

Doctoral Dissertation

Enhancement of hydrogen production using the blend of two algal biomass with casamino acids as nitrogen source by *Clostridium butyricum* RAK25832.

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博士論文

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Enhancement of hydrogen production using the blend of two algal biomass with casamino acids as nitrogen source by *Clostridium butyricum* RAK25832.

2種類の藻類バイオマスと窒素源としてのカザミノ酸を原料とした場合の *Clostridium butyricum* RAK25832 による水素生産の向上

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ABSTRACT

Global energy requirements are heavily depend on fossil fuels such as oil, coal and natural gas. As the depletion of limited fossil fuels is inevitable, there is an urgency to search for replacement source of energy. The extensive use of fossil fuel has also created an environmental issue where emission of carbon dioxide during combustion of fossil fuels has caused a global warming effect. For these reasons, researches are looking at alternative fuels that combat both the mentioned problems. Hydrogen is one of the most abundant elements in the universe in its ionic form. It is an odorless, colorless, tasteless and non-poisonous gas. When hydrogen is used as a fuel, it generates no pollutants but produces water. In comparison with fossil fuel, hydrogen has a higher energy yield However, due to the high renewable hydrogen production and storage costs, the potential of hydrogen as alternative fuel of future has yet to be realized.

This research focus on biohydrogen production through dark fermentation. The *Clostridium butyricum* RAK25832 was isolate from Wajiro beach at the low tide conditions in Japan. It was used as fermentative inoculum at pH 8 and temperature 30 °C. it was so important to reach optimal condition and achieve a defined medium for this microorganism. Several carbon sources were tested at a fixed concentration of 10 g substrate/L, with a casamino acids concentration of 1.67 g/L under a constant temperature 30 C. It was found these bacteria could adapt with various type of carbon source but they prefer glucose, fructose, xylose, sucrose, and lactose sugars. *Clostridium butyricum* strain RAK25832 shows slight or no growth in media containing inorganic nitrogen as the sole nitrogen source but grows well in the presence of an organic nitrogen source such as yeast extract. However, the use of yeast extract as the nutrient source for bacterial culture has the main disadvantage of unpredictability, as it may contain several

types of nutrients, vitamins, and amino acids such as glutamic acid, making it very difficult to define the media.

The ability of *Clostridium butyricum* RAK25832 to use casamino acids as a nitrogen source was investigated. Casamino acids mainly contain amino acids, strain RAK25832 showed the preferred final concentration of casamino acids was 26.67 g/L, with a cumulative hydrogen production, production rate, and yield of 2505 mL H₂/L, 160 mL/h, and 1.81 mol H₂/mol glucose, respectively.

Vitamins and nutrient supplementation for hydrogen production including macro and micro nutrients are very important. As, metals and minerals have effects on cell growth as enzyme cofactor, transport processes and dehydrogenases. Therefore, reaching the important elements could minimize the inputs and therefore decrease the supplement cost. Eighteen metal elements were screened to identify the most important metals for biohydrogen production, and four elements were optimized. The optimal medium composition was MgCl₂·6H₂O (0.1 g/L), K₂HPO₄·3H₂O (6.67 g/L), NaHCO₃ (2.6 g/L), and FeCl₂·4H₂O (0.002 g/L). Vitamin supplementation of the medium showed no significant effect on hydrogen production. Under the optimized conditions, cumulative hydrogen production reached 3074 mL H₂/L.

Renewable biomass is the most versatile non-petroleum based resource that is generated from various industries as waste materials. Lignocellulosic materials such as wood and wood products, food and starch-based materials, organic industry wastewater, household wastewater and biodiesel industry waste could be potential resources for biohydrogen production and as a cost effective energy production process.

Producing hydrogen with high yields and production rates are very important for promoting the commercial biohydrogen production process in a sustainable manner. Algal waste biomass include *Chlorella fusca* and *Ulva lactuca* were used as substrates by the *Clostridium butyricum* RAK25832 for biohydrogen production. Finding the blend ratio that achieve high yield was the goal for this work. Twelve blend ratio were investigated and the ratio 4:1 were the preferred gave a yield 86 ml H₂/VS and rate 37 ml/hr. The supplementation elements with FeCl₂, NaHCO₃, MgCl₂, and K₂HPO₄ were played a vital role to improve the yield to 107 ml H₂/VS. Casamino acids supplementation were studied with every single substrate. The substrates *C. fusca* required a supplementary of 13.33 g/L casamino acids and *U. lactuca* required 6.67 g/L. Therefore, mixing two types of waste save the requirement of casamino acids as it required only 3.33 g/L. Accordingly in general, blended feedstocks from different sources will reduce additional supplements of nutrients, resulting in cost reduction and effective energy production.

学位論文要旨

昨今、世界のエネルギー事情が、特に化石燃料（石油、石炭、天然ガス等）に関して切迫してきている。一方で過剰な化石燃料の消費は、地球温暖化に代表されるように大きな地球規模の環境問題を引き起こしている。したがって、再生可能な（言い換えるならばカーボンニュートラルな）代替エネルギーの開発が急務である。

水素は、無色無臭かつ人体に無害で、エネルギーとして使用する際には水しか排出しないこと、またその高いエネルギー密度（122 kJ/g）から理想的なエネルギー源として注目されている。しかしながらまだその生産コストや保管のためのコストが高額であるため、未だ実用段階とは言えない。

そこで、本研究は、この水素生産を嫌気性発酵により行うためのプロセスを開発することに焦点を当てた。水素生産菌として、福岡市の和白干潟からスクリーニングした *Clostridium butyricum* RAK25832（中温菌で至適生育温度が 30°C、pH が 8）を用いた。まず、この *Clostridium butyricum* RAK25832 の最適水素生産条件を検討した。

Clostridium butyricum RAK25832 は様々な糖を炭素源として用いることができるが、特に glucose, fructose, xylose, sucrose, and lactose を好むことが明らかとなった。また、窒素源として無機態のものを与えた場合ほとんど増殖せず、酵母エキスのような有機態の窒素を与えた場合に良好に増殖した。しかしながら、酵母エキスを窒素源として用いると、酵母エキスが様々な栄養塩類やミネラル類、ビタミン類を豊富に含むことから、*Clostridium butyricum* RAK25832 の増殖に何が大きく影響を及ぼすのかを実験的に把握することが困難となる。

そこで、窒素源としてカザミノ酸を用いることができるかを実験的に検討した。カザミノ酸はカゼインを加水分解して得られるもので、主な構成成分はアミノ酸である。*Clostridium butyricum* RAK25832 はカザミノ酸を窒素源として順調に生育できることが明らかとなり、良好に生育するカザミノ酸の濃度は 26.67 g/L であることがわかった。このときの水素生成量、水素生産速度、水素収率はそれぞれ 2505 mL H₂/L, 160 mL/h, 1.81 mol H₂/mol glucose であった。

一方で、ビタミン類や栄養塩類も水素生産に大きな影響を及ぼす。そこで、どの成分が水素生産に大きな影響を及ぼすのかを実験的に検討した。18種の栄養塩類から最終的には4つの栄養塩類が水素生産に大きな影響を及ぼすことがわかった。これら4種の栄養塩について個別に最適化を行い、 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1 g/L), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (6.67 g/L), NaHCO_3 (2.6 g/L), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.002 g/L)が得られた。一方で、ビタミン類は水素生産にほとんど影響を及ぼさなかった。上記の最適栄養塩類濃度の設定下で水素生産量 3074 mL H_2 /L が得られた。

再生可能バイオマス（木材や食品廃棄物や有機性汚泥等の有機性廃棄物、食品工場から排出される有機性残渣等）は代替エネルギーの原料として重要な存在である。これらは水素生産の材料としてそのコストの安さから有望視されている。

