulate volatile fatty acid inhibition. *International Journal of Hydrogen Energy*, *39*(14), 7604–7615. https://doi.org/10.1016/j.ijhydene.2014.01.196

Chastain, J. P. (2017). Solids and plant nutrient content, and settling characteristics of milking centre wastewater on a grazing dairy. *Chemical Engineering Transactions*, *58*, 757–762. https://doi.org/10.3303/CET1758127

Chen, Y., Cheng, J. J., & Creamer, K. S. (2008). Inhibition of anaerobic digestion process: A review. In *Bioresource Technology* (Vol. 99, Issue 10, pp. 4044–4064). https://doi.org/10.1016/j.biortech.2007.01.057

Chi, J., & Yu, H. (2018). Water electrolysis based on renewable energy for hydrogen production. *Cuihua Xuebao/Chinese Journal of Catalysis*, *39*(3), 390–394. https://doi.org/10.1016/S1872-2067(17)62949-8

Chojnacka, K., & . F.-J. M. (2003). Kinetic and Stoichiometric Relationships of the Energy and Carbon Metabolism in the Culture of Microalgae. *Biotechnology(Faisalabad)*, *3*(1), 21–34. https://doi.org/10.3923/biotech.2004.21.34

Clark, D. P. (1989). The fermentation pathways of Escherichia coli. *FEMS Microbiology Letters*, *63*(3). https://doi.org/10.1016/0378-1097(89)90132-8

Cole, J. K., Hutchison, J. R., Renslow, R. S., Kim, Y. M., Chrisler, W. B., Engelmann, H. E., Dohnalkova, A. C., Hu, D., Metz, T. O., Fredrickson, J. K., & Lindemann, S. R. (2014). Phototrophic biofilm assembly in microbial-mat-derived unicyanobacterial consortia: Model systems for the study of autotroph-heterotroph interactions. *Frontiers in Microbiology*, *5*(APR). https://doi.org/10.3389/fmicb.2014.00109

Das, D., & Veziroglu, T. N. (2008). Advances in biological hydrogen production processes. *International Journal of Hydrogen Energy*, *33*(21), 6046–6057. https://doi.org/10.1016/j.ijhydene.2008.07.098

Dutta, D., De, D., Chaudhuri, S., & Bhattacharya, S. K. (2005). Hydrogen production by Cyanobacteria. *Microbial Cell Factories*, *4*, 36. https://doi.org/10.1186/1475-2859-4-36

Fakhimi, N., Dubini, A., Tavakoli, O., & González-Ballester, D. (2019). Acetic acid is key for synergetic hydrogen production in Chlamydomonas-bacteria co-cultures. *Bioresource Technology*, *289*. https://doi.org/10.1016/j.biortech.2019.121648

Fakhimi, N., Gonzalez-Ballester, D., Fernández, E., Galván, A., & Dubini, A. (2020). Algae-Bacteria Consortia as a Strategy to Enhance H2 Production. In *Cells* (Vol. 9, Issue 6). https://doi.org/10.3390/cells9061353

Fakhimi, N., Tavakoli, O., Marashi, S. A., Moghimi, H., Mehrnia, M. R., Dubini, A., & González-Ballester, D. (2019). Acetic acid uptake rate controls H2 production in Chlamydomonas-bacteria co-cultures. *Algal Research, 42*. https://doi.org/10.1016/j.algal.2019.101605

Fang, H. H. P., Zhu, H., & Zhang, T. (2006). Phototrophic hydrogen production from glucose by pure and co-cultures of Clostridium butyricum and Rhodobacter sphaeroides. *International Journal of Hydrogen Energy*, *31*(15), 2223–2230. https://doi.org/10.1016/j.ijhydene.2006.03.005

Ferreira, A., & Gouveia, L. (2020). Microalgal biorefineries. In *Handbook of Microalgae-Based Processes and Products: Fundamentals and Advances in Energy, Food, Feed, Fertilizer, and Bioactive Compounds* (pp. 771–798). https://doi.org/10.1016/B978-0-12-818536-0.00028-2

Garcia, A. B., De Almeida Engler, J., Iyer, S., Gerats, T., Van Montagu, M., & Caplan, A. B. (1997). Effects of osmoprotectants upon NaCl stress in rice. *Plant Physiology*, *115*(1), 159–169. https://doi.org/10.1104/pp.115.1.159

