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Green Hydrogen Production from Residual Lignocellulosic Biomass via Dark Fermentation: Maximizing Hydrogen Yield via Optimal Pretreatment Method and Substrate-to-Inoculum Ratio

By

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Abstract

This thesis presents an investigation into the biohydrogen production potential of Lignocellulosic Aquatic Residue (LAR), a byproduct of an industrial process. A detailed examination of the substrate and inoculum characterization, pretreatment methods, biohydrogen production via dark fermentation at different Substrate-to-Inoculum Ratios (SIRs), and kinetics modelling was conducted. The study aims to illustrate that LAR can serve as an effective substrate for renewable biohydrogen production via dark fermentation.

After mild acid hydrolysis and lipid extraction pretreatment, LAR showed a high carbohydrate and lipid content. However, the pretreatment process needs to be optimized to avoid the introduction or release of inhibitory compounds since no gas production was observed from those pretreated LAR. Further examination revealed an optimal SIR of 2.7, where Hydrogen Yield (HY) of LAR reached around 280 mL H_2 g^{-1} VS.

A Continuous Flow Stirred-Tank Reactor (CFSTR) was built to upscale the biohydrogen production, which produced promising preliminary results. Energy output estimation indicated that biohydrogen production from LAR could contribute between 2.6 to 3.5 TWh per year, equating to 1.2 to 1.6 % of Norway's total energy demand. This approach turns an otherwise waste product into a source of renewable energy.

These findings suggest that the utilization of LAR for biohydrogen production via dark fermentation holds significant potential for future green energy solutions. Continued research is necessary to optimize pretreatment methods, operational conditions, and to fully understand this unique biomass resource.

Keywords: *Lignocellulosic Biomass, Dark Fermentation, Biohydrogen, Biohydrogen Potential (BHP), Pretreatment, Substrate-to-Inoculum Ratio, AMPTS, CFSTR*

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Table of contents

List of figures

List of tables

Acronyms

ALK Alkalinity. 19, 20, 29, 37

AMPTS Automated Methane Potential Test System II. 3, 19, 21, 23–25, 27, 31, 32, 40–43

BHP Biohydrogen potential. 3, 21, 42

CCUS Carbon Capture, Utilization and Storage. 5

CFSTR Continuous Flow Stirred-Tank Reactor. ii, 3, 20, 25–27, 39–41, 43

COD Chemical Oxygen Demand. 16, 18–20, 27, 29, 30, 33, 37, 38, 40, 42

DLAR Defatted Lignocellulosic Aquatic Residue. 17, 18, 23, 30–32, 42, 56

FS Fixed Solid. 16, 17, 19, 29, 30, 42

HLAR Hydrolyzed Lignocellulosic Aquatic Residue. 17, 18, 23, 30–32, 42, 56

HP Hydrogen Productivity. 35, 36, 42

HRT Hydraulic Retention Time. 12, 27

HY Hydrogen Yield. ii, 35, 36, 42

LAR Lignocellulosic Aquatic Residue. ii, 16–19, 23, 27, 28, 30–34, 36–40, 42, 43, 56

LCB Lignocellulosic Biomass. 1, 2, 13, 39

ORP Oxidation Reduction Potential. 26, 27, 40

SIR Substrate-to-Inoculum Ratio. ii, 3, 21, 23, 32–38, 42

SNJ Sentralrenseanlegg Nord Jæren. 19

SRT Solid Retention Time. 12

TDS Total Dissolved Solid. 20, 37

TRL Technology Readiness Level. 10

TS Total Solid. 16, 17, 19, 29, 30, 56

VFA Volatile Fatty Acid. 7, 12, 19, 20, 29, 37

VS Volatile Solid. 16, 17, 19, 29, 30, 42

Chapter 1

Introduction

This chapter provides an overview of the potential of lignocellulosic for renewable energy. It also presents the objectives and the structure of this thesis.

1.1 Background

The increasing global population and industrialization are driving the demand for energy. The primary sources of energy have traditionally been fossil fuels, but their non-renewable nature, along with the environmental problems caused by their extensive use, makes the search for alternative energy sources crucial. Hydrogen stands out among these alternatives because of its renewable nature and high energy content. It can be derived from both natural and bioresources [1], thereby making biological processes and techniques for biohydrogen production more relevant in the current era. The key benefits of these processes include renewable energy production, resource recovery, and waste management.

Lignocellulosic Biomass (LCB) represents a vast resource of renewable carbon which could support carbon sequestration and provide an alternative to fossil fuel-derived raw materials, which in turn could reduce our reliance on such finite resources and reduce greenhouse gas. LCB residues such as spent coffee grounds, jatropha waste, coconut husk, potato peel, empty fruit bunches, sugarcane bagasse, wheat straw, etc., are all abundant in cellulose, hemicellulose, and lignin. These components can be converted into a wide range of valuable bioproducts encompassing biofuels, chemicals, and bioenergy. Additionally, this can also yield value-added products like biopolymers, acids, and pigments. This

transformation is made possible through a holistic biorefinery approach that incorporates processes like hydrolysis, fermentation, and extraction [2].

Given these facts, biohydrogen produced from LCB can theoretically significantly contribute to the global hydrogen supply. However, the complex and diverse characteristics of LCB pose substantial obstacles for those conversion techniques. Around 4.6 billion tons of lignocellulosic biomass waste are generated from agricultural residues annually, and merely a quarter of these residues are being effectively utilized [3]. Pretreatment technologies aim to tackle these challenges by degrading lignin and hemicellulose, thereby enhancing the accessibility of cellulose for further conversion [4].

In Europe, it is considered unpleasant to handle undesirable forms of a type of LCB, those washed ashore, known as seaweed or macroalgae, to prevent them from becoming an environmental issue [5]. This aquatic LCB is, in fact, a critical resource, serving as the principal source of hydrocolloids such as alginate, agar, and carrageenan, which are widely used as thickeners in the food industry. Beyond their role in food production, these marine plants are also harvested for their invaluable compounds, which find diverse applications. These include being processed into food additives, integrated into animal feed, and utilized as ingredients in health products and bio-medicines. Furthermore, extracts from seaweeds are used as bio-stimulants and fertilizers, and they are increasingly being explored as potential sources of biofuels. Seaweed cultivation is becoming increasingly attractive to businesses due to high growth rate, low resource requirements, various applications and potential mitigation for climate change [6][7]. Norway, blessed with the second-longest coastal line in the world, is the biggest macroalgae producer in Europe with total annual production of more than 160,000 tons [8].

Seaweed needs to go through several processes to be applicable or ready for elemental extraction. However, such additional steps are usually not optimized, hence generating a large amount of residue which needs to be treated as waste. According to Phyconomy database [9], only 27 over 1203 recorded companies are able to fully utilize 100 % of the macroalgal biomass. In addition, Porse and Rudolph [10] reported that more than 236,000 tons (dry weight) of alginate-bearing seaweeds harvested in 2015 were transformed into approximately 93,000 tons of products, which accounted for only 40 % of the feedstock,

while the rest has been turned out to be waste. This tremendous volume of organic waste poses challenges in both waste and resources management, which requires the industry to improve their utilization of the substrate to minimize the discarded biomass, recover more resources and earn better income.

1.2 Research objectives

The main aim of this research is to assess the Biohydrogen potential (BHP) of residual seaweed biomass obtained from an industrial entity. This primary objective can be divided into several sub-objectives as follows:

- Investigate the BHP of residual seaweed biomass as a feedstock for biohydrogen production via dark fermentation using the Automated Methane Potential Test System II (AMPTS).
- Determine the optimal substrate pre-treatment method and Substrate-to-Inoculum Ratio (SIR) for dark fermentation of this biomass based on the experimental results.
- Build and operate a Continuous Flow Stirred-Tank Reactor (CFSTR) under the optimal conditions determined from findings of the AMPTS experiments to upscale the biohydrogen production.
- Apply the modified Gompertz model to the experimental data and discuss biohydrogen production performance via kinetics modelling parameters.
- Estimate and discuss the theoretical annual energy output based on the experimental results.

1.3 Brief overview of the thesis structure

This thesis is divided into five chapters and is structured as follows:

Chapter 1: Introduction - This chapter provides an overview of the potential of lignocellulosic biomass for renewable energy, setting the context of the research, and outlines the objectives of this thesis.

Chapter 2: Literature Review - This chapter presents a comprehensive review of the existing literature on the topic of biohydrogen production from lignocellulosic biomass.

Chapter 3: Materials and Methods - In this chapter, the research design and methodology adopted for this study are explained in detail.

Chapter 4: Results and Discussion - This chapter presents the findings from the experiments conducted. The results are analyzed, discussed, and compared with relevant studies in the field of biohydrogen production.

Chapter 5: Conclusion and Outlook - The final chapter summarizes the key findings of the research and their implications. It also provides recommendations for future research in this area.

Chapter 2

Literature review

2.1 Biohydrogen production using lignocellulosic biomass

In 2021, the global hydrogen production reached approximately 94 million tonnes (MT), but the majority of this production heavily relied on fossil fuels. Unfortunately, this reliance on such finite resouces led to the release of more than 900 MT of CO_2 into the atmosphere. Both green hydrogen, produced through electrolysis, and blue hydrogen, generated from fossil fuels with Carbon Capture, Utilization and Storage (CCUS), accounted for less than 1 % of the total annual production [11].

Biomass typically refers to any type of organic material. They are renewable and fossil-free compounds formed naturally or anthropogenically. As shown in Figure 2.1, biomass can be classified into first-, second- and third-generation. Food crops such as corn, potato, sugarcane, cereals are considered first-generation biomass. Utilization of these feedstocks to produce biofuels often faces controversies related to food security and land use changes. Lignocellulosic biomass e.g. wood, straw, grasses is the main ingredient for production of second-generation biofuels, but the process primarily faces the problems of limited cultivation area and high content of lignin - a rigid natural polymer that shapes plant tissues and defends against insect and microbial attacks [12]. Third-generation biomass includes micro- and macro-algae. As mentioned in previous sections, algae have numerous advantages over other types of substrates like greater productivity, no land competition, low resource requirement, high CO_2 fixation capacity, wide range of applications, and zero to very low lignin content.

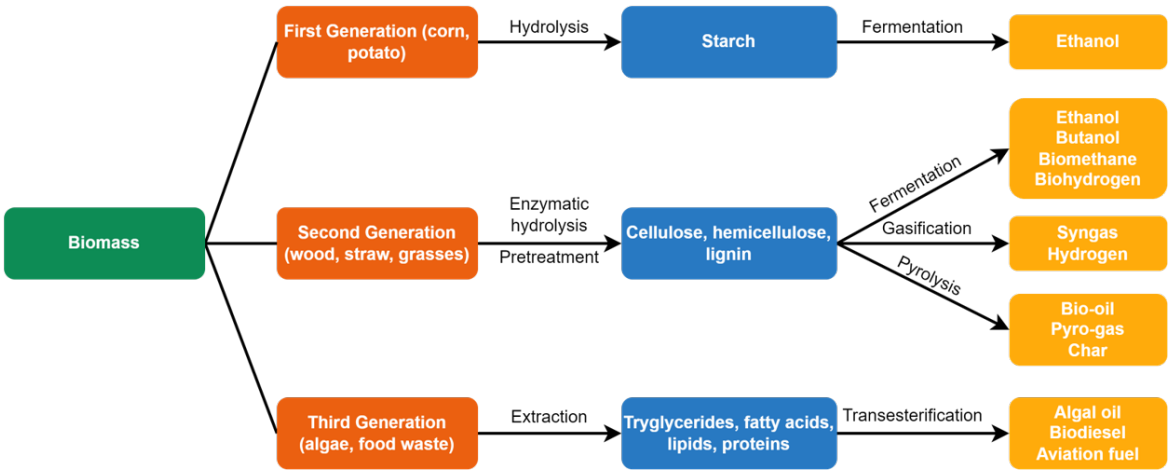

Figure. 2.1 A holistic overview of the first-, second-, and third-generation biomass and their respective fundamental processes for biofuel production. From [12]

Additionally, different types of organic solid waste from municipalities, industrial complexes, livestock farms, fish farms, slaughterhouses, sewage etc. are also considered third-generation biomass. Interestingly waste biomass is further arranged into three groups depending on the amount of energy released through a complete combustion process or so-called calorific value [13]. High potential biomass is defined as having high calorific values from 24 to 40 MJ/kg, typically including industrial waste, algal biomass and biomedical waste. While biomass with calorific values ranging from 19 - 22 MJ/kg and from 2 - 4.3 MJ/kg are called medium (e.g. food waste, municipal solid waste, agricultural residues) and low potential waste (e.g. animal manure) respectively.