嫌気性発酵による水素生産プロセスを実用化するに当たっては、水素収率が高く、その生産速度も高いことと同時にコストを抑えることが要求される。低コストという面で有機性廃棄物は魅力的である。有機性廃棄物の中でも海藻類や微細藻類はアミノ酸を豊富に含むため、窒素源も含有した炭素源（窒素源の節約が可能）として有用と考えられる。そこで本研究では藻類（海藻類、微細藻類）を原料として用いた場合の水素生産において、海藻と微細藻類の混合比率を変化させ、できるだけ窒素源としてのカザミノ酸、ビタミン類や微量元素（ミネラル）類などの使用量を減じることができる条件を実験的に調査した。海藻類、微細藻類として *Chlorella fusca* と *Ulva lactuca* を選択した。*Chlorella fusca* と *Ulva lactuca* の混合比率を12通りに変化させ、水素生産量を最大にできる比率を調査した。実験結果から最適な比率は *Chlorella fusca* が4に対して *Ulva lactuca* が1であった。その際の水素収率と水素生産速度はそれぞれ 86 ml H_2 /VS と 37 ml/hr であった。先に述べた4種の栄養塩 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1 g/L), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (6.67 g/L), NaHCO_3 (2.6 g/L), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.002 g/L)を添加した場合には、水素収率は 107 ml H_2 /VS まで向上した。上述の *Chlorella fusca* が4に対して *Ulva lactuca* が1の比率に必要なカザミノ酸の量は 3.33 g/L であり、それぞれ単体で必要とされるカザミノ酸の量（*C. fusca* で 13.33 g/L、*U. lactuca* で 6.67 g/L）を大きく下回り、2つの藻類を混合する利点（コスト減を含めて）が明らかとなった。

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LIST OF ABBREVIATION

HPB	Hydrogen-producing bacteria
PCR	Polymerase chain reaction
SMP	Soluble metabolite product
VFA	Volatile fatty acids
FID	Flame ionization detector
rRNA	Ribosomal ribonucleic acid
NCBI	National Center for Biotechnology Information
HAc	Acetic acid
HPr	Propionic acid
i-HBu	Isobutyric acid
n-HBu	N-butyric acid
EtOH	Ethanol
OD ₆₂₀	Optical density at 620 nm
EDTA	Ethylenediaminetetraacetate
V _{H,i}	Cumulative hydrogen gas volume at the current (i) time
V _{H,i-1}	Cumulative hydrogen gas volume at the previous (i – 1) time
V _{G,i}	Total biogas volume at the current time interval
V _{G,i-1}	Total biogas volume at the previous time interval
C _{H,i}	Hydrogen gas fraction in the headspace at the current time interval
C _{H,i-1}	Hydrogen gas fraction in the headspace at the previous time interval
H	Cumulative hydrogen production (mL)
λ	Lag time (hr)
P	Hydrogen production potential (mL)

R_m	Maximum hydrogen production rate (mL/hr)
e	Constant, 2.718281828
Fd	Ferredoxin
CAA	Casamino Acids

CHAPTER I: INTRODUCTION

1.1 Background

The variety consumption of fossil fuels like coal has resulted in serious environmental pollution and energy crisis (John et al., 2011). Hydrogen is considered as a promising energy source because of its clean combustion as the end product is water and high energy content around 122 kJ/g (Argun & Kargi, 2011). Biohydrogen production from biomass got a potential industrial applications because of its environment-friendly, energy-saving, and carbon-neutral characteristics which relatively lacking in many conventional hydrogen-producing processes, such as steam reforming and water electrolysis (Cheng et al., 2012a).

Algae biomass is a potential source for biohydrogen production for its fast growth and global distribution (Cheng et al., 2012b). Algae are primitive photosynthetic microorganisms and a primary producers in any ecosystem. Photoautotrophic, meaning that they could utilize sunlight to fix CO₂ and convert them into organic compounds and biomass. Some are heterotrophic, requiring organic carbon sources and can be cultivated without of light (Chen et al., 2011). Bacteria that utilize carbon sources via an anaerobic pathway are known to produce hydrogen as a by-product and this has been known for decades for biohydrogen production. Hydrogen bacteria utilize protons as terminal electron acceptors releasing hydrogen, as the electrical neutrality of the cells needs to be maintained for ATP generation (Lee et al., 2011). Biohydrogen production could be a promising way of waste valorization and many industrial waste can be used as a substrate for dark fermentation. *Clostridium butyricum* is well known as an anaerobic bacteria as well as hydrogen producer (Zigová et al., 1999). Vitamins and metals seems to be essential factors for biohydrogen bacteria. Although, a higher concentration of metals may inhibit the activity of hydrogen producer

bacteria, a trace level of metal is required for hydrogen production. As an example, Fe^{2+} is the most investigated metal for biohydrogen production, which may be essential for hydrogenase activity (Zhao et al., 2012). Thus, more investigations are needed to determine the effect of the concentration of other metals on fermentative hydrogen production.

1.2 Dissertation objectives

The aim of this study to develop effective dark fermentation hydrogen process with low cost by mesophilic bacteria *Clostridium butyricum* RAK25832, isolated from Wajiro beach Fukouka using algal waste substrate. In order to accomplish the goal of the study, the study purpose divided to direction:

- The first is to reach a defined medium required for the microorganism *Clostridium butyricum* RAK25832 as an optimal condition. Therefore, finding suitable carbon sources, casamino acids as nitrogen source, the use of vitamins and metal important for hydrogen production.
- The second is the application by using algal substrate and fined effective production at low cost by reach to the blend ratio to reduce the required supplementation of casamino acids and minerals.

1.3 Structure of dissertation

The dissertation included six chapters and listed as follow:

- **Chapter I Introduction** states the problem, aims of the research, and structure of the dissertation.
- **Chapter II Literature review** the chapter reviews the literature of the previous studies and introduces the background knowledge of biohydrogen.

- **Chapter III** Identification of factors that accelerate hydrogen production by *Clostridium butyricum* RAK25832 using casamino acids as a nitrogen source. This chapter demonstrate the important carbon source utilized by the microorganism, use the casamino acids as nitrogen source, and reach to the important mineral element and vitamins for hydrogen production.
- **Chapter IV** Enhancement of hydrogen production by combining a blend ratio for two types of algal waste biomass and reducing casamino acids supplementation by *Clostridium butyricum* RAK25832. In this chapter according to chapter III, the importunate element of hydrogen production optimized with algal waste substrate to reduce the use of casamino acids as it is the most expensive elements required for hydrogen production.
- **Chapter V Conclusion** summarize the finding and overall results of this study.

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CHAPTER II: LITERATURE REVIEW

2.1 Introduction

After the disaster of Fukushima in March 2012. Around 19,000 people were killed and towns were destroyed. This accident showed the risk of using nuclear energy. Before this disaster, 30% of Japanese electricity produce by nuclear power. This disaster did not only left the Japanese government thinking of renewable energy as alternatives, but it also warning to other nations to find renewable greener alternatives and safer to replace the fossil fuels. Biohydrogen seems to be one of the more promising alternatives to the fossil fuels. Icreasment of the human population and climate change let the renewable energy technologies get the challenge as an alternate for fossil fuel (Cohen, 2001). The renewable energy sources such as biomass, hydropower, wind, solar, geothermal, marine, and hydrogen will play an important roles for the energy crisis. The advantages of renewables are the availability, end use has no greenhouse gas emission, the cost is economical, generate new employment opportunities.

Hydrogen energy considered as a clean and efficient renewable sources to replace fossil fuels. Hydrogen produces only water as a by-product which is friendly to the environment and it has the highest energy per unit mass (Table 2.2). During 2009 the annual worldwide hydrogen consumption is about of 400–500 billion Nm³ (Demirbas, 2009). In addition for the environment friendly advantage for the biohydrogen production processes – it is inexhaustible (Benemann, 1997; Miyake et al., 1990). Biohydrogen production has several advantages over fossil fuels as it is produced naturally by microorganisms, no greenhouse emission, and the sustainability as it produced from a variety of substrates include waste. Moreover, compared to other fuel, biohydrogen conducts one of the highest energy per unit mass (Table 2.1). Biohydrogen

microorganisms include direct and indirect biophotolysis, photofermentative bacteria, and dark fermentation by fermentative bacteria.

Table 2. 1 Comparison of different fuel types with energy content and carbon dioxide emission

Fuel Type	Carbon (%)	Calorific value (J/kg)	Carbon Emission (kg C/kg Fuel)
Hydrogen	0	141.90	0.00
Ethanol	52	29.90	0.50
Biodiesel	77	37.00	0.50
Methanol	37.5	22.30	0.50
Natural gas	75	50.00	0.46
Gasoline	74	47.40	0.86

2. 2 Dark fermentative bacteria

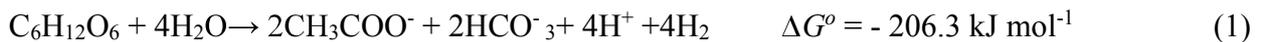
Dark fermentative bacteria could use to different substrates and temperatures. These bacteria metabolized energy by partial oxidation of organic materials using the organic intermediates as electron donors and electron acceptors instead of oxygen using the NADH to produce metabolic by-products, organic acids, and alcohols, including hydrogen. Family Clostridia are mesophilic use the complex carbohydrates to produce hydrogen (Penfold et al., 2003; Hawkes et al., 2007 and; Cheng et al., 2008).