Gérard, P., & Rabot, S. (2010). GI Bacteria Changes in Animal Models Due to Prebiotics. In *Bioactive Foods in Promoting Health: Probiotics and Prebiotics* (pp. 553–570). https://doi.org/10.1016/B978-0-12-374938-3.00033-5

Ghirardi, M. L., Dubini, A., Yu, J., & Maness, P. C. (2009). Photobiological hydrogen-producing systems. *Chemical Society Reviews*, *38*(1), 52–61. https://doi.org/10.1039/b718939g

Ghirardi, M. L., Zhang, L., Lee, J. W., Flynn, T., Seibert, M., Greenbaum, E., & Melis, A. (2000). Microalgae: A green source of renewable H2. *Trends in Biotechnology*, *18*(12), 506–511. https://doi.org/10.1016/S0167-7799(00)01511-0

Ghosh, D., & Hallenbeck, P. C. (2009). Fermentative hydrogen yields from different sugars by batch cultures of metabolically engineered Escherichia coli DJT135. *International Journal of Hydrogen Energy*, *34*(19), 7979–7982. https://doi.org/10.1016/j.ijhydene.2009.08.004

Grechanik, V., Naidov, I., Bolshakov, M., & Tsygankov, A. (2021). Photoautotrophic hydrogen production by nitrogen-deprived Chlamydomonas reinhardtii cultures. *International Journal of Hydrogen Energy*, *46*(5), 3565–3575. https://doi.org/10.1016/j.ijhydene.2020.10.215

Guo, L., Zong, Y., Lu, M., & Zhang, J. (2014). Effect of different substrate concentrations and salinity on hydrogen production from mariculture organic waste (MOW). *International Journal of Hydrogen Energy*, *39*(2), 736–743. https://doi.org/10.1016/j.ijhydene.2013.10.127

Hao, X., Zhou, M., Yu, H., Shen, Q., & Lei, L. (2006). Effect of sodium ion concentration on hydrogen production from sucrose by anaerobic hydrogen-producing granular sludge. *Chinese Journal of Chemical Engineering*, *14*(4), 511–517. https://doi.org/10.1016/S1004-9541(06)60106-7

Hay, J. X. W., Wu, T. Y., Juan, J. C., & Md. Jahim, J. (2013). Biohydrogen production through photo fermentation or dark fermentation using waste as a substrate: Overview, economics, and future prospects of hydrogen usage. *Biofuels, Bioproducts and Biorefining*, *7*(3), 334–352. https://doi.org/10.1002/bbb.1403

Higgins, B. T., Gennity, I., Samra, S., Kind, T., Fiehn, O., & VanderGheynst, J. S. (2016). Cofactor symbiosis for enhanced algal growth, biofuel production, and wastewater treatment. *Algal Research*, *17*, 308–315. https://doi.org/10.1016/j.algal.2016.05.024

Hiremath, P., Nuguru, K., & Agrahari, V. (2018). Material attributes and their impact on wet granulation process performance. In *Handbook of Pharmaceutical Wet Granulation: Theory and Practice in a Quality by Design Paradigm*. https://doi.org/10.1016/B978-0-12-810460-6.00012-9

Javed, M. A., Zafar, A. M., Aly Hassan, A., Zaidi, A. A., Farooq, M., El Badawy, A., Lundquist, T., Mohamed, M. M. A., & Al-Zuhair, S. (2022). The role of oxygen regulation and algal growth parameters in hydrogen production via biophotolysis. In *Journal of Environmental Chemical Engineering* (Vol. 10, Issue 1). https://doi.org/10.1016/j.jece.2021.107003

Jeckelmann, J. M., & Erni, B. (2020). Transporters of glucose and other carbohydrates in bacteria. *Pflugers Archiv European Journal of Physiology*, *472*(9), 1129–1153. https://doi.org/10.1007/s00424-020-02379-0

Jun, Y. S., Yu, S. H., Ryu, K. G., & Lee, T. J. (2008). Kinetic study of pH effects on biological hydrogen production by a mixed culture. *Journal of Microbiology and Biotechnology*, *18*(6), 1130–1135.