The major interest of resource recovery has indeed lied in second- and third-generation biomass, especially the latter, as they offer better utilization of resources and potential alternatives to energy generation solutions without compromising food security, biodiversity and land usage. Those organic matters are widely available anywhere and carry a variety of fermentable sugars, but a thoughtful and systematic process design is required to produce biohydrogen efficiently. Kumar et al. [14] suggested an oversimplified synergetic step-by-step procedure for biofuels generation as follows: 1/ pre-treat raw material to disintegrate the refractory polymeric structure of the lignocellulosic complex; 2/ hydrolyze the unbound cellulose and hemicellulose fractions to obtain monosaccha-

rides; 3/ convert monomeric sugars into biofuels using adequate microorganism culture in optimally controlled bioprocesses. In this case, using third-generation waste streams also has another advantage that the biomass could have gone through some form of process (e.g. extraction, size reduction), hence reducing the cost and effort for the pre-treatment step.

2.2 Dark fermentation under mesophilic and thermophilic conditions

Biological techniques for hydrogen synthesis using different types of bioresources come into play to potentially replace the current carbon-intensive hydrogen production processes. Fermentation process is known to be effective as an environmentally friendly process that does not rely on hydrocarbons and offers enhanced resource utilization and recovery. This biological conversion process is generally categorized into dark fermentation and photofermentation. Photofermentation uses phototrophic microorganisms whose light is an energy source for splitting water molecules into hydrogen and oxygen. Despite the benefits of increased biomass productivity and reduced atmospheric carbon dioxide, its low hydrogen yield remains the main obstacle which requires more research on strain selection, light intensity, and oxygen accumulation to become an efficient and profitable method [14]. On the other hand, dark fermentation method involves several light-independent steps i.e. hydrolysis (hydrolyze carbohydrates into monosaccharides), acidogenesis (acidify sugars into Volatile Fatty Acids (VFAs) under enzymes activities) and acetogenesis (convert VFAs into acetate and hydrogen). It is necessary to collect the biohydrogen produced here and avoid its ultimate conversion into methane via methanogenesis. The liquor mixture contains a variety of VFAs, solvents (e.g. acetic acid, butyric acid, lactic acid, etc.) and alcohol (e.g. ethanol). Since these compounds can be used as immediate feedstocks for other processes, putting dark fermentation into a biorefinery concept can possibly generate more value-added products. Note that the mixture composition varies depending on the type of utilized culture and operational conditions, and this could be challenging for post-treatment processes. Despite the limitations, dark fermentation

is often deemed more practical than the phototrophic process. This is largely due to its operational independence from external energy sources and the elimination of the need for a vast surface area to harness light, making it more feasible for integration into existing infrastructure. It leverages current reactor technologies to process organic wastes effectively, serving a dual purpose of energy generation and waste management. One of the significant benefits of the dark fermentation process is its versatility in handling diverse feedstocks, including particulate organic matter. Unlike phototrophic processes that require consistent light exposure, dark fermentation can run continuously, irrespective of the time of day, which enhances its overall productivity and efficiency. This unique characteristic makes dark fermentation a favorable approach in harnessing bioenergy, especially in settings with particulate organic feedstocks. It thus presents a sustainable and practical strategy for renewable energy production and resource recovery [15].

In principle, dark fermentation harnesses the activity of obligate and/or facultative anaerobic microorganisms in an oxygen-free environment for hydrogen production. Primarily, hydrogen is generated through the acetate and butyrate pathways. The final fermentation solution usually consists of a variety of compounds derived from multiple pathways. Thus, instead of obtaining the maximum of 12 mol H_2/mol glucose—which would be the theoretical yield if only the most efficient pathway were utilized—the practical yield is often significantly lower. For instance, using the acetate pathway, the theoretical maximum H_2 yield is 4 mol H_2/mol glucose consumed and 3.33 mol H_2/mol pentose consumed. These yields correspond to 550 and 275.4 ml H_2/g COD removed, respectively, as shown in Equations 2.1 and 2.2. However, in reality, the yield achieved is usually even lower than these theoretical maximums. This discrepancy can be attributed to various factors, such as sub-optimal conditions for the microorganisms, the presence of inhibitors, or the occurrence of side reactions that compete with hydrogen production.

$$C_6H_{12}O_6 + 6\,H_2O \longrightarrow 2\,CO_2 + 2\,CH_3COOH + 4\,H_2 \tag{2.1}$$

$$C_6H_{12}O_6 + 6\,H_2O \longrightarrow 2\,CO_2 + CH_3CH_2CH_2COOH + 2\,H_2 \tag{2.2}$$

Dark fermentation using biomass as a substrate for hydrogen production can be carried out by mixed, co- or pure cultures usually at mesophilic temperatures ($20 - 40^oC$) [16]. But thermophilic ($40 - 60^oC$) and hyperthermophilic ($>60^oC$) cultures have also been studied [16][17][18][19][20] as high temperature operation is one of the proposed techniques to increase its net energy production [15].

Studies on combined dark and photo-fermentation were carried out to explore the feasibility of biological H_2 production. In dark fermentation, glucose was used as substrate. This was followed by a photo-fermentation process utilizing the spent medium from the dark process. This combination could achieve higher yields of H_2 by complete utilization of the chemical energy stored in the substrate. The effects of the threshold concentration of acetic acid, light intensity and the presence of additional nitrogen sources in the spent effluent on the amount of H_2 produced during photofermentation were was found to be higher compared to a single stage process [21]. Despite the higher hydrogen yield exceeding the maximum theoretical yield (4 mol H_2/mol glucose), the net energy gain was negative [15] due to high energy input demand, especially in large-scale systems [22]. Therefore, more experimental studies in mesophilic dark fermentation have been recently conducted [16].

Table 2.1 shows numerous studies of dark fermentative hydrogen conducted with pure, co- and mix cultures using different types of substrate under various temperature ranges and operating parameters. Simple substrates are often used for isolated culture setups where the biohydrogen potentials of those particular strains are investigated. While co- or mixed cultures are often used to consume more structurally complex materials e.g. food waste, sludge, animal manure, algal biomass, etc. Members of such consortia are capable of communicating with each other by exchanging molecular signals to separate the tasks of degrading different compounds, hence enhanced substrate utilization is observed in numerous studies. Additionally, they are also more adaptable to varying environmental parameters like pH or temperature, whereas an isolated strain may be more susceptible to contamination under nonsterile environment which is the case in most industrial operations [23].

Levin et al. [24] compared a number of processes and dark fermentation has appeared promising to become a practically viable method with high hydrogen yield and a reasonable bioreactor size. In another study, dark fermentation with biomass as the substrate is also considered one of the most promising hydrogen production methods, with the net energy ratio (renewable energy output/non-renewable energy input) of 1.9 as compared to 0.64 of steam methane reforming [25]. In recent years, numerous projects on dark fermentative hydrogen production have been done to improve its yield, performance and efficiency via different perspectives e.g. feedstock usage, strain selections, bioreactor engineering, downstream hydrogen recovery and purification [14]. A large number of studies reported that dark fermentation is currently the best-understood process among available biotechnological methods. A study conducted by Fan et al. [26] meticulously gathered and evaluated results from numerous studies that spanned a spectrum of Technology Readiness Levels (TRLs) - a measure used to assess the maturity of evolving technologies. In this scale, TRL1 signifies the lowest level of readiness, primarily referring to conceptual research conducted at the laboratory benchtop level. In contrast, TRL9 indicates the highest readiness level, marking a technology as commercially available and mature. In this comprehensive evaluation, dark biohydrogen fermentation was categorized under TRL7. Fan et al. [26] essentially forecast that it may take less than a decade for this method to be both technically and economically viable for large-scale implementation. Such positioning in the TRL scale highlights the potential of dark biohydrogen fermentation as a promising bioenergy technology. It underscores the importance of further research and development in this field, aiming to bridge the gap between the lab-scale prototypes and commercially viable technologies. Consequently, it would be interesting to investigate how different factors, such as reactor design, substrate type, pretreatment methods, microbial inoculum, and process conditions, could influence the efficiency of biohydrogen production via dark fermentation. This could pave the way for the transition from the current TRL status to a higher, commercially ready state.

Table 2.1 Dark fermentative biohydrogen potential of different pure, co- and mix cultures under various substrates and operational conditions. Modified from [27]

Microorganism	Substrate	Temp. (^oC)	pH	Reactor type	Optimum H_2 yield	References
C. thermocellum DSM 1237 and C. thermopalmarium DSM 5974	Cellulose	55	7.0	Batch	1.36 mol/mol glucose eqv.	[28]
Enterobacter cloacae IIT-BT08	Glucose	36	6.0	Batch	2.2 mol/mol glucose eqv.	[29]
Thermotoga neapolitana DSM 4349	Hydrolyzed potato steam peels	80	6.9	Batch	3.3 mol/mol glucose eqv.	[30]
Caldicellulosiruptor saccharolyticus DSM 8903	Hydrolyzed potato steam peels	70	6.9	Batch	3.4 mol/mol glucose eqv.	[30]
Wastewater treatment plant sludge	Rice straw	55	6.5	Batch	24.8 mL/g TS	[31]
Thermophilic acidogenic culture	Food waste	55	4.5	Batch	46.3 mL/g VS added	[32]
Anaerobic digester sludge	Untreated de-oiled algae cake	29	6.0	Batch	66 mL/g BM	[33]
Anaerobic digester sludge	Food waste and sewage sludge	35	5.0 – 6.0	Batch	122.9 mL/g COD	[34]
Enriched cow dung composts	Corn stalk wastes with acidification pretreatment	50	7.0	Batch	149.69 mL/g VS added	[35]
Clostridium thermocellum 7072	Corn stalk	55	7.2	Continuous	1.2 mol/mol glucose eqv.	[36]
Clostridium thermolacticum DSM 2910	Lactose	58	7.0	Continuous	1.5 mol/mol glucose eqv.	[37]
Enterobacter cloacae DM 11	Malt, yeast extract & glucose	37	6.0	Continuous	3.9 mol/mol glucose eqv.	[38]
Mesophilic methanogenic sludge	Pig slurry	70	6.7	Continuous	3.65 mL/g VS added	[39]
Cow dung compost	Wheat straw	36	6.5	Continuous	68.1 mL/g VS added	[40]
Organic fraction of municipal solid waste	Non-anaerobic inocula (soil, pig excreta)	38	5.6	Continuous	99 mL/g VS removed	[41]
Heat shock treated anaerobic sludge	Food waste	37	5.5 – 7.0	Continuous	310 mL/g VS added	[42]
Kitchen waste compost	Vegetable kitchen waste	55	6.0	Semi-continuous	38 mL/g COD	[43]

2.3 Process parameters optimization and enhancement for biohydrogen production

As many different factors can alter the fermentative hydrogen yield, study objectives are often to optimize those parameters to achieve the yield close to the theoretical maximum. Apart from substrate-related factors like structure, composition, concentration, pretreatment and detoxification methods, hydrogen production process can be influenced by 1/ types of cultures, microbial strains, inoculum preparation methods; 2/ environmental conditions such as temperature, initial pH or fixed pH, Hydraulic Retention Time (HRT), Solid Retention Time (SRT) and hydrogen partial pressure; 3/ type of bioreactors and the operating mode e.g. batch, semi-continuous or continuous [44]. pH and temperature are the most critical variables which determining the metabolic pathways of hydrogen production and hydrogen consumption processes. Initial pH of 5.5 has been reportedly effectively inhibiting methanogenesis and homoacetogenesis, which are hydrogen-consuming, in both mesophilic and thermophilic conditions [45]. It should be noted that the optimal operational pH value varies with substrate types and organic loading rates. The continuous production of VFAs resulting in decreased pH can hinder the hydrogen production because the alcohol pathway is favoured at pH value lower than < 4.5 [27]. To address this issue, buffer solution shall be supplemented in an appropriate amount in order to maintain the initial pH value which encourages hydrogen generation.

Selection of operating temperature directly affects the microbial community and their enzymatic activities. The optimum temperature relies on the type of inoculum and the utilized substrate. Evidently, high temperature helps accelerate the hydrolysis process of complex biomass such as agricultural residues or food waste, Due to the increased activity of hydrolytic enzymes and the loosened biomass structure. It should also be noted that hydrogenase works most efficiently in the thermophilic range and hydrogen solubility drastically decreases as temperature increases [46]. All these factors could sufficiently explain the greater hydrogen yield under thermophilic conditions. On the contrary, readily biodegradable substrates are effectively consumed and converted under mesophilic conditions [27].

Elevated hydrogen gas concentration can inhibit the substrate conversion process of the bacterial community as non-hydrogen-producing pathways are thermodynamically preferred. Therefore, immediate removal of hydrogen gas is necessary. Application of different methods such as enhanced agitation, CO_2 and N_2 sparging, selective membranes and vacuum pumps has effectively lifted hydrogen yield. However, these methods all have their own issues related to cost-benefits and/or process controls [44].