Clostridia is Gram-positive found in sewage, soil, and animals' intestines. Species *C. butyricum* and *C. pasteurianum* have the ability to fix the nitrogen (Chen et al., 2001). Clostridia are used for the production of alcohols and it has high hydrogen production yield through a

reversible hydrogenase enzyme (Calusinska et al., 2010). Clostridia could produce hydrogen via large number of carbohydrates, like xylose, arabinose, glucose, cellobiose, fructose, sucrose and galactose as well as the waste (Suzuki & Karube, 1981; Taguchi et al., 1993). *Clostridium* sp. produced efficiently hydrogen from xylose and arabinose than from glucose. However, other isolate produced xylanase and converted xylan to hydrogen (Taguchi et al., 1994). The clostridia end products sugars fermentation was butyric acid and acetic acid, carbon dioxide, and hydrogen, along with small portion of ethanol (Ren et al., 2008).

2. 3 Mechanism of dark fermentation hydrogen production

Dark fermentation biohydrogen is produced by anaerobic bacteria under anaerobic conditions (Nandi & Sengupta, 1998). Different organic substances could use as substrates, like sugars, carbohydrates, lipids and proteins. The theoretical yields estimation of hydrogen production from glucose towards acetate is used as reference. Thauer et al., 1977 mentioned that the maximum theoretical biohydrogen yield from glucose fermentation is 4 mol H₂/mol glucose as in eq. (1):



Also can be achieved through two steps by fermentation of glucose to acetate and formate as following (Voordouw, 2002) eq. (2a and 2b)



the maximum theoretical yield in the case of acetate- butyrate byproduct is 2mol H₂ /mol of glucose eq. (3) (Thauer et al., 1977):



Glucose metabolite to pyruvate eq. (4);



Depending on the enzymatic reactions the acetylCoA could generate either by eq. (5 or 6).



eq. (5) is reaction catalyzed by the pyruvate-Fd_{ox} oxidoreductase enzyme, where Fd_{ox} acts as electron receiver (Uyeda & Rabinowitz, 1971; Buchanan, 1972).

When, acetate is the final byproduct, during glycolysis one mole of H₂ will generate from the reduction of one mole of NADH, to NAD⁺, leading to a yield 4 mol H₂/mol glucose. When, butyrate is the final byproduct, the total final hydrogen yield is 2 mol H₂/mol glucose. Depending on the type of microorganism produce of acetate and butyrate as byproducts a hydrogen yield of 2 and 4 could achieve like in clostridia such as *C. butyricum* (Daesch & Mortenson, 1968) and *Clostridium pasteurianum* (Jungermann et al., 1973).

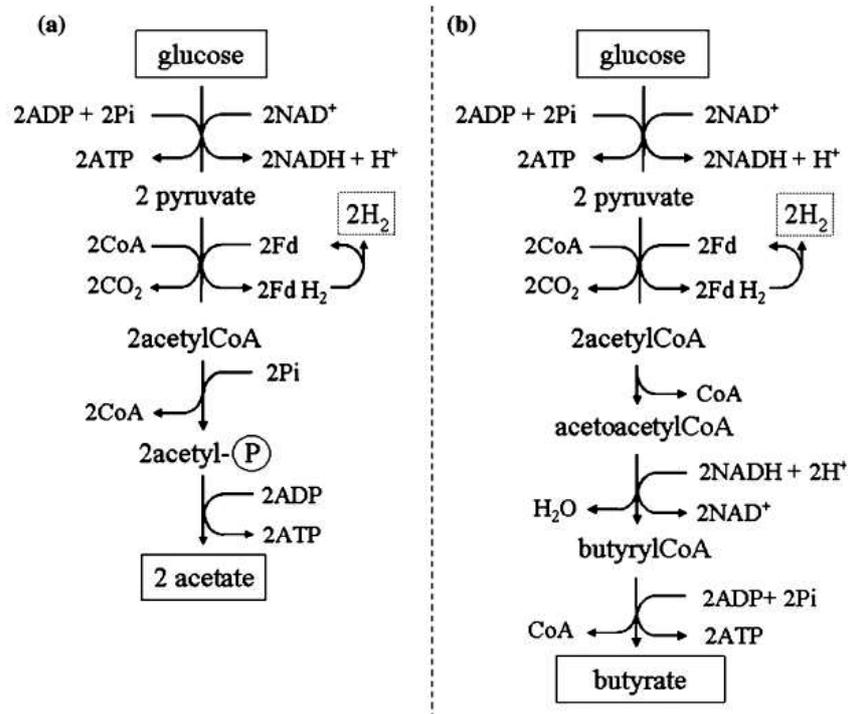


Fig. 2. 1 Glycolysis hydrogen production through acetate (a) and butyrate (b) pathway

2.4 Dark fermentation challenges and advantages

There are many challenges facing the dark fermentation for example the low yields and the final product contains a mixture of CO₂ and H₂, which leads to additional separation cost. However, dark fermentation has advantages by comparing to the other biological processes, it has the highest yields. It could utilize a high variety of carbon source and can use waste as substrate and not require light.

2.5 Biohydrogen substrates

A wide range of substrate could utilize for hydrogen production (Fig. 2.2). Dark fermentation hydrogen production investigation was almost produced by 80% from pure sugars: mono, di, or polysaccharides. However, for a cost effective production, renewable substrates should use (Show

et al., 2012). Biomass including plants, seaweed, and algae in addition to, the agricultural waste, food waste, livestock and other industrial waste could be sources for biohydrogen substrates. In a theoretical way, it could illustrate that any organic substrate have high concentration of fats, carbohydrates and proteins could use as substrate for biohydrogen production (Kapdan & Kargi, 2006).

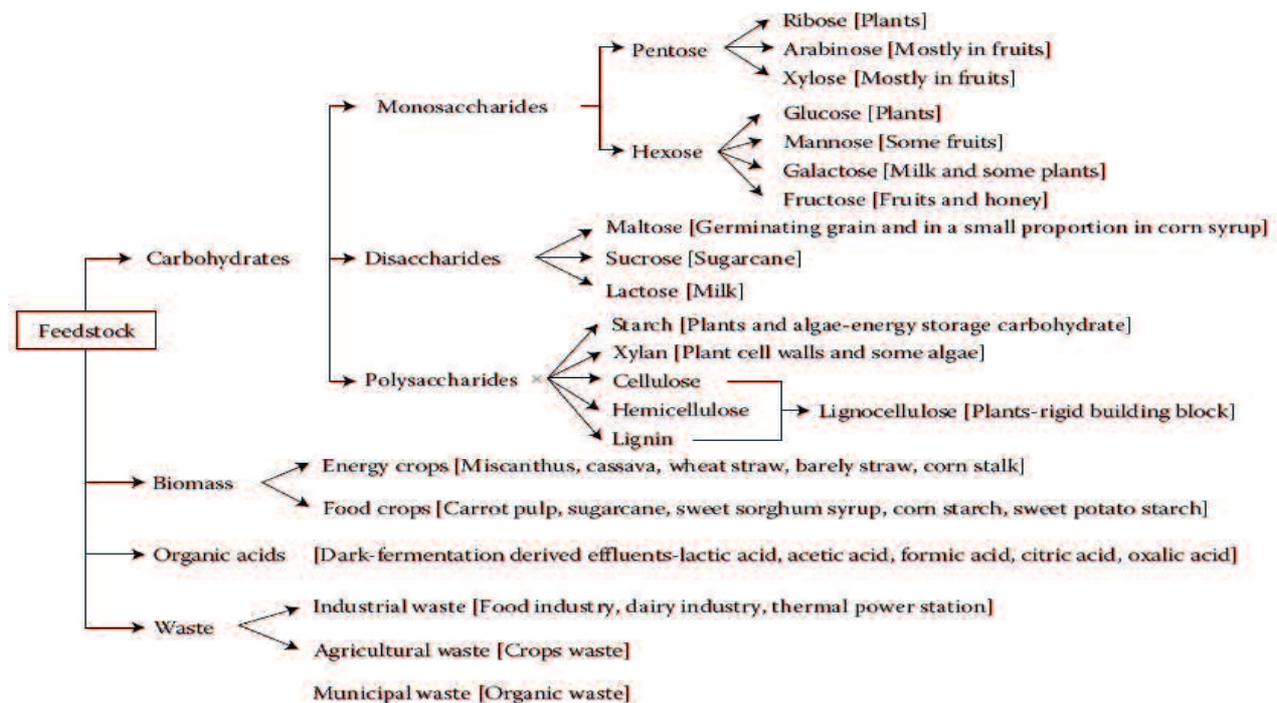


Fig. 2. 2 Substrate sources used for biohydrogen production

2.6 Algae source for hydrogen production

Algae is a primitive organisms for understanding the photosynthesis basics in higher plants. Recently algae become a potential source of biofuels as it is rich in carbohydrates and lipids (Show et al., 2017). Algae have very short cycles life, and compared to terrestrial plants it has high biomass productivity (Brennan & Owende, 2010). Microalgae preferred for biofuels as they do not