Kim, D. H., Kim, S. H., Jung, K. W., Kim, M. S., & Shin, H. S. (2011). Effect of initial pH independent of operational pH on hydrogen fermentation of food waste. *Bioresource Technology*, *102*(18), 8646–8652. https://doi.org/10.1016/j.biortech.2011.03.030

Koku, H., Erolu, I., Gündüz, U., Yücel, M., & Türker, L. (2002). Aspects of the metabolism of hydrogen production by Rhodobacter sphaeroides. *International Journal of Hydrogen Energy*, *27*(11–12), 1315–1329. https://doi.org/10.1016/S0360-3199(02)00127-1

Kothari, R., Singh, D. P., Tyagi, V. V., & Tyagi, S. K. (2012). Fermentative hydrogen production - An alternative clean energy source. *Renewable and Sustainable Energy Reviews*, *16*(4), 2337–2346. https://doi.org/10.1016/j.rser.2012.01.002

Kraemer, J. T., & Bagley, D. M. (2007). Improving the yield from fermentative hydrogen production. *Biotechnology Letters*, *29*(5), 685–695. https://doi.org/10.1007/s10529-006-9299-9

Lazaro, C. Z., & Hallenbeck, P. C. (2019). Fundamentals of Biohydrogen Production. In *Biohydrogen*. https://doi.org/10.1016/b978-0-444-64203-5.00002-2

Lee, M.-J., Kim, T.-H., Min, B., & Hwang, S.-J. (2012). Sodium (Na+) concentration effects on metabolic pathway and estimation of ATP use in dark fermentation hydrogen production through stoichiometric analysis. *Journal of Environmental Management*, *108*, 22–26. https://doi.org/10.1016/j.jenvman.2012.04.027

Li, L., Zhang, L., & Liu, J. (2021). Proteomic analysis of hydrogen production in Chlorella pyrenoidosa under nitrogen deprivation. *Algal Research*, *53*. https://doi.org/10.1016/j.algal.2020.102143

Li, N., Xia, Y., He, X., Li, W., Yuan, L., Wu, X., Qin, Y., Yuan, R., & Gong, X. (2021). Glucose addition enhanced the advanced treatment of coking wastewater. *Water (Switzerland)*, *13*(23). https://doi.org/10.3390/w13233365

Li, X., Huang, S., Yu, J., Wang, Q., & Wu, S. (2013). Improvement of hydrogen production of Chlamydomonas reinhardtii by co-cultivation with isolated bacteria. *International Journal of Hydrogen Energy*, *38*(25), 10779–10787. https://doi.org/10.1016/j.ijhydene.2013.02.102

Lin, C. Y., & Lay, C. H. (2005). A nutrient formulation for fermentative hydrogen production using anaerobic sewage sludge microflora. *International Journal of Hydrogen Energy*, *30*(3). https://doi.org/10.1016/j.ijhydene.2004.03.002

Liu, J. Z., Ge, Y. M., Sun, J. Y., Chen, P., Addy, M., Huo, S. H., Li, K., Cheng, P. F., & Ruan, R. (2019). Exogenic glucose as an electron donor for algal hydrogenases to promote hydrogen photoproduction by Chlorella pyrenoidosa. *Bioresource Technology*, *289*. https://doi.org/10.1016/j.biortech.2019.121762

Luo, Y. H., & Mitsui, A. (1994). Hydrogen production from organic substrates in an aerobic nitrogen-fixing marine unicellular cyanobacterium Synechococcus sp. strain Miami BG 043511. *Biotechnology and Bioengineering*, *44*(10), 1255–1260. https://doi.org/10.1002/bit.260441013

Manish, S., & Banerjee, R. (2008). Comparison of biohydrogen production processes. *International Journal of Hydrogen Energy*, *33*(1), 279–286. https://doi.org/10.1016/j.ijhydene.2007.07.026

Margarites, A. C., Volpato, N., Araújo, E., Cardoso, L. G., Bertolin, T. E., Colla, L. M., & Costa, J. A. V. (2017). Spirulina platensis is more efficient than Chlorella homosphaera in carbohydrate productivity. *Environmental Technology (United Kingdom)*, *38*(17), 2209–2216. https://doi.org/10.1080/09593330.2016.1254685