2.4 An industrial connection to the hydrogen economy

In industrial settings, the utilization of LCB typically requires multiple processing steps to prepare the material for subsequent elemental extraction. However, these processes are often not perfectly optimized, leading to the generation of significant amounts of residual waste material. Porse and Rudolph [10] reported that more than 236,000 tons (dry weight) of seaweed biomass harvested in 2015 were transformed into approximately 93,000 tons of products. This translates to less than 40 % of the original feedstock being utilized, while the remainder was disposed of as waste. Such a high volume of residual waste not only creates challenges in waste management but also poses significant concerns regarding resource utilization efficiency. The industrial sectors dealing with LCB thus face an imperative need to improve their extraction and processing procedures. By enhancing feedstock utilization, they could minimize the volume of discarded biomass, promote more efficient resource recovery, and potentially increase their overall economic returns.

Table 2.2 consists of some roughly estimated values based on the available data collected via the internet.

Table 2.2 Rough estimation of total carbohydrate remaining in the residue biomass of an industry in 2019

	Production	Product	Residue
Carbohydrates (% dry weight)	45^a	-	23.5^c
Total dry weight (tons)	$236,820^b$	$93,035^b$	143,785
Total carbohydrates (tons, dry)	106,569	-	33,790

a - from [47]

b - from [10]

c - from this study (section 4.1)

Therefore, total dry weight of products equals 40 % of total dry weight of this biomass. As a result, 143,785 tons of residual biomass was generated annually.

Annual carbohydrates (dry) in fresh biomass = 236,829 × 45 % = 106,569 (tons)

Annual carbohydrates (dry) in residual biomass = 143,785 × 23.5 % = 33,790 (tons)

According to Lorenzo et al., most monosaccharides in this biomass are hexose (C_6). Assuming that optimization of bioprocesses in a biorefinery scheme, dark fermentation of this residue for hydrogen production achieves maximum theoretical yield (550 ml H_2/g COD), the total amount of hydrogen produced is calculated as follows:

$$\frac{550\,m^3\,H_2}{ton\,COD} \times 33,790\,tons\,c_6 \times \frac{1.07\,ton\,COD}{ton\,c_6} = 19,885,415\,m^3\,H_2\,@STP = 1.8MT\,H_2$$

Standard Temperature and Pressure (0^oC & 1 *atm*)

Normal Temperature and Pressure (20^oC & 1 *atm*)

Total energy generation: 1.8 MT H_2 × 33.33 MWh/T H_2* = 60 TW

* Lower heating value of hydrogen. From [48].

By applying these projections, it is estimated that biohydrogen production from this particular seaweed biomass could potentially contribute up to 60 TWh of energy. To offer a sense of scale, this amount of energy could supply more than a quarter of the entire energy demand of Norway for a year, given that the total energy consumption for the country was reported to be 223 TWh in 2021 [49]. This is a considerable fraction of the national energy demand, and it emphasizes the immense potential that biohydrogen production holds for contributing to our energy requirements. Furthermore, adopting

biohydrogen as an alternative energy carrier to fossil fuels is not just about energy production. This process could play a significant role in addressing some of the most pressing environmental concerns of current time. The most immediate benefit is the significant reduction in greenhouse gas emissions. Unlike fossil fuels, which release large amounts of harmful gases such as carbon dioxide when burned, biohydrogen is clean and sustainable, producing only water as a byproduct when used as a fuel. Moreover, the implementation of this process could offer a viable solution to the increasingly challenging issue of waste management. The seaweed biomass used in the process is essentially a form of waste from the industry, and its utilization in biohydrogen production thus provides a valuable method for waste reduction and resource recovery. Instead of disposing of this organic waste, which can contribute to environmental pollution and the overfilling of landfills, it can be transformed into a valuable source of renewable energy.

While the theoretical potential of biohydrogen production from seaweed biomass is indeed promising, there are several practical considerations and challenges that might limit the actual energy production. The actual efficiency of the biohydrogen production process can be significantly lower than the theoretical maximum due to various factors such as incomplete conversion of biomass to hydrogen, energy losses in the process, and the energy required for the process itself. Scaling up lab-scale processes to industrial scales often comes with numerous technical challenges. Overcoming these obstacles can be time-consuming and expensive [50]. Moreover, gathering, handling, and transporting this large volume of biomass from various sources to processing facilities can pose logistical challenges and additional costs, which could make the process less economically viable. [51] estimated the cost of producing biohydrogen to be between \$10/GJ and \$20/GJ, which is not even close to competitive with gasoline (\$0.33/GJ). The recognized potential of hydrogen as a green energy source brings with it significant transitional challenges, especially when it comes to shifting from existing fossil fuel-focused infrastructure to one that's hydrogen-centric. Current energy systems, encompassing production, storage, and distribution, are primarily engineered around fossil fuels. This implies substantial financial investments would be required to retrofit or rebuild this infrastructure to accommodate hydrogen. In conclusion, while the theoretical potential for energy generation from seaweed biomass is high, the practical implications and obstacles mean that actual energy production could be significantly lower.

Chapter 3

Materials and methods

3.1 Analysis methods

3.1.1 Substrate

In this research, a form of tertiary lignocellulosic biomass sourced from a local industrial collaborator with the University of Stavanger. To maintain the privacy and confidentiality of the partner, this substrate will be referred to as Lignocellulosic Aquatic Residue (LAR) throughout this study. The LAR was cut into small pieces, cooked for several hours at pH 12 under atmospheric pressure. Then it entered a pressurized vessel (approximately 7.5 bar) to continue the process. Finally, the mixture was filtered and decanted with a bowl separator. The major extracted components are monosaccharides and polysaccharides. Due to confidentiality constraints, specific information about the origin and exact nature of the LAR cannot be disclosed. However, it should be noted that it is a byproduct of an industrial process, and its utilization for biohydrogen production aligns with the principles of waste valorization and circular economy.

The characterization of the LAR was conducted to understand its composition and potential for biohydrogen production. Detailed analysis was conducted on this LAR to determine its COD, TS, VS, FS, moisture, lipid, and carbohydrate content. The measurements for TS, VS, FS, and moisture content were performed according to the guidelines provided in Section "2540 Solids" listed in [52]. Ceramic crucibles were pre-treated before the experiment by being burned in a muffle oven at 550^oC for 3 hours, then cooled in a desiccator before being weighed (m_{cup}), ensuring that any potential traces of organic matter were effectively removed. The LAR samples were weighed ($m_{wet\ sample}$) into these crucibles and then dried in a muffle oven at 105^oC for 12 hours. After cooling in a desiccator, the samples were weighed again ($m_{dried\ total}$), then ignited in a muffle

oven at 550^oC for 6 hours. Finally, they were weighed once more ($m_{ash\,total}$) after being cooled down to room temperature. The TS, moisture, VS, and FS were calculated using the following formulas:

$$TS\,(\%) = \frac{m_{dried\,total} - m_{cup}}{m_{wet\,sample}} \times 100 \tag{3.1}$$

$$Moisture\,(\%) = 100\,\% - TS\,(\%) \tag{3.2}$$

$$FS\,(\%) = \frac{m_{ash\,total} - m_{cup}}{m_{wet\,sample}} \times 100 \tag{3.3}$$

$$VS\,(\%) = \frac{m_{dried\,total} - m_{ash\,total}}{m_{wet\,sample}} \times 100 \tag{3.4}$$

Sample preparation involved spreading the LAR evenly on a baking paper sheet and drying it at 60^oC in an atmospheric pressure oven overnight until the moisture content dropped below 10 % weight. The dried LAR was then ground to a size of 200 μm using a classic mortar and pestle. The ground biomass was hydrolyzed and defatted as per the recommended procedure [53] utilizing the Soxtec 8000 Extraction System and Hydrotec 8000 Hydrolysis System manufactured by FOSS, Denmark. The resulting product from the hydrolysis process was referred to as Hydrolyzed Lignocellulosic Aquatic Residue (HLAR), while the output of the defatting process was called Defatted Lignocellulosic Aquatic Residue (DLAR) throughout this study.

The lipid extraction procedure involved several steps. First, 1.5 - 2.0 g sample was weighed into each thimble (m_{sample}). Then all thimbles were placed in a capsule holder and put into the beaker of the Hydrotec 8000 Hydrolysis System. After hydrolized in 3M HCl solution and rinsed, the thimbles were dried overnight in a 55^oC oven. A thin wad of de-fatted cotton was placed on top of the sample in each thimble, carefully pushing it down in the center to prevent overflow of biomass. All the thimbles were then attached onto the sample holders, and dry and cool extraction cups (m_{cup} before) containing 85 mL of solvent were docked onto the hotplate of the Soxtec 8000 Extraction Sytem. The extraction program was then started. The samples were cooked in the solvent, and the lipids were extracted into the cups. After extraction, the cups were dried and cooled before weighing and recording the weight (m_{cup} after). The lipid content was calculated using the following formula:

$$Lipid\,(\%) = \frac{m_{cup\,after} - m_{cup\,before}}{m_{sample}} \times 100 \tag{3.5}$$

Table 3.1 provides the detailed configuration of the extraction process.

Table 3.1 Configuration of the extraction process using the Soxtec 8000 and Hydrotec 8000 by FOSS

Programs and instruments	Configurations
Hydrolysis program	Hydrolysis acid: HCl 3M
	Boiling Power: 70 %
	Boiling time: 60 min
	Cooling: OFF
	Numbers of rinses: 15
Glass extraction cups	Drying time (minutes)
	Before extraction, dry at at 103^oC for >= 50 min
	After extraction, dry at at 103^oC for 50 min
	Cool in desiccator with cup stand for 75 min
Lipid extraction program	Solvent: Petroleum Ether $40 - 60^oC$
	Solvent volume: 85 mL
	Temperature: 16^oC
	Boiling/Rinsing/Recovery time: 30/45/15 min

To determine the carbohydrate content, samples of LAR, HLAR, and DLAR were prepared in accordance with section 10.1 and 10.3 of NREL's Laboratory Analytical Procedure [54]. Initially, 25 mg of each sample was digested with 250 μL of a 72 % (w/w) H_2SO_4 solution in a 30^oC water bath. After one hour, 7 mL of deionized water was added to each tube to dilute the acid concentration to 4 %. Following this, the tubes were capped and autoclaved for one hour at 121^oC using the liquid setting. After the autoclaving process, the carbohydrate content was measured by subjecting the hydrolyzed samples to the phenol-sulfuric acid method proposed by [55]. This involved reacting the samples with a 5 % phenol solution and concentrated sulfuric acid, resulting in an orange-yellow color of which absorbance was then measured at 490 nm using a standard spectrophotometer. A standard glucose curve (Appendix A) was previously constructed as a reference for the quantification of sugar content.

For the COD analysis, methodologies from the novel studies by [56][57] were referred to. The dried LAR was initially milled using Fritsch's Pulverisette 7 planetary mill with 2 mm grinding balls and 2 zirconium oxide grinding bowls. The mill's rotational speed was set at 800 rpm with a process involving 25 cycles of 20-minute grinding periods, each

followed by a 10-minute pause. From the resultant fine LAR powder, approximately 7 mg was combined with 0.1 mL of deionized water and added into a Spectroquant COD cell test kit #1.01797.0007 (5000 - 90000 mgCOD/L). This experiment was conducted six times to ensure accuracy. The COD measurements were subsequently determined using a Spectroquant Prove 300 spectrophotometer.

3.1.2 Inoculum

Seed sludge was originated from a local municipal wastewater treatment plant, known as Sentralrenseanlegg Nord Jæren (SNJ), operated by IVAR in Randaberg, Norway.

For the AMPTS batch 1, the sludge underwent a thermal pre-treatment at 105^oC for 24 hours and was subsequently ground to a particle size of 200 μm, following the methodology proposed by [58][59]. For the second batch, the inoculum was heated in a 95^oC water-bath for 45 minutes, following the approach used in many previous studies [34][60]. The aim of employing these treatments was to restrict the activity of hydrogenotrophic methanogens, while simultaneously promoting the enrichment of endospore-forming bacteria that are efficient in biohydrogen production.

The sludge was also subjected to several analyses to determine its pH, COD, TS, VS, FS, ALK and VFA. The measurements for TS, VS, FS, and moisture content were performed according to the guidelines provided in Section "2540 Solids" listed in [52]. A similar procedure to the one used for LAR sample was adopted here. The only difference is that 25 mL (V_{sample}) of the sludge was added to each ceramic crucible. The corresponding TS, VS, FS formulas are as follows:

$$TS\ (mgTS/L) = \frac{m_{dried\ total} - m_{cup}}{V_{sample}} \tag{3.6}$$

$$VS\ (mgVS/L) = \frac{m_{dried\ total} - m_{ash\ total}}{V_{sample}} \tag{3.7}$$

$$FS\ (mgFS/L) = \frac{m_{ash\ total} - m_{cup}}{V_{sample}} \tag{3.8}$$

The COD values were estimated using the COD test kit #1.09773.0001 (100 - 1500 mgCOD/L). This involved pipetting 2 mL of well-mixed sludge into the vials provided in the kit. The COD measurements were subsequently determined using a Spectroquant Prove 300 spectrophotometer.