required for pretreatments as they do not contain lignin (John et al., 2011). Microalgae contain lipids, carbohydrates and proteins. In addition to, the residual biomass after extraction can be used for anaerobic fermentation (Chen et al., 2015). Bio-refinery is the best choice to have cost effective biofuels production. The cultivation conditions and nutritional stress lead to a carbohydrate accumulation or lipid accumulation (Ho et al., 2013).

Seaweeds, include brown, red, and green algae. Macroalgae are the main resource for agar, alginate, and carrageenan (Jensen, 1993; Tseng, 2001). The advantages of using seaweeds as biomass source were: they not required land, and yields are higher than crop land (Klass, 1974). Brown algae cell wall contain alginates, cellulose and protein (Kloareg et al., 1986). Hydrogen production potential were investigated for various marine macro-algae, including *Gelidium amansii*, *Codium fragile*, *Gracilaria verrucosa*, *Porphyra tenera*, *Undaria pinnatifida*, *Laminaria japonica*, *Ecklonia stolonifera* *Hizikia fusiforme* results indicated that *L. japonica*, the best substrate (Jung et al., 2011).

2.7 Dark fermentation process important factors

Hydrogen production pathway is affected by various parameters in the bioreactor. The major parameters include: Inoculum, pH, Temperature, hydraulic retention time, alkalinity, H₂ and CO₂ partial pressure and effect of micronutrients.

2.8 Effect of nutrients on the hydrogen production

During the biohydrogen fermentation the bacterial metabolism required essential nutrients like zinc, magnesium, sodium and iron due to bacteria required these elements for enzyme's cofactor, dehydrogenases, and transport processes. Iron seems to be important element for hydrogen production. As, it is catalyzed by hydrogenase enzyme, which contains either a [Fe-Fe]

hydrogenase or a [Ni-Fe] hydrogenase. Ferredoxin is work as a donor to let the enzyme receive the electrons make the pyruvate oxidation. Media supplementation with iron enhances hydrogen production.(Vaňáčová et al., 2001). Hydrogen production increased by equal molar ratio of nickel and iron salts supplementation (Kim et al., 2010; Ferchichi et al., 2005; Wang & Wan, 2008). Hydrogen production increased as the metabolic pathway changed from lactic acid to butyric acid fermentation after FeSO₄ supplementation (Lee et al., 2008). The strain *E. cloacae* IIT-BT metabolic pathway changed to increased acetic acid (Khanna & Das, 2013). Hydrogen production decreased by increasing Nikle concentration (Fan et al., 2008).

Magnesium is also, essential elements for microorganisms. It is required for membranes, cell walls constituent, ribosomes, work as enzymes cofactor and work as an activator for synthetases. Most of the glycolytic enzymes mostly, require magnesium as cofactor such as phosphofruktokinases, as hexokinase and enolases (Wang et al., 2007). The supplementation of Mg, Fe, Na, and Zn have a significant effect for hydrogen production and magnesium was the most significant. It produce 3.52 mol H₂/mol consumed sucrose at 120 kg/m³ MgCl₂ concentration (Lin & Lay, 2005).

Industrial wastewaters may contain a significant concentrations of heavy metals and cause difficulties for wastewater treatment process (Lester et al., 1983; Stronach et al., 1986; Fang & Chan, 1997) . The toxicity of heavy meatless in wastewater for H₂ production were carried on up flow reactor using sucrose, it was found Cu was the most toxic and Pb was the least toxic (Li & Fang, 2007).

2.9 Effect of carbon and nitrogen on biohydrogen

The C/N ratio is important to find the appropriate composition of the substrate for efficient production. Anaerobic microorganisms need a 20–30:1 ratio of C to N. Mixing waste contain a low carbon with high nitrogen will balance the C/N ratio required for optimal hydrogen production. Inorganic nitrogen compounds such as nitrate, nitrite and ammonia inhibit hydrogen production rate and activity (Lambert et al., 1979; Madamwar et al., 2000).

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CHAPTER III:

Identification of factors that accelerate hydrogen production by *Clostridium butyricum* RAK25832 using casamino acids as a nitrogen source

3.1 Introduction

Since fossil fuels are the main source of greenhouse gases, there is increasing global interest for identifying feasible clean energy alternatives. One such alternative is hydrogen, as the final combustion results in the formation of water with almost no other emissions. Hydrogen also has a high calorific value of 242 kJ/mol. The conventional processes for hydrogen production such as gasification, water electrolysis, water gas shift reaction, and steam methane reforming are efficient, and contribute to most of the produced hydrogen worldwide (Obradović et al., 2013; Obradović et al., 2013). Nevertheless, these processes require a large energy input from fossil fuels. Therefore, there has been recent attention paid to the possibility of biological hydrogen (biohydrogen) production from renewable resources such as waste biomass, as a promising process that combines energy recovery and waste minimization (Wang & Wan, 2009). Biohydrogen production has already been investigated with various species of microorganisms (Benemann, 1996; Kumar & Das, 2000) Fermentative hydrogen production has been shown to be a promising approach because it has the advantages of independence on the availability of light, higher hydrogen production rates, and a wide range of possible carbon sources such as low-cost wastes, organic compounds, and cellulosic substrates (Lalaurette et al., 2009; Vardar-Schara et al., 2008). The isolation and identification of hydrogen-producing bacteria (HPB) with high yields and production rates are very important for promoting the commercial biohydrogen production process in a sustainable manner. Fermentative hydrogen production can be carried out through a wide range of microorganisms

(Wang & Wan, 2009), including species of *Clostridium* (Taguchi et al., 1995), *Enterobacter* (Fabiano & Perego, 2002), and *Bacillus* (Kotay & Das, 2007).

Sewage sludge, food residue, manure, agricultural waste, and algae blooms are examples of waste biomass substrates that can be utilized in fermentative hydrogen production (Lee et al., 2010; Yang et al., 2011). The principal organic components used in fermentation are carbohydrates and proteins. Carbohydrates, including starch and cellulose, are better organic substances that are utilized for fermentative biohydrogen production than proteins (Dong et al., 2009). Carbohydrates can be readily hydrolyzed for reducing sugars such as xylose and glucose, which are also easily utilized by HPB for fermentative hydrogen production (Su et al., 2009).

Although several microbial strains have been shown as feasible candidates for biohydrogen production, this always requires the use of a complex medium. Various nitrogen sources have been investigated in biotechnological studies to optimize the growth and metabolite production rates of microorganisms. Proteins are among the main components in waste biomass. For example, the protein content was reported to reach up to 72% of the dry weight in *Spirulina* biomass (Dismukes et al., 2008). The most widely studied organic sources for this purpose are peptone, yeast extract, and casamino acids (Wang et al., 1971). Yeast extract was found to be the favored nitrogen source for the growth of *Clostridium butyricum* W5 using glucose as the substrate (Wang et al., 2008). The amino acids derived from proteins cannot easily be used by HPB to directly produce hydrogen (Lay et al., 2003; Xia et al., 2013). The generally low yield of biohydrogen production has led researchers to focus on seeking high-yielding hydrogen-producing microorganisms, target genes for genetically modifying existing microorganisms, or fermentation process optimization (Gavala et al., 2006; Minnan et al., 2005). Members of the class Clostridia have been confirmed as the main HPB in many hydrogen-production processes (Chang et al., 2006; Jo et al., 2007). Urea and KNO_3

appeared to not be favored nitrogen sources by *Clostridia* spp. (Wang et al., 2008). However, there is little information available on the role of nitrogen source in the hydrogen production rate and formation of associated by-products in hydrogen fermentation. Therefore, detailed investigations for identifying suitable and new nitrogen sources, and to evaluate the optimal nitrogen concentrations in the fermentation broth for hydrogen fermentation by certain bacteria are clearly necessary.