Marquez, F. J., Sasaki, K., Kakizono, T., Nishio, N., & Nagai, S. (1993). Growth characteristics of Spirulina platensis in mixotrophic and heterotrophic conditions. *Journal of Fermentation and Bioengineering*, *76*(5), 408–410. https://doi.org/10.1016/0922-338X(93)90034-6

Masset, J., Calusinska, M., Hamilton, C., Hiligsmann, S., Joris, B., Wilmotte, A., & Thonart, P. (2012). Fermentative hydrogen production from glucose and starch using pure strains and artificial co-cultures of Clostridium spp. *Biotechnology for Biofuels*, *5*. https://doi.org/10.1186/1754-6834-5-35

Maza-Márquez, P., Martinez-Toledo, M. V., Fenice, M., Andrade, L., Lasserrot, A., & Gonzalez-Lopez, J. (2014). Biotreatment of olive washing wastewater by a selected microalgal-bacterial consortium. *International Biodeterioration and Biodegradation*, *88*, 69–76. https://doi.org/10.1016/j.ibiod.2013.12.009

Melis, A., & Melnicki, M. R. (2006). Integrated biological hydrogen production. *International Journal of Hydrogen Energy*, *31*(11), 1563–1573. https://doi.org/10.1016/j.ijhydene.2006.06.038

Melitos, G., Voulkopoulos, X., & Zabaniotou, A. (2021). Waste to Sustainable Biohydrogen Production Via Photo-Fermentation and Biophotolysis − A Systematic Review. *Renewable Energy and Environmental Sustainability*, *6*. https://doi.org/10.1051/rees/2021047

Min, M., Wang, L., Li, Y., Mohr, M. J., Hu, B., Zhou, W., Chen, P., & Ruan, R. (2011). Cultivating chlorella sp. in a pilot-scale photobioreactor using centrate wastewater for microalgae biomass production and wastewater nutrient removal. *Applied Biochemistry and Biotechnology*, *165*(1), 123–137. https://doi.org/10.1007/s12010-011-9238-7

Mohan, S. V., Mohanakrishna, G., Veer Raghavulu, S., & Sarma, P. N. (2007). Enhancing biohydrogen production from chemical wastewater treatment in anaerobic sequencing batch biofilm reactor (AnSBBR) by bioaugmenting with selectively enriched kanamycin resistant anaerobic mixed consortia. *International Journal of Hydrogen Energy*, *32*(15 SPEC. ISS.), 3284–3292. https://doi.org/10.1016/j.ijhydene.2007.04.043

Mohd Yasin, N. H., Rahman, N. A., Man, H. C., Mohd Yusoff, M. Z., & Hassan, M. A. (2011). Microbial characterization of hydrogen-producing bacteria in fermented food waste at different pH values. *International Journal of Hydrogen Energy*, *36*(16), 9571–9580. https://doi.org/10.1016/j.ijhydene.2011.05.048

Mona, S., Kumar, S. S., Kumar, V., Parveen, K., Saini, N., Deepak, B., & Pugazhendhi, A. (2020). Green technology for sustainable biohydrogen production (waste to energy): A review. *Science of the Total Environment*, *728*. https://doi.org/10.1016/j.scitotenv.2020.138481

Monfet, E., & Unc, A. (2017). Defining wastewaters used for cultivation of algae. In *Algal Research* (Vol. 24, pp. 520–526). https://doi.org/10.1016/j.algal.2016.12.008

Mu, Y., Wang, G., & Yu, H. Q. (2006). Response surface methodological analysis on biohydrogen production by enriched anaerobic cultures. *Enzyme and Microbial Technology*, *38*(7), 905–913. https://doi.org/10.1016/j.enzmictec.2005.08.016

Muñoz, R., & Guieysse, B. (2006). Algal-bacterial processes for the treatment of hazardous contaminants: A review. *Water Research*, *40*(15), 2799–2815. https://doi.org/10.1016/j.watres.2006.06.011

Nakamura, H. (1977). Genetic determination of sensitivity to salt hypertonicity in Escherichia coli K12. *Journal of General Microbiology*, *102*(1), 221–222. https://doi.org/10.1099/00221287-102-1-221