3.1.3 Post-fermentation broth

Post-fermentation broths were subjected to an array of measurements including pH, COD, TDS, conductivity, ALK, and total VFA. COD measurements were conducted in the same manner as with the sludge mentioned previously. TDS measurements were performed in accordance with the guidelines provided in Section “2540 Solids" listed in [52]. The broth was centrifuged at 5000 rpm for 20 minutes using an Eppendorf Centrifuge 5804R, equipped with a rotor radius of 155 mm. Then, the supernatants were filtered through 2 μm filters. 5 mL of each supernatant was added to pre-weighed aluminum dishes (m_{dish}) and subjected to a 2-hour drying process at 180^oC to determine TDS. The dishes were then cooled to room temperature and weighed again ($m_{dried\,total}$). TDS was calculated with the following formula:

$$TDS\,(mgTDS/L) = \frac{m_{dried\,total} - m_{dish}}{V_{sample}} \tag{3.9}$$

The same samples were analyzed for Alkalinity (ALK) via an automatic titration using the TitroLine 5000, equipped with a WTW SenTix 21 pH Electrode and 40 mM HCl as the titrant. 5 mL of each sample was diluted into 20 mL of deionized water. The titration process was initiated, and the volumes of acid used at pHs 6.7, 5.9, 5.2, and 4.3 were recorded.

Conductivity values of these supernatants were measured using the VWR phenomenal CO 3100L equipped with a CO11 probe. ALK and total VFA were determined using a 5-point titration method as proposed by [61], aided by the supplementary software “TITRA 5”. This software automatically calculates VFA and ALK concentrations after the relevant variables such as pH, TDS, conductivity, volume of the titrant, and temperature have been specified.

For samples obtained from the CFSTR (at day 5, 10 and 16), where the initial pH was below 6.7, 3N NaOH was used to raise the pH to above 6.7. Following this pH adjustment, the samples underwent titration as described previously.

The titration involves diluting 5 mL of samples into 20 mL of deionized water, the titration commenced and the volumes of acid used at pHs 6.7, 5.9, 5.2, and 4.3 were noted.

3.2 Experimental setup and reactor configuration

3.2.1 BHP assay (AMPTS)

BHP was determined using an AMPTS produced by Bioprocess Control Instruments. This system represents a state-of-the-art solution for conducting biochemical hydrogen/methane potential tests, delivering reliable and precise measurements of gas production.

In our experiment, the AMPTS system was configured specifically to evaluate the BHP of the investigated substrate. The operational principle of the AMPTS for BHP testing revolves around an integrated system where the produced gas is collected and measured in real-time using a highly sensitive flowmeter.

The sample substrate is loaded into 500-mL Schott glass bottles as reactors of the system, which are subsequently sealed to maintain anaerobic conditions necessary for dark fermentation. Each bottle was added 22.6 mL nutrients and buffer solution (Table 3.2), prepared as guided in [58], to ensure a stable environment for the microorganisms. Fifteen 360mL-filled Schott bottles were each installed a rotating motor head on top and were submerged in a 37^oC water bath (Figure 3.1). The process is initiated by inoculating the substrate with a specific amount of microbial culture. For this study, two sequential batches were conducted, the details of which will be further elaborated in the following tables (Table 3.3). The objective of Batch 1 was to investigate the impact of various pretreatment methods on the substrate, whereas Batch 2 aimed to examine higher SIR (from 6 - 90), using the optimally pretreated substrate as identified in Batch 1.

Table 3.2 Nutrients and buffers solution [58]

Reagents	Added amount to 1L of deionized water (mg)
NH_4Cl	41,600
2-(N-Morpholino) ethanesulfonic acid (MES)	19,520
$MgCl_2 \bullet 6\,H_2O$	2,000
$FeSO_4 \bullet 7\,H_2O$	1,600
$CoCl_2 \bullet 6\,H_2O$	40
$MnCl_2 \bullet 4\,H_2O$	40
KI	40
$NiCl_2 \bullet 6\,H_2O$	8
$ZnCl_2$	8

Table 3.3 AMPTS Batch 1 & 2 compositions

Caldicellulosiruptor saccharolyticus DSM 8903	Reactor	Name	Substrate	*Substrate weight (gTS)*	*Inoculum weight (gVS)*	*SIR (gTS/gVS)*
Batch 1	1	Blank B1	-	0.00	0.67	-
	2	Blank B2	-	0.00	0.67	-
	3	Blank B3	-	0.00	0.67	-
	4	Control C1	Glucose	1.80	0.67	2.7
	5	Control C2	Glucose	1.80	0.67	2.7
	6	Control C3	Glucose	1.80	0.67	2.7
	7	Defatted DF1	DLAR	2.94	1.09	2.7
	8	Defatted DF2	DLAR	2.94	1.09	2.7
	9	Defatted DF3	DLAR	2.94	1.09	2.7
	10	Hydrolized HD1	HLAR	4.06	1.51	2.7
	11	Hydrolized HD2	HLAR	4.06	1.51	2.7
	12	Hydrolized HD3	HLAR	4.06	1.51	2.7
	13	Wet W1	LAR	7.71	2.85	2.7
	14	Wet W2	LAR	7.71	2.85	2.7
	15	Wet W3	LAR	7.71	2.85	2.7
Batch 2	1	Blank B1	-	0.00	2.47	-
	2	Blank B2	-	0.00	1.23	-
	3	Blank B3	-	0.00	0.25	-
	4	Blank B4	-	0.00	0.12	-
	5	Blank B5	-	0.00	0.08	-
	2	Blank $B6^a$	-	0.00	1.60	-
	3	Blank $B7^a$	-	0.00	3.19	-
	4	Blank $B8^a$	-	0.00	3.99	-
	5	Blank $B9^a$	-	0.00	4.79	-
	6	SIR 6.3-1^b	LAR	7.71	1.23	6.25
	7	SIR 6.3-2^b	LAR	7.71	1.23	6.25
	8	SIR 31.3-1	LAR	7.71	0.25	31.25
	9	SIR 31.3-2	LAR	7.71	0.25	31.25
	10	SIR 31.3-3	LAR	7.71	0.25	31.25
	11	SIR 62.5-1	LAR	7.71	0.12	62.5
	12	SIR 62.5-2	LAR	7.71	0.12	62.5
	13	SIR 62.5-3	LAR	7.71	0.12	62.5
	14	SIR 93.8-1^b	LAR	7.71	0.08	93.75
	15	SIR 93.8-2^b	LAR	7.71	0.08	93.75

a - replaced blanks 2, 3, 4, 5 after they failed to produce any gas.
b - bottles with SIR 6.3 and 93.8 were done only in duplicates due to the limited capacity of AMPTS.

As the microorganisms begin to consume the substrate, hydrogen is continuously generated. The gas produced in each bottle passes through an individual vial containing 80 mL 3N NaOH solution, which serves to remove any CO_2 and H_2S that could interfere with the accurate measurement of biohydrogen. The thymolphthalein pH indicator in the alkaline solution will change from blue to colorless when the CO_2 absorption capacity of the NaOH solution drops below the optimum. At 22^oC, approximately 2.9 L of CO_2 can

be absorbed in each vial before the alkaline solution needs to be changed [58]. However, the slight overestimation of gas volume was reported due to the incomplete absorption of carbon dioxide [62].

The cleaned gas then enters a Gas Volume Measuring Device, which is capable of measuring gas volume using a wet gas flow measuring device with a multi-flow cell arrangement. This measuring device works according to the principle of liquid displacement & buoyancy and can monitor ultra-low gas flows; a digital pulse is generated when a defined volume of gas flows through the device.

The experiment setup is demonstrated in Figure 3.1.

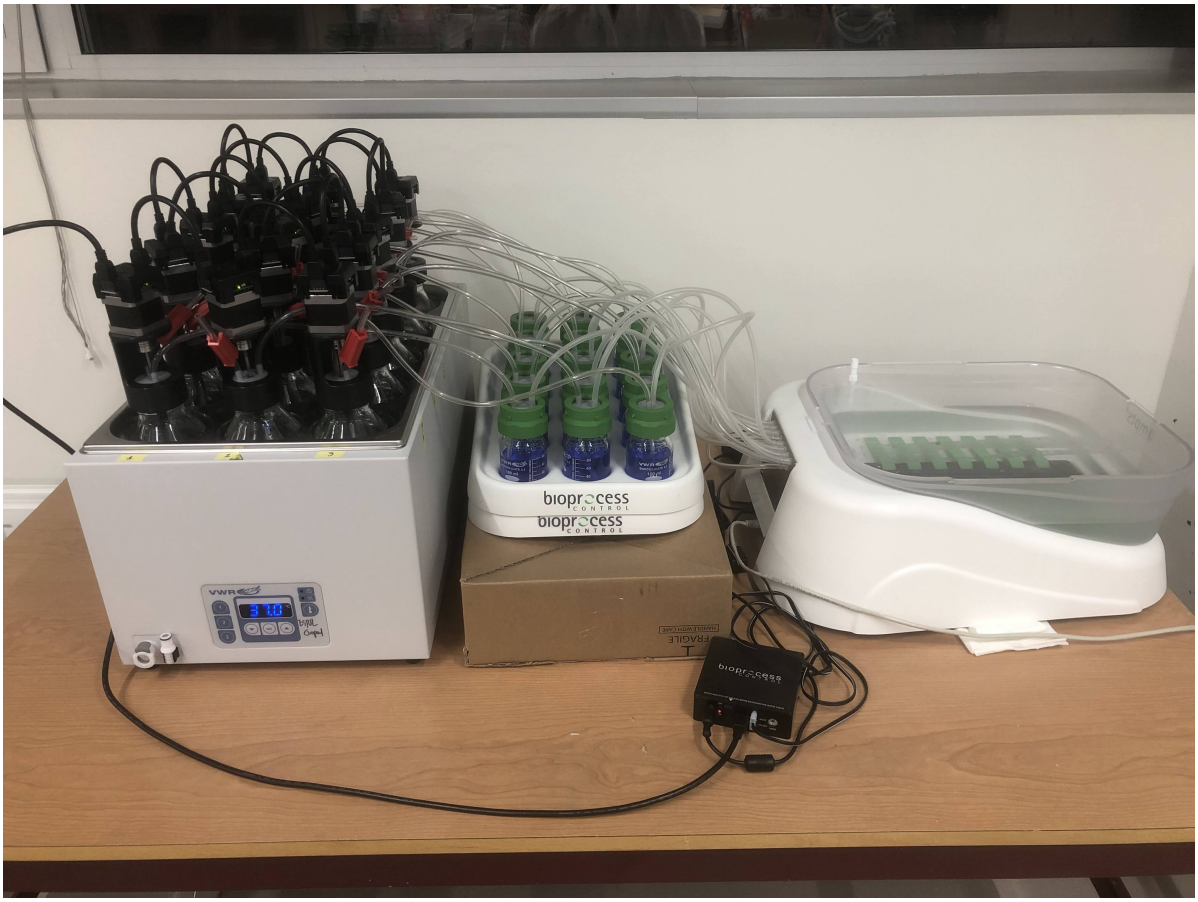

Figure. 3.1 AMPTS experiment setup (from left to right: Sample Incubation Unit – CO_2 Absorbing Unit – Gas Measuring Unit)

Gas volume data is automatically recorded by an integrated data acquisition system and transmitted to a connected computer, where it can be monitored and analyzed

in real time. The AMPTS's automated nature not only reduces the risk of manual errors but also allows for continuous monitoring and high-resolution data, facilitating detailed kinetic analyses of the process. The AMPTS allowed for systematic evaluation of the biohydrogen production potential of the substrates under consistent and controlled conditions, leading to accurate and reliable results that provide key insights into the mechanisms and efficiencies of biohydrogen production.

3.2.2 CFSTR

As part of the efforts to upscale biohydrogen production, a CFSTR was set up. The reactor, with a working volume of 2740 L, was operated at a constant temperature of 37^oC with a water jacket. A schematic representation and the bench-top model of the reactor configuration is presented in Figure 3.2 and Figure 3.3. Additionally, the required components with some specifications are listed in Table 3.4.