Clostridium butyricum is well known as an anaerobic bacterium as well as hydrogen produce (Zigová et al., 1999) and butyric acid producer (Zhang et al., 2009). Accordingly, several strains of *C. butyricum* have long been used for microbial industrial applications, especially for butyric acid production (He et al., 2005b; Wang & Jin, 2009; Zigová et al., 1999). Glucose is metabolized to pyruvate via the Embden–Meyerhof–Parnas pathway and produces two moles of ATP and NADH, respectively. The butyrate-producing *Clostridia* produce butyrate concomitantly with acetate, H₂, CO₂, and trace lactate and other products (Zhang et al., 2009) (Fig. 3.1). A theoretical investigation showed that the maximum yield of 4 mol H₂/mol glucose can be produced when acetic acid is the only volatile fatty acid (VFA), and a maximum yield of 2 mol H₂/mol glucose can be produced when butyric acid is the VFA product, using Eqs. (1) and (2) below (Das et al., 2008) . However, a lower yield is usually obtained in practice as glucose is not only used for biohydrogen production but also to support microbial growth.



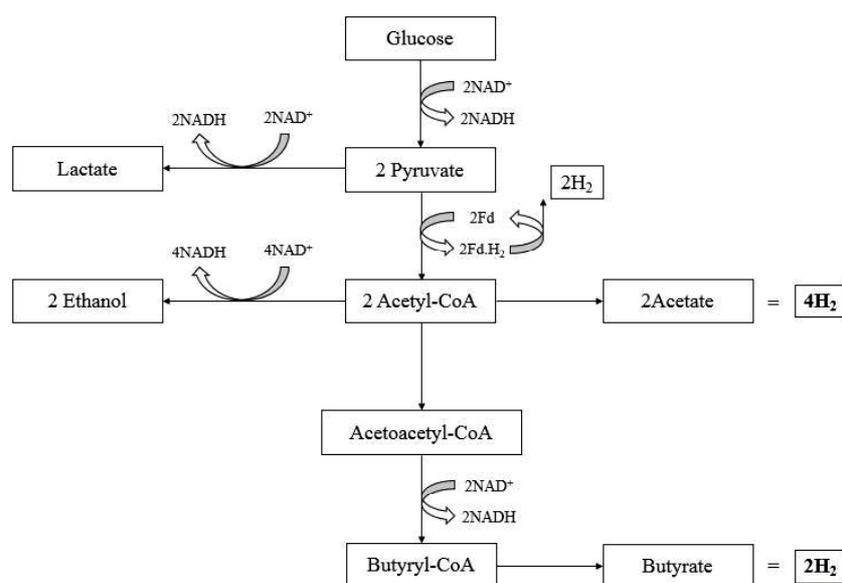


Fig 3. 1 A simplified metabolic pathway for glucose breakdown and the various metabolites produced using dark fermentation in *Clostridium*. Each arrow indicates one or several reactions in series along the pathways. In addition, the amount of hydrogen produced by the [Fe-Fe]-hydrogenase during the fermentation of each metabolite is also shown. Adapted from Chin et al. (Chin et al., 2003).

Clostridium butyricum strain RAK25832 shows slight or no growth in media containing inorganic nitrogen as the sole nitrogen source but grows well in the presence of an organic nitrogen source such as yeast extract. However, the use of yeast extract as the nutrient source for bacterial culture has the main disadvantage of unpredictability, as it may contain several types of nutrients, vitamins, and amino acids such as glutamic acid, making it very difficult to define the media. Bacteria digest and break down proteins into simple compounds, i.e., amino acids and peptides. Casamino acids are mainly comprised of amino acids as they are derived from the acid hydrolysis of casein. Therefore, casamino acids might be a suitable, simpler alternative source of amino acids to yeast extract. Indeed, casamino acids are commonly used as a supplement in culture media or

as an enrichment broth on its own, providing a suitable source of nutrients for enhancing the growth of certain bacteria (Lev, 1977; Lindsay & Murrell, 1983; Nolan, 1971).

In addition, vitamins and metal ions have been shown to be essential factors for biohydrogen production. Although a higher concentration of metals ions may inhibit the activity HPB, a trace level of metal ions is required for hydrogen production. Fe^{2+} is the most widely investigated metal ion for fermentative hydrogen production, which may be related to the fact that its presence is essential for hydrogenase activity (Dhar et al., 2012; Liu et al., 2009; Wang & Wan, 2008; Zhao et al., 2012). Thus, more investigations are needed to determine the effect of the concentration of other metals on fermentative hydrogen production. In addition, few studies have focused on identifying the vital ions and vitamins that play a role in increasing the cell concentration and hydrogen production in dark fermentation using *C. butyricum*.

Accordingly, the aim of this chapter was to evaluate the possibility of using casamino acids as a nitrogen source for biohydrogen production with the isolate *C. butyricum* RAK25832, and determine the optimal concentration of casamino acids. In addition, we aimed to identify the main components in the medium that would enhance hydrogen productivity in *C. butyricum* RAK25832 by first screening the ion medium composition and then optimizing the target components at different concentrations. Finally, we determined the effect of vitamin supplementation in the medium on hydrogen production and cell growth.

3.2 Material and methods

3.2.1 Bacteria isolation and medium preparation

The isolated bacteria were obtained from the Wajiro marine sediment in Fukouka, Japan, at a low tide condition. The physical properties of the site are a temperature of 27°C and pH of 7.2. The sediment was collected and enriched in reinforced Clostridia medium (Daigo, Nihon Seiyaku

391-01781) for general testing. After growth of the bacteria, the supernatants were placed in anaerobic bottles, sealed with rubber and an aluminum cap, and flushed with nitrogen gas. Different dilutions were prepared, streaked on petri dishes, and incubated under anaerobic condition to obtain single colonies. Anaerobic culture was carried out in serum bottles flushed with nitrogen. The composition of the enriched nutrient solutions used for biohydrogen production is shown in Table S1 (Fangkum & Reungsang, 2011). Each medium with a different composition was prepared as a separate stock solution. All chemicals were obtained from Wako Japan. The initial inoculum was developed with a final optical density at 620 nm (OD_{620}) of 0.1 in the final exponential phase. The cell concentration was determined by measuring the absorbance at 620 nm with a Hitachi U-1800 spectrophotometer (Pan et al., 2008). The volume of biogas was measured by releasing the pressure in the serum bottle using wetted glass syringes ranging from 50 mL to 100 mL (Owen et al., 1979). The biogas volume was checked every 6 hr, and the compositions of the liquid phase were determined at the end of the fermentation process for hydrogen production.

3.2.2 Overall experimental design for medium optimization in dark fermentation

The dark fermentation was performed in 75-mL bottles for batch tests with a working volume of 30 mL. The initial inoculum was developed at a final OD_{620} of 0.1 at the late exponential phase. For adjustment of the carbon source applied for this experiment, the effects of 10 g/L of different sugars were examined during fermentation at a constant temperature of 30°C with an initial pH of 8. To study the effect of the nitrogen source, the yeast extract in the medium was replaced by casamino acids (392-00655; Wako Chemical Industries, Ltd.), which was tested at concentrations of 3.33, 6.66, 13.33, 26.67, and 53.33 g/L. The optimal medium composition for hydrogen production was determined by studying the effect of the elimination of single elements.

After setting the final element composition, the effect of vitamin supplementation (Table S2) on hydrogen production was investigated.