Nakashimada, Y., Rachman, M. A., Kakizono, T., & Nishio, N. (2002). Hydrogen production of Enterobacter aerogenes altered by extracellular and intracellular redox states. *International Journal of Hydrogen Energy*, *27*(11–12), 1399–1405. https://doi.org/10.1016/S0360-3199(02)00128-3

Neuer, G., & Bothe, H. (1985). Electron donation to nitrogenase in heterocysts of cyanobacteria. *Archives of Microbiology*, *143*(2), 185–191. https://doi.org/10.1007/BF00411045

Ni, M., Leung, D. Y. C., Leung, M. K. H., & Sumathy, K. (2006). An overview of hydrogen production from biomass. *Fuel Processing Technology*, *87*(5), 461–472. https://doi.org/10.1016/j.fuproc.2005.11.003

Nikolaidis, P., & Poullikkas, A. (2017). A comparative overview of hydrogen production processes. *Renewable and Sustainable Energy Reviews*, *67*, 597–611. https://doi.org/10.1016/j.rser.2016.09.044

Opeolu, B. O., Bamgbose, O., Arowolo, T. A., & Adetunji, M. T. (2010). Utilization of biomaterials as adsorbents for heavy metals' removal from aqueous matrices. *Scientific Research and Essays*, *5*(14), 1780–1787.

Pachapur, V. L., Sarma, S. J., Brar, S. K., Bihan, Y. Le, Buelna, G., & Verma, M. (2017). Hydrogen production from biodiesel industry waste by using a co-culture of Enterobacter aerogenes and Clostridium butyricum. *Biofuels*, *8*(6), 651–662. https://doi.org/10.1080/17597269.2015.1122471

Parmar, A., Singh, N. K., Pandey, A., Gnansounou, E., & Madamwar, D. (2011). Cyanobacteria and microalgae: A positive prospect for biofuels. In *Bioresource Technology* (Vol. 102, Issue 22). https://doi.org/10.1016/j.biortech.2011.08.030

Patel, S. K. S., Kumar, P., Mehariya, S., Purohit, H. J., Lee, J. K., & Kalia, V. C. (2014). Enhancement in hydrogen production by co-cultures of Bacillus and Enterobacter. *International Journal of Hydrogen Energy*, *39*(27), 14663–14668. https://doi.org/10.1016/j.ijhydene.2014.07.084

Perera, I. A., Abinandan, S., Subashchandrabose, S. R., Venkateswarlu, K., Naidu, R., & Megharaj, M. (2019). Advances in the technologies for studying consortia of bacteria and cyanobacteria/microalgae in wastewaters. *Critical Reviews in Biotechnology*, *39*(5), 709–731. https://doi.org/10.1080/07388551.2019.1597828

Perez-Garcia, O., de-Bashan, L. E., Hernandez, J. P., & Bashan, Y. (2010). Efficiency of growth and nutrient uptake from wastewater by heterotrophic, autotrophic, and mixotrophic cultivation of chlorella vulgaris immobilized with azospirillum brasilense. *Journal of Phycology*, *46*(4), 800–812. https://doi.org/10.1111/j.1529-8817.2010.00862.x

Pierra, M., Trably, E., Godon, J. J., & Bernet, N. (2014). Fermentative hydrogen production under moderate halophilic conditions. *International Journal of Hydrogen Energy*, *39*(14), 7508–7517. https://doi.org/10.1016/j.ijhydene.2013.08.035

Poggi-Varaldo, H. M., & Oleszkiewicz, J. A. (1992). Anaerobic co-composting of municipal solid waste and waste sludge at hlgh total solids levels. *Environmental Technology (United Kingdom)*, *13*(5), 409–421. https://doi.org/10.1080/09593339209385169

Posadas, E., García-Encina, P. A., Domínguez, A., Díaz, I., Becares, E., Blanco, S., & Muñoz, R. (2014). Enclosed tubular and open algal-bacterial biofilm photobioreactors for carbon and nutrient removal from domestic wastewater. *Ecological Engineering*, *67*, 156–164. https://doi.org/10.1016/j.ecoleng.2014.03.007

Pugazhendhi, A., Shobana, S., Nguyen, D. D., Banu, J. R., Sivagurunathan, P., Chang, S. W., Ponnusamy, V. K., & Kumar, G. (2019). Application of nanotechnology (nanoparticles) in dark fermentative hydrogen production. *International Journal of Hydrogen Energy*, *44*(3), 1431–1440. https://doi.org/10.1016/j.ijhydene.2018.11.114