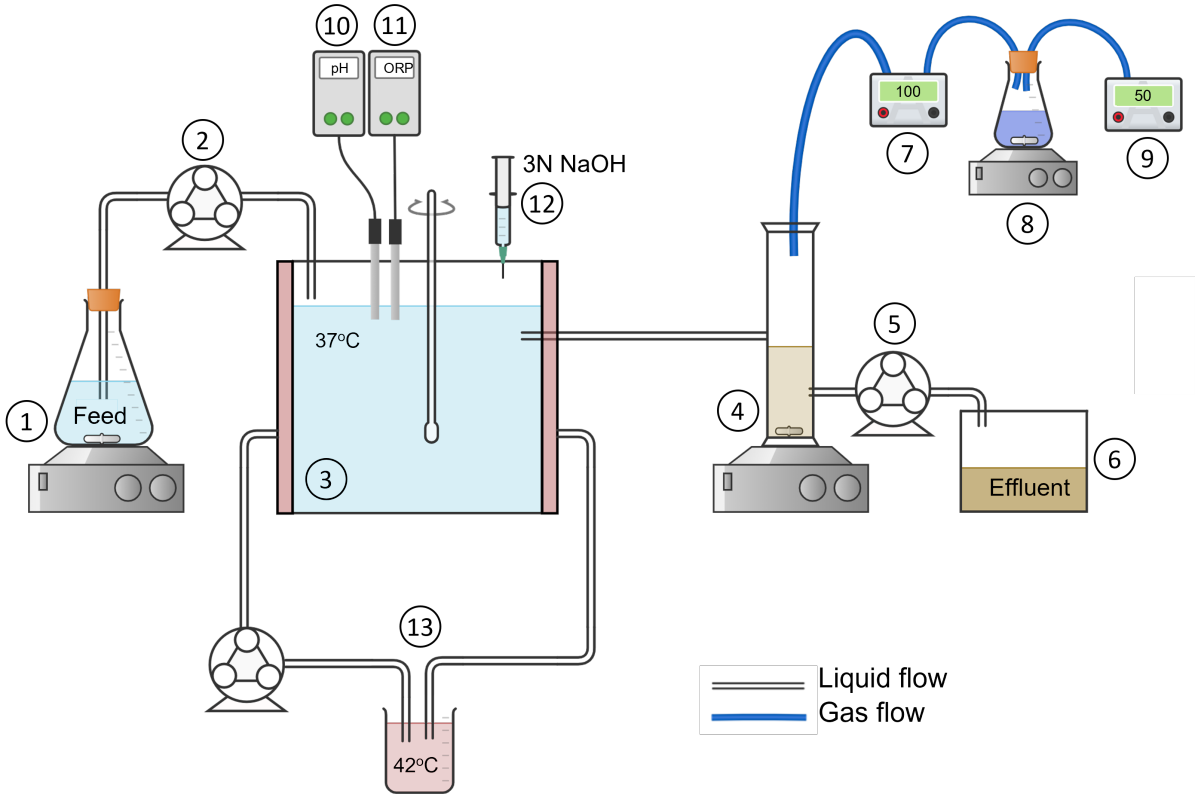

Figure. 3.2 Schematic diagram of the CFSTR

1. Feed bottle
2. Feed pump

3. Bioreactor
4. Liquid/gas separator
5. Effluent pump
6. Effluent tank
7. Total gas counter
8. CO_2 absorber
9. Biohydrogen counter
10. pH meter
11. ORP meter
12. Needle for pH adjustment
13. Water jacket system

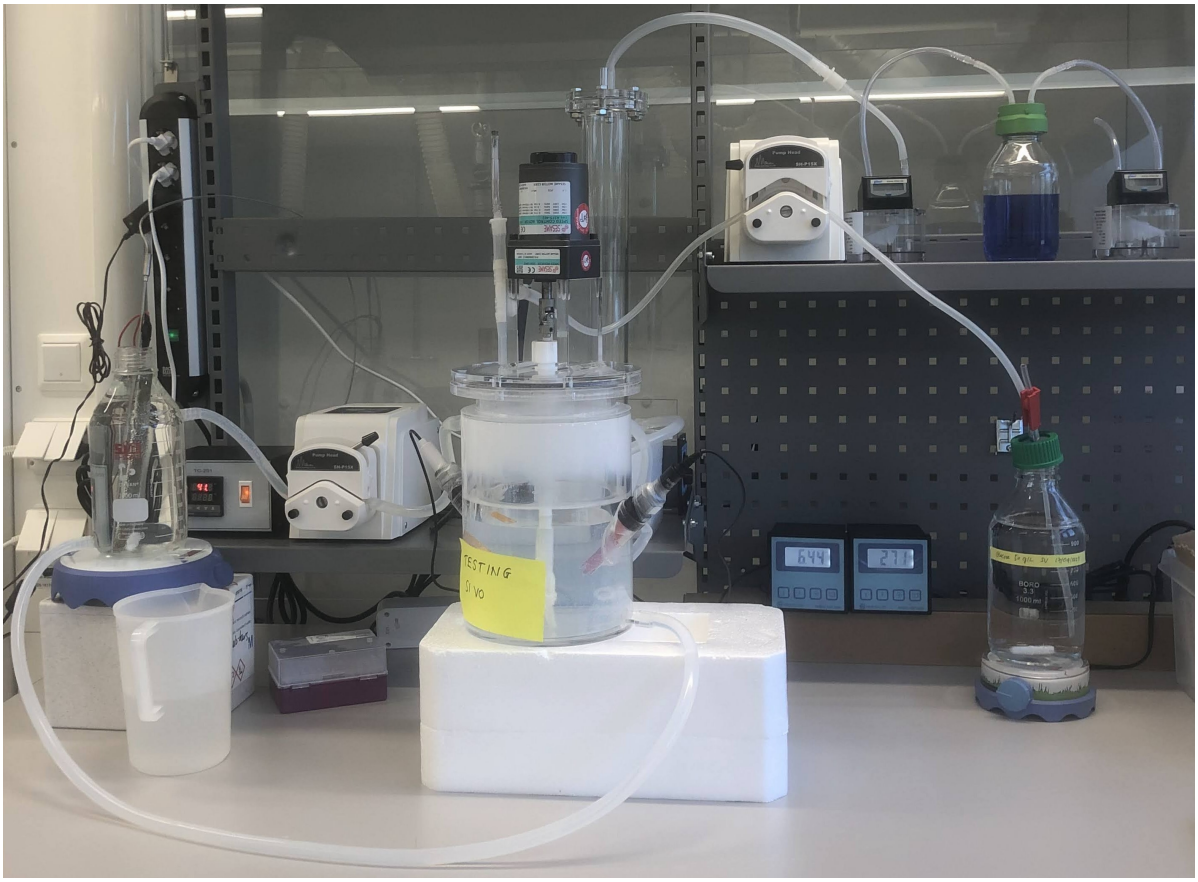

Figure. 3.3 CFSTR setup at the lab

Table 3.4 Bench-top CFSTR setup components and their specifications

Equipment	Manufacturer	Specification
Feed/discharge pump	Shishin Technology	Type: peristaltic pump Model: SH-P100 Flowrate: 0.0002 - 380 mL/min
Gas counter	Ritter	Model: MGC-1 V3.4 PMMA Flowrate: 1 - 1000 mL/h Pressure: 8 - 100 mbar Packing liquid: HCl 1.8 %
Heating coil	N/A	N/A
Temperature controller	Walfront	N/A
Water jacket pump	Behr Labor-Technik GmbH	Type: peristaltic pump Model: PLP 1000 Flowrate: 250 - 1000 mL/min
pH probe and meter	Kidd Co., Ltd	N/A
ORP probe and meter	Kidd Co., Ltd	N/A
Agitator motor and controller	Sesame Motor	Model: M315 – 402 Output: 15W Speed range: 90 - 1700 rpm

The substrate feed was pumped from a continuously stirred Schott bottle into the reactor using a peristaltic pump with adjustable flow rates. The generated gas traveled through a liquid/gas separator to a total gas counter and, subsequently, to a carbon absorber and, finally, a biohydrogen gas counter. The liquid/gas separator and the carbon absorber were continuously stirred with magnetic stirrers in order to enhance gas release and CO_2 absorption, respectively. Monitoring of pH and ORP was ensured through two separated probes attached to the reactor.

The reactor was inoculated with 500 mL of pretreated inoculum and was fed with the same nutrient and buffer solution as was introduced to the 500 ml reactors in the AMPTS assay. To maintain the pH value above 5.5, the reactor was manually fed with 3N NaOH solution.

Initially, the reactor was fed with 580 mL of a 50 g/L glucose solution and was acidified to pH 5.5 to upstart the microorganism activities. The plan was to maintain in batch mode for 2 days then transition to a continuous mode from day 3 with a HRT of 24 hours, with an estimated feed/discharge rate of 90 mL/h. However, due to various practical challenges, the reactor's flow stabilization was achieved only after 2 weeks.

The discharge was managed by pumping out from the gas-liquid separator, maintaining a smooth flow throughout the process. Upon reaching a stable flow, an altered substrate feed, integrating a blend of LAR and glucose was made. This mixture was designed to ensure that the theoretical COD remained on par with the 50 g/L glucose solution. This strategy was intended to prevent drastic changes in organic loading and to maintain the

previous flow rate. However, the integration of LAR presented unexpected challenges, leading to changes in the feed solution's properties, such as pH, viscosity (based on personal observation), and consistency. As a result, the task of controlling the flow rate had to be redone and adjusted accordingly.

3.3 Kinetics modelling

The modified Gompertz model, proposed by [63] is a nonlinear regression model that has been widely used to describe microbial growth, specifically in the context of cumulative biogas or biohydrogen production during anaerobic digestion [64][65].

The model's equation is as follows:

$$H(t) = H_{max} exp\left\{-exp\left[\frac{e \times R_{max}}{H_{max}}(\lambda - t) + 1\right]\right\} \tag{3.10}$$

where:

H represents the cumulative biohydrogen production at time t (mL),

H_{max} is the maximum cumulative biohydrogen production capacity (mL),

R_{max} is the maximum biohydrogen production rate (mL/day),

λ is the lag phase time (day), and

e is the base of the natural logarithm (approximately equal to 2.71828).

In this equation, the parameters H_{max}, R_{max}, and λ are estimated based on experimental data, and they provide insights into the biohydrogen production process. H_{max} indicates the maximum potential for biohydrogen production from the substrate. The R_{max} parameter indicates the maximum rate of gas production, which can provide an indication of the kinetics of the microbial process. The lag phase time, λ, is the period before the microbes adapt to the conditions in the reactor and start producing biogas/biohydrogen at a significant rate. In this thesis, GraphPad Prism 9 and Microsoft Excel were utilized to perform the nonlinear regression needed to estimate the parameters in this model.

Chapter 4

Results and discussion

All experiments in this study were conducted in triplicate, unless stated otherwise. The results are presented as ***mean*** ± ***standard deviation***.

4.1 Substrate and inoculum characterization

Table 4.1 Substrate and inoculum characterization

Parameter	Unit	*Substrate (residual seaweed)*	*Inoculum (anaerobic sludge)*
TS	a, b	23.85 ± 0.23^a	22495.4 ± 132.0^b
VS		13.74 ± 0.17^a	15950.9 ± 102.8^b
FS		10.11 ± 0.17^a	6544.5 ± 35.2^b
Moisture		76.15 ± 0.23^a	-
VS/TS ratio	-	0.58	0.71
COD	c, d	0.96 ± 0.01^c	34433.3 ± 625.2^d
pH	-	-	7.31 ± 0.05
ALK	$mgCaCO_3/L$	-	3813.6 ± 123.1
VFA	$mgCH_3COOH/L$	-	480.5 ± 72.3
VFA/ALK ratio	-	-	0.13

a - % weight of wet sample

b - mg/L

c - gCOD/gTS

d - mgCOD/L

Table 4.2 Substrate compositions comparison [27][66][67]

Parameters	LAR	Spent coffee ground	Food waste	Buffalo manure	Olive pomace	Potato waste	Pumpkin waste
TS (% wet matter)	23.85	38.31	10	7.03	28.8	16.87	11.6
VS (% wet matter)	13.74	37.61	8.8	5.76	25.2	15	8.4
FS (% wet matter)	10.11	0.71	1.2	1.27	3.6	1.87	3.2
VS/TS	0.58	0.98	0.88	0.82	0.88	0.89	0.72
COD (gCOD/gTS)	0.96	1.49	0.99	-	-	-	-

The LAR and inoculum was analyzed for several key parameters as displayed in Table 4.1.