3.2.3 Strain identification

Bacterial genomic DNA was extracted from the cell pellets by ISOIL Beating Kit for beads (No. 319-06201; Nippon Gene Co., Ltd., Japan). The 16S rRNA gene was polymerase chain reaction (PCR)-amplified using the universal consensus primers 16SF (5'AACGCGAAGAACCTTAC-3') and 16SR (5'-ACGGGCGGTGTGTRC-3') with a target size of 1.5 kb, as described by Nielsen et al. (Nielsen et al., 1999). The obtained product was subjected to PCR using EmeraldAmp Max PCR Master Mix (2X Premix) (Takara, Japan) (Van Niel et al., 2002). The amplification reaction consisted of an initial denaturing step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 40 sec, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. All PCR steps were performed in an automatic thermal cycler (iCycler™, Bio-Rad, USA). The PCR products were then sequenced and compared with sequences in the GenBank database through a BLAST similarity search. Phylogenetic trees of *C. butyricum* RAK25832 as a biohydrogen-producing strain and its close relatives were constructed based on the full 16S rRNA gene sequences. The tree was based on Jukes-Cantor distance and constructed using a neighbor-joining method with 1000 bootstrap resampling replicates using MEGA 6.06 to estimate the confidence of the tree topologies. *Escherichia coli* was selected as an outgroup species for the tree.

3.2.4 Analytical method

Biogas volume was analyzed using gas chromatography (GC-8APT, Shimadzu, Japan) with a thermal conductivity detector and a Porapak Q stainless-steel column packed with an

activated carbon 60/80 column (1.5 m × 3.0 mm internal diameter). Argon was used as the carrier gas at a flow rate of 6.5 mL/min. The operation temperatures for the injector, column, and detector were 50°C, 60°C, and 50°C, respectively. The concentrations of VFAs were detected using gas chromatography with the same chromatographer as above along with a flame ionization detector (FID) and an 8-foot glass column packed with 10% PEG-20M and 2% H₃PO₄ (80/100 mesh). The temperatures of the injection port, FID detector, and oven were 250°C, 140°C, and 140°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 20 mL/min. The ethanol concentration was determined by high-performance liquid chromatography (LC_10AD) equipped with a Shim pack SPR-PB column (Shimadzu, Japan); the oven temperature was 80°C, and deionized water was used as the mobile phase at a flow rate of 0.6 mL/min with a refraction index detector.

3.2.5 Kinetic modeling

The amount of hydrogen gas produced was determined according to the measurement of vial headspace gas composition and the total biogas volume at each time interval using the following mass equation:

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H(C_{H,i} - C_{H,i-1}) \quad (3)$$

where $V_{H,i}$ and $V_{H,i-1}$ are the cumulative hydrogen gas volumes at the current (i) and previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes at the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ are the hydrogen gas fractions in the headspace at the current and previous time intervals, and V_H is the total headspace volume of vials (Logan et al., 2002).

The cumulative volume of hydrogen produced in the batch experiments was determined according to the modified Gompertz equation (Lay et al., 1997):

$$H = P \exp\{-\exp[\frac{R_m e}{P} (\lambda - t) + 1]\} \quad (4)$$

Where H is the cumulative hydrogen production (mL), λ is the lag time (hr), P is the hydrogen production potential (mL), R_m is the maximum hydrogen production rate (mL/hr), and e is the constant 2.718281828. The P, R_m , and λ values for each batch were estimated using the solver function of Sigmaplot (version 12) with a Newtonian algorithm. The hydrogen yield (mol H₂/mol glucose) was calculated as the total molar amount of hydrogen (mol H₂) divided by the molar amount of glucose (mol substrate).

3.3 Results and discussion

3.3.1 Isolation, screening, and identification

Among the 11 strains isolated, a preliminary experiment showed that only two (RAK25832 and RAK25833) efficiently produced hydrogen from the reinforced medium for Clostridia. The cumulative hydrogen production for strain RAK25832 and strain RAK25833 was 933 mL H₂/L and 614 mL H₂/L, respectively. Therefore, strain RAK25832 was further examined for its hydrogen production capability.

3.3.2 Characterization of strain RAK25832

The partial 16S rRNA gene sequence (1.5 kb) of strain RAK25832 was deposited in GenBank under accession no. KY582833. Comparison of the 16S rRNA gene sequences with those in the NCBI database revealed that strain RAK25832 has high sequence similarity (100%) with strains of *Clostridium butyricum*. Phylogenetic analysis of the 16S rRNA gene sequence placed strain RAK25832 in the cluster comprising other strains of *Clostridium butyricum* (Fig. 3.2). *C. butyricum* is well known as an anaerobic bacterium as well as a producer of hydrogen (Chen et al., 2005) and butyric acid (He et al., 2005a). *C. butyricum* RAK25832 showed growth

ability with various types of carbohydrates as the sole carbon and energy source under a mesophilic condition (Table 3.1), which is a characteristic of Clostridia species (Yokoi et al., 1998).

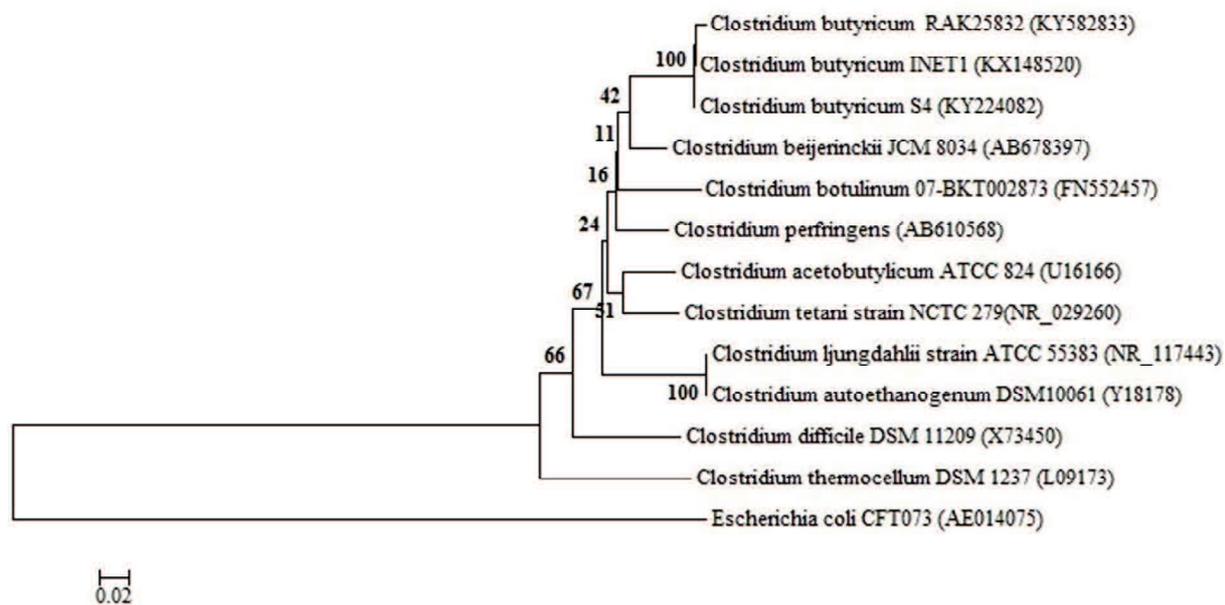


Fig 3. 2 Phylogenetic tree showing the relationships between *Clostridium butyricum* RAK25832 and related species based on the 16S rRNA gene. The scale bar represents 0.02 substitutions per nucleotide position. Bootstrap values are indicated at the nodes. Reference sequences in the dendrogram were obtained from NCBI (accession numbers are in parentheses).

3.3.3 Effect of carbon source on hydrogen production and cell growth

Several carbon sources were tested at a fixed concentration of 10 g substrate/L, with a casamino acids concentration of 1.67 g/L under a constant temperature 30°C. As shown in Table 3.1, *C. butyricum* RAK25832 could utilize various types of carbon sources, including monosaccharides (glucose, galactose, and mannose), disaccharides (sucrose, trehalose, lactose, and maltose), and polysaccharides (starch, inulin, and glycogen). The sugars inulin, arabinose, and

xylose are widely present in plants. Therefore, the present results indicate that this strain could effectively utilize the hydrolysate of agricultural waste but not sugar alcohols such as inositol as a carbon source. Rhamnose, maltose, xylan, cellulose, dextran, glycerol, melizitose, glycine, mannitol, sorbitol, and xylitol could not be utilized by *C. butyricum* RAK25832.

Table 3. 1 Growth of *Clostridium butyricum* RAK25832 with different carbon sources.