Qian, C. X., Chen, L. Y., Rong, H., & Yuan, X. M. (2011). Hydrogen production by mixed culture of several facultative bacteria and: Anaerobic bacteria. *Progress in Natural Science: Materials International*, *21*(6), 506–511. https://doi.org/10.1016/S1002-0071(12)60090-2

Rafrafi, Y., Trably, E., Hamelin, J., Latrille, E., Meynial-Salles, I., Benomar, S., Giudici-Orticoni, M. T., & Steyer, J. P. (2013). Sub-dominant bacteria as keystone species in microbial communities producing bio-hydrogen. *International Journal of Hydrogen Energy*, *38*(12), 4975–4985. https://doi.org/10.1016/j.ijhydene.2013.02.008

Rajta, A., Bhatia, R., Setia, H., & Pathania, P. (2020). Role of heterotrophic aerobic denitrifying bacteria in nitrate removal from wastewater. *Journal of Applied Microbiology*, *128*(5), 1261–1278. https://doi.org/10.1111/jam.14476

Rashid, N., Rehman, M. S. U., Memon, S., Ur Rahman, Z., Lee, K., & Han, J. I. (2013). Current status, barriers and developments in biohydrogen production by microalgae. *Renewable and Sustainable Energy Reviews*, *22*, 571–579. https://doi.org/10.1016/j.rser.2013.01.051

Reddy PM, Spiller H, & Albrecht SL. (1996). Photodissimilation of Fructose to H(inf2) and CO(inf2) by a Dinitrogen-Fixing Cyanobacterium, Anabaena variabilis. *Appl Environ Microbiol*, 1220–1226.

Rittmann, S., & Herwig, C. (2012). A comprehensive and quantitative review of dark fermentative biohydrogen production. *Microbial Cell Factories*, *11*, 1–18. https://doi.org/10.1186/1475-2859-11-115

Ruiz-Marin, A., Canedo-López, Y., & Chávez-Fuentes, P. (2020). Biohydrogen production by Chlorella vulgaris and Scenedesmus obliquus immobilized cultivated in artificial wastewater under different light quality. *AMB Express*, *10*(1), 1–7. https://doi.org/10.1186/s13568-020-01129-w

Ryu, B. G., Kim, J., Han, J. I., & Yang, J. W. (2017). Feasibility of using a microalgal-bacterial consortium for treatment of toxic coke wastewater with concomitant production of microbial lipids. *Bioresource Technology*, *225*, 58–66. https://doi.org/10.1016/j.biortech.2016.11.029

Sagir, E., & Alipour, S. (2021). Photofermentative hydrogen production by immobilized photosynthetic bacteria: Current perspectives and challenges. In *Renewable and Sustainable Energy Reviews* (Vol. 141). https://doi.org/10.1016/j.rser.2021.110796

Sakurai, H., Masukawa, H., Kitashima, M., & Inoue, K. (2013). Photobiological hydrogen production: Bioenergetics and challenges for its practical application. *Journal of Photochemistry and Photobiology C: Photochemistry Reviews*, *17*, 1–25. https://doi.org/10.1016/j.jphotochemrev.2013.05.001

Seppälä, J. J., Puhakka, J. A., Yli-Harja, O., Karp, M. T., & Santala, V. (2011). Fermentative hydrogen production by Clostridium butyricum and Escherichia coli in pure and cocultures. *International Journal of Hydrogen Energy*, *36*(17), 10701–10708. https://doi.org/10.1016/j.ijhydene.2011.05.189

Shah, V., Garg, N., & Madamwar, D. (2001). Ultrastructure of the fresh water cyanobacterium Anabaena variabilis SPU 003 and its application for oxygen-free hydrogen production. *FEMS Microbiology Letters*, *194*(1), 71–75. https://doi.org/10.1016/S0378-1097(00)00508-5

Shah, V., Garg, N., & Madamwar, D. (2003). Ultrastructure of the cyanobacterium Nostoc muscorum and exploitation of the culture for hydrogen production. *Folia Microbiologica*, *48*(1), 65–70. https://doi.org/10.1007/BF02931278