The inoculum results were comparable to previous studies which also used anaerobic sludge from IVAR [68][67]. The LAR's TS were found to be 23.85 ± 0.23 % of the wet weight, indicative of a substantial moisture content amounting to 76.15 ± 0.23 % of the wet weight. This can be attributed to the inherent aquatic nature of the biomass and its significant alteration through industrial alkaline treatment. Comparing with other frequently studied substrates in Table 4.2, LAR's VS is relatively low, amounting to 13.74 % of wet matter, whereas its FS is substantially higher. Consequently, the VS/TS ratio, which serves as a crucial indicator of substrate biodegradability and potential biogas/biohydrogen yield, is approximated to be 0.58. This VS/TS ratio is lower than most other substrates, suggesting that LAR might offer a moderate level of degradable organic material and a potentially lower biohydrogen yield. Furthermore, the COD of LAR is also relatively low at 0.96 ± 0.01 mgCOD/L, reinforcing the assessment that LAR encompasses a significant proportion of non-biodegradable or inorganic components due to high FS content. Despite these traits, LAR constitutes an interesting substrate for additional investigation into its utilization for biohydrogen production and potentially other side-stream products.

Table 4.3 Substrate composition

Parameter	Unit	Experimental data			Composition conformed to parameters defined by [47][69]
		LAR	HLAR	DLAR	
Moisture	%	76.15 ± 0.23	-	-	70 - 85
Lipid	% of dry matter	9.78 ± 0.75	20.20 ± 0.12	-	2 - 7
Carbohydrates	% of dry matter	23.46 ± 1.97	44.33 ± 1.44	61.30 ± 0.53	45 - 60
Protein	% of dry matter	-	-	-	5 - 10
Ash	% of dry matter	42.4	-	-	15 - 25; 17 - 20

Carbohydrates content of LAR is determined to be 23.46 ± 1.97 % dry weight, much lower than reported in fresh biomass [47]. This is definitely due to the industrial extraction process which primarily aims at several mono- saccharides and poly-saccharides. As a result, lipid content turned out to increase to 9.78 ± 0.75 % dry weight. Notably, HLAR and DLAR have significantly high carbohydrates (44.33 ± 1.44 % and 61.30 ± 0.53 % respectively), while HLAR constitutes 20.20 ± 0.12 % of lipid.

4.1.1 LAR oil extraction – potential by-product stream

The LAR investigated in this research project exhibits characteristics that make it a promising feedstock for oil extraction, comparable to traditionally exploited oil-bearing materials like rice bran. The lipid content of the HLAR stands at 20.20 ± 0.12 % of dry matter, fitting comfortably within the range typically found in rice bran (15 % to 25 %), which is directly subjected to solvent extraction without prior mechanical expression for oil recovery [70]. Furthermore, the LAR displays a high carbohydrate composition, especially noticeable post hydrolysis and defatting, aligning with the high-carbohydrate nature of rice bran. This positions the LAR as a potential dual-purpose feedstock not only for energy recovery processes but also oil extraction that take advantage of its relatively high lipid and carbohydrates contents. Lastly, also similar to rice bran, the LAR is assumed to contain less than 10 % protein content [47]. On the flip side, it's important to note that while this oil source could have diverse industrial applications, its refinement for use in edible or feeding purposes may pose various challenges due to stringent requirements in those sectors [71]. This, however, does not diminish the value of the oil extracted from LAR, as it opens up possibilities for various industrial uses.

4.2 BHP assay and biohydrogen production

In the first batch of the AMPTS, various substrates were employed, including glucose, HLAR, DLAR, and LAR. However, most of these substrates, with the exception of the LAR, did not yield any appreciable gas. The non-production of gas from glucose was attributed to operational issues related to the CO_2 capture system. Due to the formation of a vacuum inside the control reactors C1, C2 and C3, a considerable volume of 3N NaOH solution from the absorption bottles was inadvertently siphoned into these control

reactors. This incident was attributed to the submersion of the hoses (Figure 4.1), leading to the unanticipated inflow of the NaOH solution.

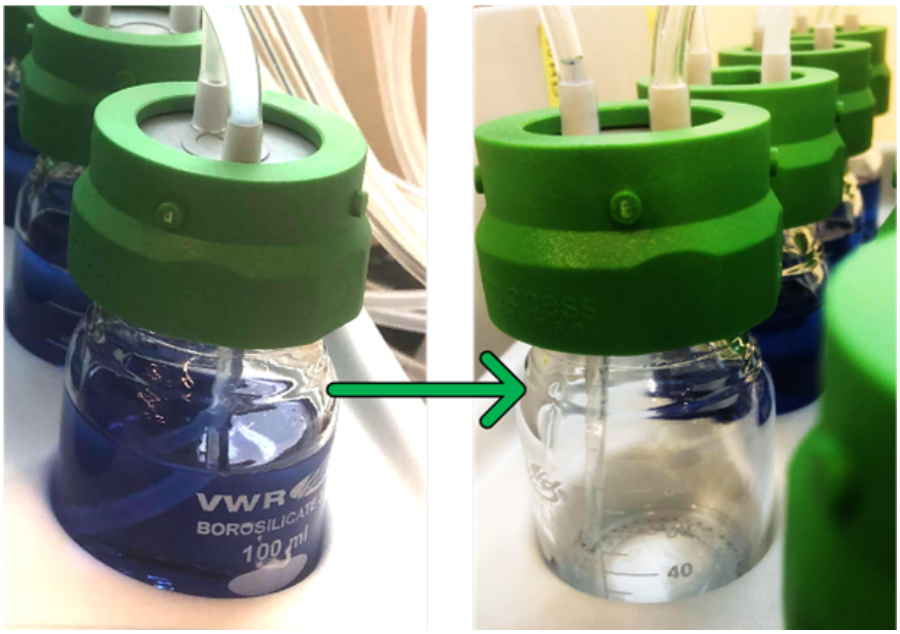

Figure. 4.1 NaOH siphoned into reactors

The unexpected lack of gas production from HLAR and DLAR samples might be attributed largely to the presence of toxic or inhibitory compounds. The processing of LAR, which involved exposure to a strong acid during hydrolysis, a solvent during defatting, and high pH environment at industrial levels, could have inadvertently introduced or released these compounds. Long-chain fatty acids hydrolyzed from lipids are the main dragging components that have been identified as the main inhibitory factor on microbial activity of anaerobic consortium [72]. While pretreatment methods are crucial in disrupting lignocellulosic structures and liberating fermentable sugars, they often culminate in the formation of undesirable byproducts, often referred to as fermentation inhibitors [14]. The principal objective of these pretreatments is to enhance the accessibility to carbohydrate fractions while simultaneously minimizing the production of these inhibitory compounds. However, it's evident that the formation of these inhibitors is inevitable but rather reduced through careful management and optimization of the pretreatment conditions [14][73]. In further studies after this thesis, a comprehensive reassessment of these parameters is definitely necessary to prevent such detrimental occurrences.

The cumulative H_2 production kinetics obtained from both AMPTS batches with LAR at different SIR and the corresponding curves followed modified Gompertz's model are

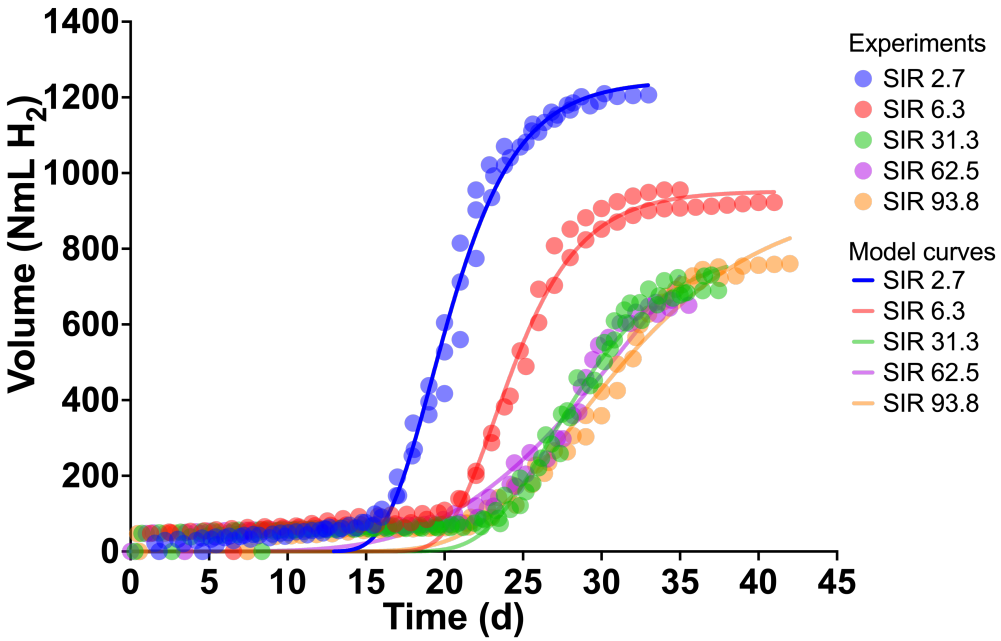

Figure. 4.2 Experimental and modeled cumulative biohydrogen production of LAR at different SIR

presented in Figure 4.2. The most significant hydrogen volume and the shortest lag phase were recorded at an SIR of 2.7, yielding a total production of 1209 NmL of hydrogen and an acclimation period of 16.2 days. As the SIR escalated, a decline in both cumulative biohydrogen production and the duration of the lag phase was observed. Notably, once SIR exceeded 30 gTS/gVS, minimal variations were found in the growth curves, with a relatively constant lag phase between 20.6 and 22.5 days, and the maximal production around 800 NmL. These observations suggest that while more substrate availability (beyond SIR 2.7) may provide such abundant 'nutrient' concentration for the microbial community that it caused signs of inhibition (less gas, longer lag). When it reached a threshold beyond 30, further substrate addition neither enhanced the energy yield nor contributed to microbial inhibition.

Figure 4.3 illustrates the daily variations in biohydrogen production rates at different SIRs. The findings are normalized to express the volume of hydrogen produced per gram of volatile solids per day (NmL H_2 g^{-1} VS d^{-1}). Each experimental run is marked by two noticeable peaks in flow rate. The initial peak, observed around day 1, indicates an immediate conversion of readily available COD. The second peak, however, emerges after a delay of approximately five days following the lag phase. This delayed response can be attributed to the time taken by the hydrogen-producing bacteria to adapt to

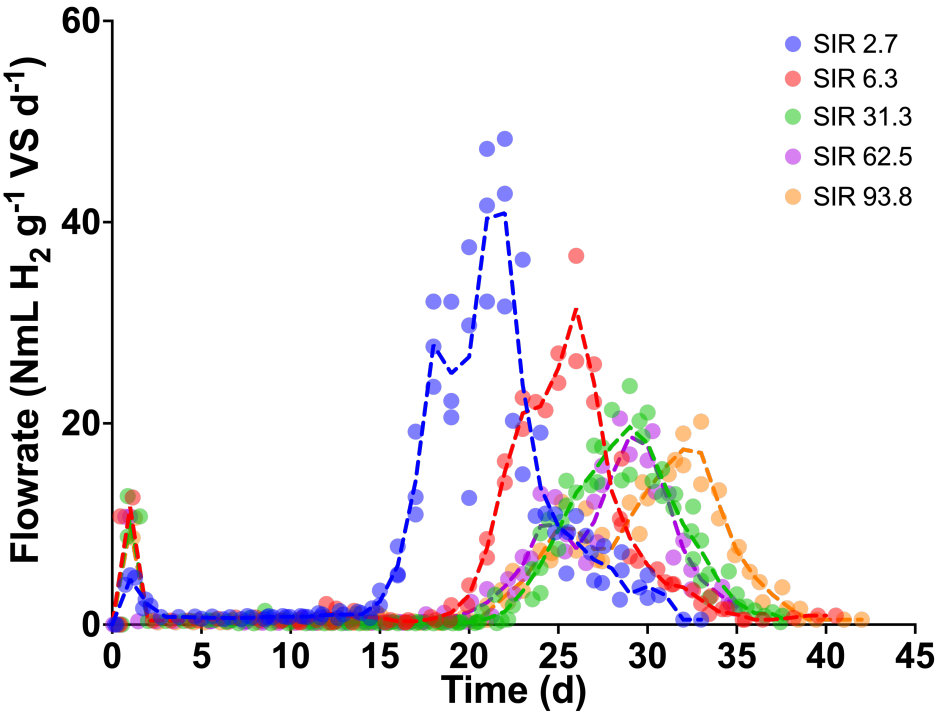

Figure. 4.3 Daily flowrate variations in biohydrogen production at different SIR

the new substrate environment, break down the complex structure of LAR into readily fermentable sugars, and initiate the biohydrogen production pathway. The maximum daily production rate was achieved at SIR 2.7, reaching a peak at 48.3 NmL H_2 g^{-1} VS d^{-1}. At SIR 6.3, the cumulative biohydrogen production reached a satisfactory level of 980 mL (Figure 4.2), with a decent flow rate peak of 36.7 NmL H_2 g^{-1} VS d^{-1}. However, as the substrate concentration was more than doubled the SIR 2.7, these production parameters began to decline. This reduction in biohydrogen production and flow rate suggest the inhibitory effects caused by the high substrate concentration. The concentration of substrate directly influences the microbial activity and the balance of metabolic processes. When this concentration is too high, it can lead to reduced conversion into biogas, and increased in production of short-chain fatty acids e.g lactic acid and its compounds [74]. The least effective scenario was observed at SIRs 93.8, where the peak daily production rate dropped to 20.2 NmL H_2 g^{-1} VS d^{-1} on the 33^{rd} day of the second batch of experiments. It shoule be noted that the SIRs beyond 30 all hovered around a peak flow rate close to 20 NmL H_2 g^{-1} VS d^{-1}. This again suggest that excessively high SIRs limiting the biohydrogen production efficiency.