Carbon source	Results
Xylose	+
L-Arabinose	+
Glucose	+
Fructose	+
Galactose	+
Mannose	+
Rhamnose	-
Sucrose	+
Lactose	+
Maltose	-
Cellobiose	+
Trehalose	+
Raffinose	+
Xylan	-
Starch	+
Dextran (Dextrin)	-
Inulin	+
Pectin	+
Glycerol	-
Salicin	+
Hepatin	ND
Melizitose	-
Chitin	ND
Glycine	-
Mannitol	-
Sorbitol	-
Xylitol	-
Glycogen	+
Cellulose	-

ND = not detected, +; could utilize, -; cannot utilize

The H₂ yield (mol H₂/mol substrate), produced hydrogen (mL/L), and concentrations of soluble metabolite products (SMPs) as fermentation by-products during 96 hr of incubation when

using the carbon sources found to be most suitable for the growth of strain *C. butyricum* RAK25832 (glucose, fructose, xylose, sucrose, and lactose) are shown in Table 3.2. The results showed that the highest hydrogen yield was realized when lactose was the carbon source, followed by xylose, glucose, fructose, and then sucrose. The main VFA identified was butyric acid, followed by acetic acid; others were nominally present. The final pH decreased because of the SMPs. The characteristics reported of different *C. butyricum* isolates vary to a certain extent, even for the same strain, partly owing to variations among studies in culture conditions (Chong et al., 2009; Hu et al., 2013; Yin & Wang, 2017). Based on these results, glucose was selected as the carbon source for further analysis.

Table 3. 2 Production of hydrogen by *Clostridium butyricum* RAK25832.

Carbon source (g/L)	Hydrogen yield (mol H ₂ /mol substrate)	Final pH	Cell concentration (OD ₆₂₀)	Kinetics parameters estimated by the modified Gompertz model				VFA (mg-COD/L) SMPs				
				p (mL)	Rm (mL/hr)	λ (hr)	R ²	HAc	HPr	i-HBu	n-HBu	EtOH
Glucose	0.91	4.77	1.60	1262	146	28	0.9899	160	11	0	451	50
Fructose	0.86	4.84	1.55	1182	149	16	0.9959	328	60	21	677	40
Xylose	1.06	4.88	1.39	1457	52	13	0.9920	168	0	0	643	10
Sucrose	0.82	4.78	2.26	1136	155	17	0.9960	166	0	0	508	15
Lactose	1.13	4.72	2.42	1559	89	28	0.9986	190	0	0	648	19

VFA, volatile fatty acids; SMPs, soluble metabolite products; HAc, acetic acid; HPr, propionic acid; i-HBu, isobutyric acid; n-HBu, N-butyric acid; EtOH, ethanol.

3.3.4 Effect of casamino acids concentration on hydrogen production and SMPs

The effect of replacing yeast extract with casamino acids was examined using 10 g/L glucose as the carbon source. Fig. 3.3 shows the plots of cumulative hydrogen production fitted to the modified Gompertz equation for various casamino acids concentrations ranging from 3.33 to 53.33 g/L. The results showed that the maximum hydrogen production was achieved with a

casamino acids concentration of 26.67 g/L. The kinetic parameters are shown in Table 3.3. Casamino acids had a significant effect on hydrogen production, with a concentration-dependent increase in cumulative hydrogen production until 26.7 g/L of casamino acids. Similarly, increasing the casamino acids concentration resulted in an increase in cell density until reaching an OD₆₂₀ of 9.89 (Table 3.3).

Table 3. 3 Kinetics parameters of hydrogen production by *Clostridium butyricum* RAK25832 using casamino acids as the nitrogen source.

Casamino acids concentration (g/L)	Final pH	Cell concentration (OD ₆₂₀)	Kinetics parameters estimated by the modified Gompertz model			
			P (mL)	Rm (mL/hr)	λ (hr)	R ²
3.33	4.26	2.06	1311	161	28	0.9905
6.67	4.24	4.11	1576	147	23	0.9998
13.33	4.36	5.49	1473	116	16	0.9997
26.67	4.43	8.26	2505	160	4	0.9938
53.33	4.49	9.89	2233	129	5	0.9811

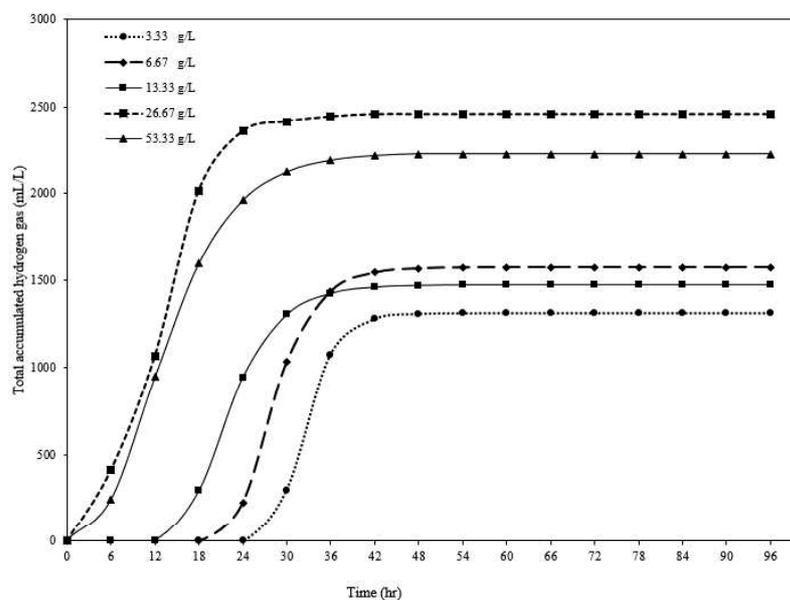


Fig 3. 3 Cumulative hydrogen production by *Clostridium butyricum* RAK25832 at different casamino acids concentrations with 10 g/L glucose as the carbon source.

The nitrogen source is well known to have a significant effect on hydrogen production. Although many researchers have investigated the associations of different nitrogen sources, including inorganic and organic sources, there is limited information on the optimal casamino acids concentration as a nitrogen source for *C. butyricum*. Fig. 3.4 shows the effect of different casamino acids concentrations on the VFAs produced by *C. butyricum* RAK25832 according to fermentation time and hydrogen yield. The main VFA produced appeared to be N-butyric acid followed by acetic acid, confirming that *C. butyricum* RAK25832 is a producer of butyric acid. The production of acetate and butyrate, as by-products of the dark fermentation process, as well as the final pH decreased in all conditions due to the active formation of SMPs. The maximum hydrogen yield was identified with a casamino acids concentration of 26.67 g/L at 1.81 mol H₂/mol glucose.

Casamino acids have also been investigated as a potential protein substrate source. Yokoyama et al. (Yokoyama et al., 2010) indicated that casamino acids could not be used in a cow

manure mixed culture as they were readily decomposed by the bacteria without production of hydrogen. Other researchers reported that barely any hydrogen was produced from proteins and lipids (Lay et al., 2003; Noike & Mizuno, 2000). Another study replaced yeast extract with casamino acids plus proline and vitamins in the medium of *C. saccharolyticus*, resulting in similar yields of hydrogen production with sucrose; however, the production rate was reduced by about 30% (Van Niel et al., 2002).