Sharma, A., & Arya, S. K. (2017). Hydrogen from algal biomass: A review of production process. *Biotechnology Reports*, *15*, 63–69. https://doi.org/10.1016/j.btre.2017.06.001

Show, K. Y., Lee, D. J., Tay, J. H., Lin, C. Y., & Chang, J. S. (2012). Biohydrogen production: Current perspectives and the way forward. *International Journal of Hydrogen Energy*, *37*(20), 15616–15631. https://doi.org/10.1016/j.ijhydene.2012.04.109

Silva, J. S., Mendes, J. S., Correia, J. A. C., Rocha, M. V. P., & Micoli, L. (2018). Cashew apple bagasse as new feedstock for the hydrogen production using dark fermentation process. *Journal of Biotechnology*, *286*, 71–78. https://doi.org/10.1016/j.jbiotec.2018.09.004

Srivastava, N., Srivastava, M., Malhotra, B. D., Gupta, V. K., Ramteke, P. W., Silva, R. N., Shukla, P., Dubey, K. K., & Mishra, P. K. (2019). Nanoengineered cellulosic biohydrogen production via dark fermentation: A novel approach. In *Biotechnology Advances* (Vol. 37, Issue 6). https://doi.org/10.1016/j.biotechadv.2019.04.006

Subashchandrabose, S. R., Ramakrishnan, B., Megharaj, M., Venkateswarlu, K., & Naidu, R. (2011). Consortia of cyanobacteria/microalgae and bacteria: Biotechnological potential. *Biotechnology Advances*, *29*(6), 896–907. https://doi.org/10.1016/j.biotechadv.2011.07.009

Tamburic, B., Zemichael, F. W., Maitland, G. C., & Hellgardt, K. (2012). A novel nutrient control method to deprive green algae of sulphur and initiate spontaneous hydrogen production. *International Journal of Hydrogen Energy*, *37*(11), 8988–9001. https://doi.org/10.1016/j.ijhydene.2012.02.043

Tiefenbacher, K. F. (2017). Technology of Main Ingredients—Sweeteners and Lipids. In *Wafer and Waffle*. https://doi.org/10.1016/b978-0-12-809438-9.00003-x

Touliabah, H. E. S., El-Sheekh, M. M., Ismail, M. M., & El-Kassas, H. (2022). A Review of Microalgae-and Cyanobacteria-Based Biodegradation of Organic Pollutants. In *Molecules* (Vol. 27, Issue 3). https://doi.org/10.3390/molecules27031141

Turon, V., Trably, E., Fouilland, E., & Steyer, J. P. (2016). Potentialities of dark fermentation effluents as substrates for microalgae growth: A review. *Process Biochemistry*, *51*(11), 1843–1854. https://doi.org/10.1016/j.procbio.2016.03.018

Van Niel, E. W. J., Claassen, P. A. M., & Stams, A. J. M. (2003). Substrate and product inhibition of hydrogen production by the extreme thermophile, Caldicellulosiruptor saccharolyticus. *Biotechnology and Bioengineering*, *81*(3), 255–262. https://doi.org/10.1002/bit.10463

Vesper, S., Sienkiewicz, N., Struewing, I., Linz, D., & Lu, J. (2022). Prophylactic Addition of Glucose Suppresses Cyanobacterial Abundance in Lake Water. *Life*, *12*(3). https://doi.org/10.3390/life12030385

Wu, S., Li, X., Yu, J., & Wang, Q. (2012). Increased hydrogen production in co-culture of Chlamydomonas reinhardtii and Bradyrhizobium japonicum. *Bioresource Technology*, *123*, 184–188. https://doi.org/10.1016/j.biortech.2012.07.055

Wu, T. Y., Hay, J. X. W., Kong, L. B., Juan, J. C., & Jahim, J. M. (2012). Recent advances in reuse of waste material as substrate to produce biohydrogen by purple non-sulfur (PNS) bacteria. *Renewable and Sustainable Energy Reviews*, *16*(5), 3117–3122. https://doi.org/10.1016/j.rser.2012.02.002