4.2.1 Kinetic modelling results

Table 4.4 Biohydrogen production performance and the modified Gompertz equation parameter values

SIR (gTS/gVS)	Max total bioH_2 (NmL)	Modified Gompertz equation parameters (GraphPad Prism 9)				Modified Gompertz equation parameters (Microsoft Excel)			
		H_{max} (NmL)	R_{max} (NmL/d)	λ (d)	R^2	H_{max} (mL)	R_{max} (mL/d)	λ (d)	R^2
2.7	1209.7	1245	153.3	16.2	0.9901	1247.2	155.4	16.3	0.9965
6.3	955.4	952.4	116.7	20.2	0.9793	970.3	113.2	20.1	0.9941
31.3	730.6	806.9	70.5	22.5	0.9644	934.4	72.6	22.5	0.9859
62.5	664	760.2	54.7	20.6	0.9596	732.6	58	20.9	0.9753
93.8	760.5	940.4	52.4	21.8	0.9741	969.2	54.3	22	0.9878

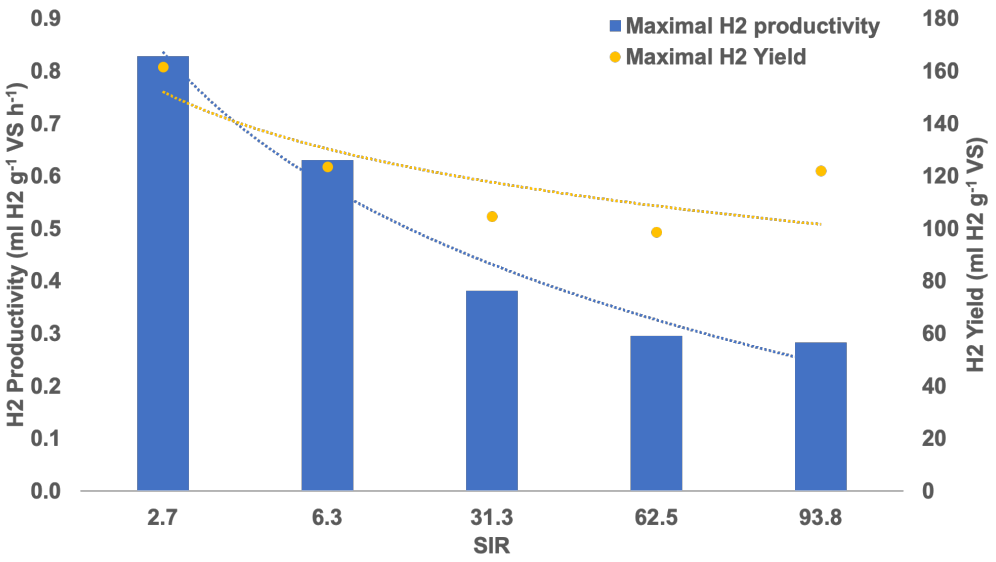

Figure. 4.4 Maximal H_2 productivity and yield

The parameters derived from the modified Gompertz model showed a good fit with microbial growth curve parameters, returning R^2 values of at least 0.9596, which indicates high reliability. The equation parameters were computed using two different software applications: GraphPad Prism 9 and Microsoft Excel. To further evaluate the conversion efficiency of the dark fermentation process, Hydrogen Yield (HY) [mL H_2 g^{-1} TS] and Hydrogen Productivity (HP) [mL H_2 g^{-1} TS h^{-1}] were calculated (as shown in Figure 4.4). These measures facilitate more straightforward comparisons with other studies and provide insights into the suitability of this substrate for biohydrogen production. Notably, as the SIR increased, both HY and HP displayed a decreasing trend, with an optimum around SIR 2.7, which was suggested in the standardized protocol by [58].

Remarkably, the peak HY, which occurred at SIR 2.7, was approximately 280 mL H_2 g^{-1} VS (equivalent to 161 mL H_2 g^{-1} TS). These figures are notably higher than most outcomes reported in previous dark fermentation investigations (in Chapter 2) that primarily utilized different substrates. Therefore, while these results are not directly comparable due to the variance in substrates, this substantial yield does suggest that the LAR could be a promising substrate for biohydrogen production via dark fermentation.

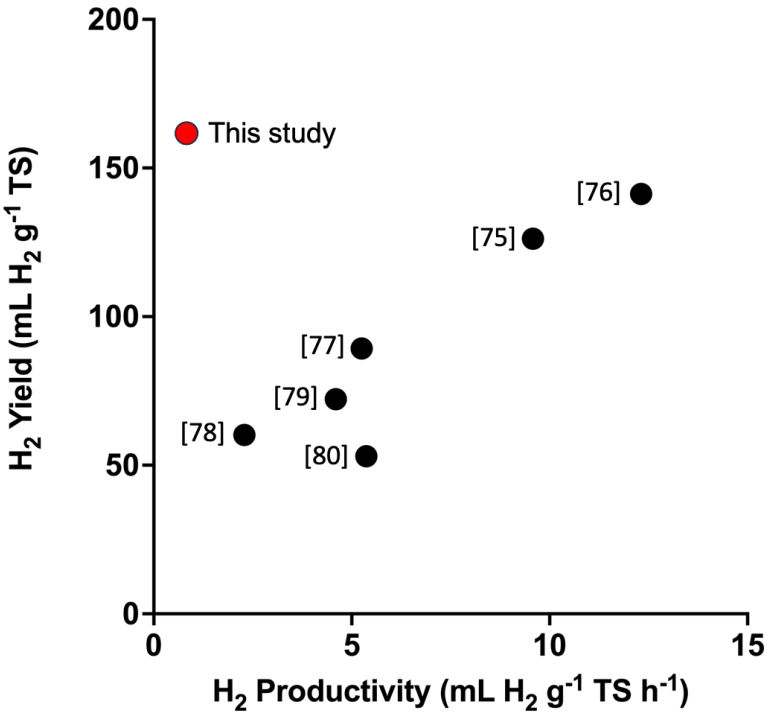

Figure. 4.5 HY and HP of previous studies on different types of lignocellulosic biomass; adopted from [14][75][76][77][78][79][80]

Although the LAR demonstrated high HY, its HP was somewhat modest. The recorded HP was 0.84 mL H_2 g^{-1} TS h^{-1} (or 1.46 mL H_2 g^{-1} VS h^{-1}), which is lower than most previous studies on other substrates (Figure 4.5). This low productivity rate could be linked to the nature of LAR and its associated inhibitors introduced during the alkaline pretreatment processes conducted before these experiments. It implies that a more comprehensive analysis of the substrate and improvement in treatment methods are needed for optimizing this fermentation process to achieve maximum production. Despite this shortcoming, the high HY establishes LAR as a potential substrate for biohydrogen production. Its other weaknesses could be addressed with better understanding and appropriate pretreatment, which can enhance the availability and digestibility of LAR's fermentable components. It is suggested that further effort should focus on refining

inoculum preparation methods, aimed at increasing the microorganisms' activity and resilience. Such enhancements would facilitate more efficient conversion of LAR into biohydrogen, thereby increasing the process's practicality and environmental sustainability. It emphasizes the necessity for continued research in this field, with a focus on optimizing the process and mitigating inhibitory effects, to fully tap into LAR's bioenergy potential.

4.3 Post-fermentative broth and COD balance

Table 4.5 Post-fermentative broth analysis

SIR	Initial pH	Adjusted pH	Final pH	TDS (mg/L)	Conductivity (mS/cm)	ALK ($mgCaCO_3/L$)	VFA (mgHAc/L)	VFA/ALK ratio	COD (mg/L)
2.7	8.12	7.53	7.21	9439	12.08	2935	159	0.05	26000 ± 2372
6.3	8.06	6.55	7.8	9693	12.48	2306	159	0.07	25575 ± 106
31.3	8.21	6.53	7.45	8989	11.1	1693	182	0.11	21500 ± 557
62.5	7.82	6.52	7.29	9508	11.07	1776	138	0.08	25500 ± 707
93.8	7.83	6.54	7.5	9140	10.9	1869	91	0.05	19875 ± 177

HAc - Acetic acid

During the fermentation process, significant pH changes are typically attributed to an increase in VFA concentration. In this study, however, as shown in Table 4.5, the estimated VFA production was quite low, which consequently led to low VFA/ALK values. As mentioned earlier, high substrate concentration commonly results in an increase in VFA. In this study, however, despite low gas production in high substrate concentration bottles, the estimated VFA production remained unexpectedly low. A possible factor is that the microorganisms may have absorbed or utilized the produced VFA, leading to a lower recorded concentration. This can also likely be explained by the high buffering capacity (high ALK) of the final liquors in this experiment, which was able to neutralize the acids produced, maintaining or even increasing the pH.

Table 4.6 COD balance

SIR	Influent			Effluent			Balance
	COD LAR (gCOD)	COD inoculum (gCOD)	Total (gCOD)	COD H_2 (gCOD)	Post-fermetative broth (gCOD)	Total (gCOD)	
2.7	7.42	N/A	7.42	0.8	9.36	10.16	1.37 ± 0.12^a
6.3	7.42	2.66	10.08	0.62	9.21	9.83	0.98 ± 0.01
31.3	7.42	0.53	7.95	0.47	7.74	8.21	1.03 ± 0.03
62.5	7.42	0.27	7.68	0.44	9.18	9.62	1.25 ± 0.03
93.8	7.42	0.18	7.59	0.5	7.16	7.65	1.01 ± 0.01

a - Batch 1's inoculum COD values were not measured due to lack of materials.

Table 4.6 presents the total COD of different inlet and outlet streams, derived from the initial inputs and the end products of the experiment. The COD mass balance in the table is fairly comprehensive, with most experiments nearing a 100 % balance. This successful mass balance offers a robust validation for the experimental methodology and the accuracy of the measurements obtained, thereby ensuring the reliability of the findings of this study.

However, an exception was observed in the case of the SIR 2.7 experiment, where the COD balance was reported as 1.37 ± 0.12. This deviation can be primarily attributed to the lack of COD measurements for the dried inoculum. The absence of this measurement might have led to an overestimation of the balance. If the COD of the dried inoculum was accounted for, it is likely that a balance close to 100 % would have been achieved.

4.4 Discussion on the theoretical energy output

Thru private communication with the industrial engineers, 30,000 T LAR could be produced per annually based on the current production activity, and that was originated from around 100,000 T per annually fresh harvested biomass.

According to gas production data, SIR 2.7 was taken as the optimal SIR for dark fermentation, while 31.3 has the least biohydrogen as increasing SIR beyond this point yielded no obvious advantage or even inhibition effects.

Table 4.7 Annual production of H_2 for 30,000 T LAR per year

SIR	Total H_2 production (metric tons)	Total energy content (TWh)	Contribution to total Norway's energy demand (%)
2.7	104,096	3.5	1.6
31.3	77,784	2.6	1.2

Table 4.7 demonstrates that, according to the results from this study, the dark fermentation of LAR could theoretically contribute between 2.6 to 3.5 TW per year, approximately accounting for 1.2 to 1.6 % of Norway's energy demand. It's important to note, however, that these estimates provide a broad overview and do not take into account the substantial energy required for the operation, transport, collection, and storage of H_2, which are

significant factors in a real-world scenario. Nonetheless, within the scope of this thesis, the results suggest that if properly utilized, LAR, or Lignocellulosic Biomass (LCB) in general, can contribute clean and renewable energy to help meet the ongoing increase in global energy demand. Furthermore, the environmental benefits of utilizing LCBs for clean energy production should not be overlooked. These include not only reductions in greenhouse gas emissions but also the potential for waste minimization and resource recovery. While there are still many challenges to overcome, the dark fermentation of LCBs, specifically LAR, presents a promising avenue for sustainable and renewable energy production, capable of contributing to global energy needs in a significant manner. Continued research and development efforts are essential to unlock the full potential of this resource.

4.5 CFSTR control and monitoring

In this study, an attempt was made to upscale hydrogen production using a CFSTR. Figure 4.6 illustrates some of the operational parameters throughout a month of operation.