Xia, A., Jacob, A., Herrmann, C., Tabassum, M. R., & Murphy, J. D. (2015). Production of hydrogen, ethanol and volatile fatty acids from the seaweed carbohydrate mannitol. *Bioresource Technology*, *193*, 488–497. https://doi.org/10.1016/j.biortech.2015.06.130

Xu, L., Cheng, X., & Wang, Q. (2017). Effect of co-cultivation of Chlamydomonas reinhardtii with Azotobacter chroococcum on hydrogen production. *International Journal of Hydrogen Energy*, *42*(36), 22713–22719. https://doi.org/10.1016/j.ijhydene.2017.06.223

Yang, G., & Wang, J. (2017). Enhanced Hydrogen Production from Sewage Sludge by Co-fermentation with Forestry Wastes. *Energy and Fuels*, *31*(9), 9633–9641. https://doi.org/10.1021/acs.energyfuels.7b02135

Yang, S. T. (2007). Bioprocessing for Value-Added Products from Renewable Resources. In *Bioprocessing for Value-Added Products from Renewable Resources*. https://doi.org/10.1016/B978-0-444-52114-9.X5000-2

Yeager, C. M., Milliken, C. E., Bagwell, C. E., Staples, L., Berseth, P. A., & Sessions, H. T. (2011). Evaluation of experimental conditions that influence hydrogen production among heterocystous Cyanobacteria. *International Journal of Hydrogen Energy*, *36*(13), 7487–7499. https://doi.org/10.1016/j.ijhydene.2011.03.078

Yodsang, P., Raksajit, W., Aro, E.-M., Mäenpää, P., & Incharoensakdi, A. (2018). Factors affecting photobiological hydrogen production in five filamentous cyanobacteria from Thailand. *Photosynthetica*, *56*(1), 334–341. https://doi.org/10.1007/s11099-018-0789-5

Yokoi, H., Tokushige, T., Hirose, J., Hayashi, S., & Takasaki, Y. (1998). H2 production from starch by a mixed culture of Clostridium butyricum and Enterobacter aerogenes. *Biotechnology Letters*, *20*(2), 143–147. https://doi.org/10.1023/A:1005372323248

Zafar, A. M., Javed, M. A., & Aly Hassan, A. (2022). Unprecedented biodesalination rates–Shortcomings of electrical conductivity measurements in determining salt removal by algae and cyanobacteria. *Journal of Environmental Management*, *302*. https://doi.org/10.1016/j.jenvman.2021.113947

Zarour, K., Vieco, N., Pérez-Ramos, A., Nácher-Vázquez, M., Mohedano, M. L., & López, P. (2017). Food Ingredients Synthesized by Lactic Acid Bacteria. In *Microbial Production of Food Ingredients and Additives* (pp. 89–124). https://doi.org/10.1016/b978-0-12-811520-6.00004-0

Zhang, X., Wang, X., Zhang, J., Huang, X., Wei, D., Lan, W., & Hu, Z. (2016). Reduction of nitrous oxide emissions from partial nitrification process by using innovative carbon source (mannitol). *Bioresource Technology*, *218*, 789–795. https://doi.org/10.1016/j.biortech.2016.07.043

Zhang, Z., Guo, L., Li, Q., Zhao, Y., Gao, M., & She, Z. (2017). Study on substrate metabolism process of saline waste sludge and its biological hydrogen production potential. *Environmental Science and Pollution Research*, *24*(19), 16383–16395. https://doi.org/10.1007/s11356-017-9222-z

Zheng, X. J., Zheng, Y. M., & Yu, H. Q. (2005). Influence of NaCl on hydrogen production from glucose by anaerobic cultures. *Environmental Technology*, *26*(10). https://doi.org/10.1080/09593332608618476

Zhou, N., & Dunford, N. T. (2017). Characterization of green microalgae and cyanobacteria isolated from the great salt plains. *Transactions of the ASABE*, *60*(2), 283–290. https://doi.org/10.13031/trans.12136

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This study aimed to produce biohydrogen using a co-culture consisting of halophytic cyanobacterium *Phormidium Keutzingianum* and activated sludge bacteria using different exogenous carbon substrates such as glucose, sorbitol, and mannitol and using different salt concentrations. The co-culture was able to produce a maximum of 1000 ml of hydrogen per one liter of the culture solution using sorbitol as carbon substrate and at salt concentration of 0 g/L.

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