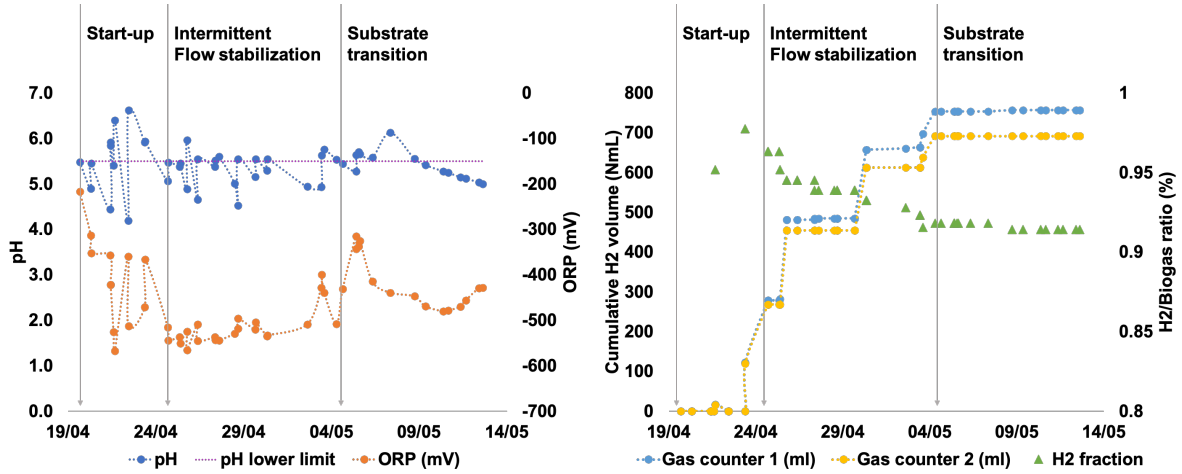

Figure. 4.6 CFSTR's monitoring recordings in three distinctive phases

During the start-up phase, the high biodegradability of glucose led to strong microbial activity, with the substrate rapidly being converted and causing multiple quickly drops in pH from 5.5 to 4.2. This necessitated pH adjustment to maintain favorable conditions for hydrogen production which reached 800 mL in total gas with very high H_2 content from 90 to 95 %. In the second phase, the reactor primarily operated in an intermittent

mode due to adjustments of the flow configuration. Despite this irregular operation, hydrogen-producing bacteria appeared to continue thriving, as indicated by the onset of biohydrogen production. However, it should be noted that the recording of gas production was probably delayed, likely due to the transfer from gas phase into the liquid phase, then to the liquid/gas separator, and finally to the gas counter. Theoretically, gas production should have initiated from day one. Throughout this second phase, the ORP remained at a notably low range of -500 - -550, indicating a highly reducing environment within the reactor. In the third phase, the substrate was altered from glucose to a mixture of glucose and LAR, with a similar theoretical COD. This transition appeared to slow down microbial activity, which could be due to the complex structure of LAR, despite it being heavily processed. As seen in two AMPTS batches, this structural complexity may have impeded the bacterial conversion process (long lag phases). It is also worth noting that the introduction of LAR increased the alkalinity and exerted a pH-increasing effect. The combined effect could explain the more gradual pH decline observed in this phase compared to previous ones. Regarding gas production, not much was recorded at the transition point. This may have resulted from the bacteria undergoing an adaptation phase to the new substrate mix. Nonetheless, continuous monitoring and analysis are necessary to optimize the conditions for maximizing hydrogen production in this setup.

4.6 Limitations and challenges

4.6.1 Limitations

1. AMPTS
    - Blanks: The study used raw experimental data without adjustment for background gas production, assuming the blanks produced negligible to zero gas. While this approach was deemed to have minimal impact on the overall data interpretation, it may have resulted in some discrepancies.
    - Possible error in pH adjustment: Due to the viscosity and alkalinity of LAR, pH adjustments may have been prone to errors. The thickness of the LAR could have caused unequal mixing during pH reading and interfered with the pH probe, leading to potentially inaccurate measurements of the true pH levels of the fermentation broth.
2. CFSTR

- Time constraints and unpredictable pump flow rates forced operation primarily in batch mode for most of the research period. This limitation may have influenced the effectiveness and efficiency of biohydrogen production.

3. Lab Equipment and Instrument
   - The inability to analyze produced gas composition due to an out-of-service gas chromatograph presented another limitation. Assuming the CO_2 adsorbers functioned as intended, recorded gas values were considered to be largely hydrogen with negligible amounts of other gases like H_2S.

4.6.2 Challenges

1. AMPTS
   - The non-production of gas from glucose was attributed to operational issues related to the CO_2 capture system. Due to the formation of a vacuum inside the control reactors C1, C2 and C3, a considerable volume of 3N NaOH solution from the absorption bottles was inadvertently siphoned into these control reactors. This incident was attributed to the submersion of the hoses (Figure 4.1), leading to the unanticipated inflow of the NaOH solution. Upon identification of this issue, immediate corrective measures were taken. The hoses were promptly removed. However, it is important to note that this sudden change in pH and composition likely disrupted the reactors' environment, skewing the experimental outcomes. This incident had me reevaluate and modify the experimental setup to prevent the recurrence of such events. Following the adjustment, the rest of batch 1 duration and the second batch of experiments proceeded smoothly without further incidents.
2. CFSTR
   - The assembly and operationalization of the CFSTR proved challenging, requiring significant time and effort. Understanding the functioning of the system, the connection of wires and connectors, and addressing potential issues were difficult tasks that contributed to the complexity of the experiment. Despite these challenges, the CFSTR was assembled and initial operations yielded promising preliminary data, indicating its potential for upscaled biohydrogen production.

Chapter 5

Conclusion and Outlook

5.1 Conclusion

This study set out to explore the potential of LAR as a source for biohydrogen production via dark fermentation. Multiple aspects were addressed, including the substrate and inoculum characterization, pre-treatment methods, BHP of LAR at different SIRs, kinetics modelling, and the estimated energy output.

Initial investigation revealed that the composition of LAR is characterized by a high FS content, a moderate VS/TS ratio, and relatively low COD and VS values. After undergoing mild acid hydrolysis and lipid extraction, the resulting HLAR and DLAR exhibited high carbohydrate and lipid contents. However, it was also noted that the presence of inhibitory compounds released during the pre-treatment processes could significantly hamper biohydrogen production. As a result, it became clear that although pre-treatment methods are critical for substrate preparation, they need to be carefully optimized to prevent the introduction of inhibitory effects.

Two batches of AMPTS examined the BHP of LAR at different SIRs yielded promising results. An optimal SIR of 2.7 yielded a significant HY of approximately 280 mL H_2 g^{-1} VS, thus confirming LAR as a viable feedstock for biohydrogen production. However, it was also observed that the HP of LAR was modest, averaging at only around 1.46 mL H_2 g^{-1} VS H_2 h^{-1}.

The estimated theoretical energy output showed the promise of this research, as the 30,000 tons of LAR, produced annually from about 100,000 tons of harvested biomass, could potentially contribute 2.6 to 3.5 TWh per year to Norway's energy demand. It's an innovative approach that effectively utilizes an otherwise waste material to generate renewable energy.

The Modified Gompertz modelling parameters fit optimally with the fermentation process, returning a regression of 95 % and higher, thereby reinforcing the validity of the model for predicting the kinetics of the biohydrogen production process as well as that of the experimental results.

In an attempt to upscale the biohydrogen production, a CFSTR was constructed and operated under optimal conditions determined from AMPTS experiments. Although the CFSTR operation and monitoring were challenging, preliminary data showed a promising start in terms of biohydrogen production.

5.2 Future work recommendation

Future work should aim to

- deepen the understanding of this unique biomass resource by analyzing specific carbohydrates content, protein, total N, total P, etc.
- refine hydrolysis and lipid extraction pre-treatment methods
- explore other pretreatment methods e.g. using fungi in solid state fermentation
- optimize operational conditions for upscaling in CFSTR

Continued investigation in these directions is critical for unlocking the full potential of LAR for biohydrogen production and contributing to a green energy future.

5.3 Outlook

The world's focus is increasingly shifting towards sustainable and renewable energy sources, and the abundant availability of lignocellulosic biomass presents a significant opportunity in this context. Such biomass has the potential to be utilized as substrates in biological hydrogen production processes, offering a renewable source for one of the cleanest forms of energy – hydrogen.

Dark fermentation for hydrogen production is a promising avenue that demands further exploration. This technology holds the potential to revolutionize green energy production within the next decade, significantly contributing to a cleaner and more sustainable energy sector. More specifically, it allows for better utilization of lignocellulosic biomass,

especially waste streams from various industries, offering an innovative solution for both waste management and renewable energy production.

The exploratory examination undertaken in this thesis, focused on using residual biomass from industrial sources, has underscored the immense potential that waste streams hold when paired with optimized dark fermentation processes for hydrogen production. From this standpoint, it's evident that these waste resources can be effectively harnessed for beneficial use, rather than ending up as environmental pollutants.

However, realizing the maximum theoretical yield of hydrogen via dark fermentation requires further research and understanding. The next stage involves meticulously investigating and fine-tuning multiple operational parameters to optimize hydrogen yield. Factors such as substrate-to-inoculum ratios, pre-treatment methods, reactor configurations, and fermentation conditions, etc. all needs further investigation. The work presented in this thesis lays a solid foundation for future studies, promising to drive us further along the path of clean, renewable, and sustainable energy generation.

This is the outlook that drives this study forward and will hopefully inspire many more in the quest for sustainable solutions to our energy needs.

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Appendix A

Glucose's standard curve

Table A.1 Phenol-Sulfuric Acid Method - Glucose's standard curve preparation

Agents and results	Vial number					
	Blank	1	2	3	4	5
Glucose stock (1g/L) (μL)	0	20	40	60	80	100
Deionized water (μL)	1000	980	960	940	920	900
Phenol 5 % (mL)	1	1	1	1	1	1
Concentrated H_2SO_4 (mL)	5	5	5	5	5	5
Glucose concentration (mg/L)	0	20	40	60	80	100
Triplicate 1 (ABS@490nm)	0	0.180	0.377	0.550	0.655	0.820
Triplicate 2 (ABS@490nm)	0	0.159	0.343	0.553	0.761	0.974
Triplicate 3 (ABS@490nm)	0	0.167	0.335	0.470	0.607	0.816
Average	0	0.169	0.352	0.524	0.674	0.870

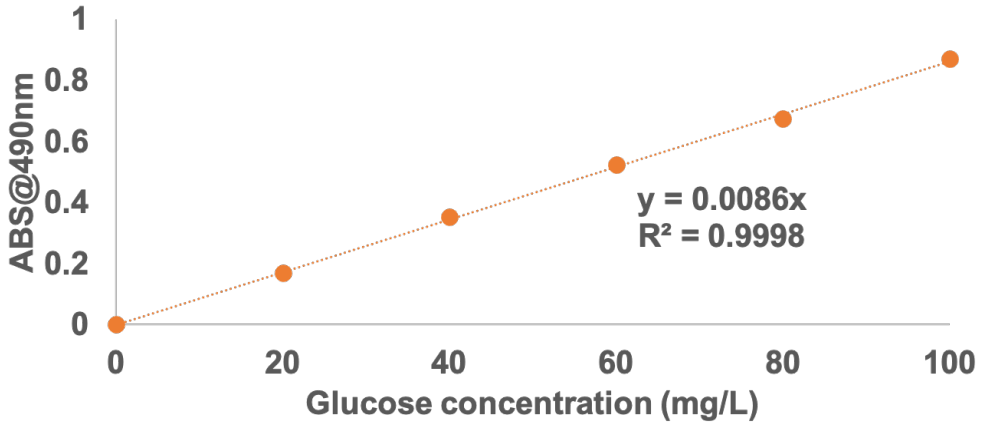

Figure. A.1 Glucose's standard curve

Table A.2 Carbohydrates content of LAR, HLAR, DLAR

Sample	Sample weight (mg)	TS (%)	Sample concentration (g/L)	Recorded ABS	Dilution factor	Carbohydrates concentration (mg/L)	Carbohydrates concentration in sample (mg/g)	Carbohydrates content (% dry matter)
LAR 1	25.86	0.24	0.428	0.409	2	94.8	221.5	22.1
LAR 2	25.38	0.24	0.420	0.408	2	94.6	225.1	22.5
LAR 3	25.64	0.24	0.424	0.471	2	109.2	257.3	25.7
HLAR 1	52.23	1	3.602	0.346	40	1604.1	445.3	44.5
HLAR 2	52.86	1	3.646	0.359	40	1664.3	456.5	45.7
HLAR 3	50.89	1	3.510	0.324	40	1502.1	428.0	42.8
DLAR 1	50.04	1	3.451	0.452	40	2095.5	607.2	60.7
DLAR 2	52.26	1	3.604	0.48	40	2225.3	617.4	61.7
DLAR 3	51.64	1	3.561	0.472	40	2188.2	614.4	61.4

Appendix B

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