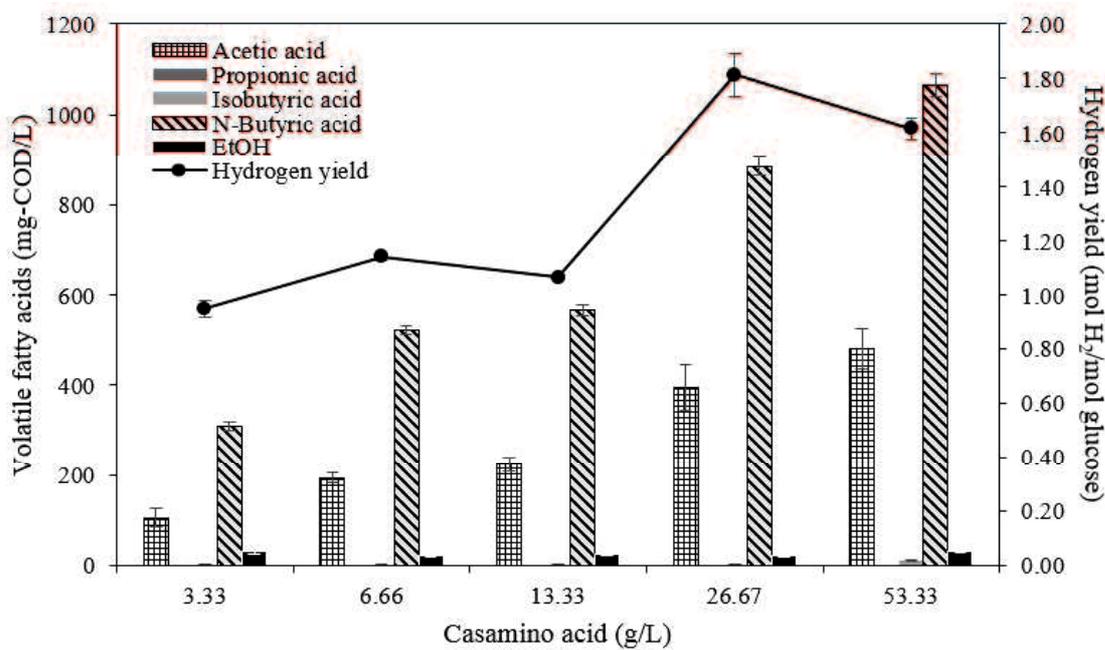


Fig 3. 4 Effect of different concentrations of casamino acids on volatile fatty acids produced by *Clostridium butyricum* RAK25832 and hydrogen yield.

3.3.5. Medium composition and optimization of target elements

Hydrogen production in dark fermentation requires essential micronutrients for bacterial metabolism. Some trace metals such as Mg, Zn, Na, and Fe have important effects on H₂ metabolism in bacterial growth since these elements are required as cofactors for the bacterial enzymes, in transport processes, and as dehydrogenases (Wang & Wan, 2009) (Ismail et al., 2010;

Sinha & Pandey, 2011). In this study, all of the medium components (Table S1) were screened to select the most important elements that could be optimized to accelerate or promote biohydrogen production by *C. butyricum* RAK25832. Table 4 shows the effect of eliminating 18 metal elements, including macro- and micro-elements, one by one with the addition of casamino acids (1.67 g/L) and glucose (10 g/L) as the nitrogen and carbon source, respectively. The results indicated that for all parameters, the deletions of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, NaHCO_3 , and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ had significant effects on the H_2 production, hydrogen production rate, and lag time. In particular, deletion of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ decreased the hydrogen production to 834 mL H_2/L compared to the control (1242 mL H_2/L), representing a 33% decrease, although there were no effects on the hydrogen production rate or lag time. Elimination of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ resulted in 759 mL H_2/L , representing a 39% decrease compared to the control; the production rate decreased until 108 mL/hr and the lag time was delayed until 52 hr. Elimination of NaHCO_3 resulted in a slight decrease in hydrogen production but had an extreme effect on the lag, with the delay increasing from 30 hr (control) to 71 hr, which was associated with a decrease in the production rate until 76 mL/hr. Elimination of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ had the most significant effect on hydrogen production at 469 mL H_2/L , representing a 62% reduction, as well as a decrease in the production rate (41 mL/hr) and an increase in the lag time (43 hr). Although other heavy metals such as Cr, Cu, and Zn have been reported to influence different biological processes such as acidogenesis and methanogenesis and other anaerobic processes (Hickey et al., 1989; Yenigün et al., 1996), in the present study, the other metal elements, including macro-nutrients (NH_4Cl , NaCl , and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) and micro-nutrients (H_3BO_3 , ZnCl_2 , $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, AlCl_3 , $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, EDTA, HCl, and $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$) did not have any effect on hydrogen production

in the dark fermentation process. The main VFAs were acetic acid and butyric acid in all cases, indicating that eliminating single components did not affect the VFA composition.

3.3.6 Optimizing the target metal elements

The results described above demonstrated that $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, NaHCO_3 , and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ are very important elements for hydrogen production. Therefore, further experiments were conducted to optimize the concentrations of these four element

3.3.6.1 Optimizing the $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ concentration

Seven concentrations of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1, 0.2, 0.4, 0.67, 1.33, 2.67, and 5.33 g/L) were investigated as shown in Fig. 3.5A, along with a control experiment without $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, to determine the optimal concentration for accelerating hydrogen production. Glucose (10 g/L) was used as the carbon source and casamino acids (26.67 g/L) was used as the nitrogen source. There was a slight increase in hydrogen production with increasing $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ concentration. The H_2 production was 2006 mL/L with 0.1 g/L and only increased to 2109 mL/L with 0.67 g/L of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (11 mg Mg^{2+}); a further increase in the concentration had no effect on hydrogen production. Therefore, it was concluded that 0.1 g/L was the optimal $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ concentration with a production rate of 223 mL/hr and a hydrogen yield of 1.45 mol H_2 /mol glucose. In addition, no further concentration increase affected the lag time (16 hr). Fig. 6A shows that the final pH was acidified because of the production of VFAs as the final fermentation products. Moreover, the cell concentration was not increased by increasing the element concentration. It has been reported that Mg has a significant effect on bacterial growth (Lin & Lay, 2005). The Mg^{2+} requirement for microorganisms is mainly for the ribosomes, and it also plays a role as a cofactor of enzymes and as one of the main constituents of the cell walls and membranes. Therefore, decreasing the Mg^{2+}

concentration would reduce cell growth to ultimately affect hydrogen production (Wang et al., 2007). Mg^{2+} was reported to enhance biogas production from organic wastes with an optimum value of 45 mg for 300 L slurry, and pure magnesium metal is toxic for biomethanation (Mandal & Mandal, 1998). Similarly, Sinha and Pandey (Sinha & Pandey, 2011) observed a maximum yield (295 mL/g of glucose consumed) and maximum hydrogen productivity (65 mL/hr) at 500 mg/L $MgSO_4 \cdot 7H_2O$, which then decreased with a further increase in the magnesium ion concentration.

Table 3. 4 Effects of eliminating different elements on hydrogen production by *C. butyricum* RAK25832.

No.	Kinetic parameters estimated by the modified Gompertz model																	Yield (mol.H ₂ /mol glucose)	VFA (mg COD/L)												
	p (mL)	Rm (mL/hr)	λ (hr)	R ²	HAc	HPr	i-HBu	n-Hbu	EtOH																						
	NH ₄ Cl	NaCl	MgCl ₂ ·6H ₂ O	CaCl ₂ ·2H ₂ O	K ₂ HPO ₄ ·3H ₂ O	NaHCO ₃	FeCl ₂ ·4H ₂ O	H ₃ BO ₃	ZnCl ₂	CuCl ₂ ·2H ₂ O	MnCl ₂ ·4H ₂ O	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	AlCl ₃	CoCl ₂ ·6H ₂ O	NiCl ₂ ·6H ₂ O	EDTA	HCl Conc.	Na ₂ SeO ₃ ·5H ₂ O	Control	Casamino acids	Glucose										
1	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1232	133	29	0.9959	0.89	470	0	0	1150	12
2	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1247	118	28	0.9945	0.90	355	0	0	1200	10
3	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	834	108	28	0.9945	0.60	454	0	0	1100	12
4	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1186	136	29	0.9959	0.86	423	9	0	1113	15
5	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	759	110	52	0.9997	0.55	270	0	0	1120	16
6	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1140	76	71	0.9827	0.83	366	0	0	980	14
7	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	469	41	43	0.9970	0.34	250	0	0	520	12
8	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	1225	179	30	0.9906	0.89	348	0	0	1202	13
9	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	1243	183	30	0.9929	0.90	444	0	0	1311	11
10	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	1228	182	30	0.9933	0.89	368	0	0	1250	15
11	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	1235	106	27	0.9947	0.89	394	0	0	1563	18
12	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	1245	142	29	0.9962	0.90	367	8	0	1114	20
13	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	1230	152	30	0.9895	0.89	356	0	0	1152	12
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	+	1265	94	26	0.9977	0.92	432	0	0	1131	13
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	1254	106	26	0.9968	0.91	458	0	0	1220	10
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	1273	136	28	0.9959	0.92	461	0	0	1168	9
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	1283	102	26	0.9968	0.93	448	0	0	1198	8
18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	1250	110	26	0.9950	0.90	389	0	0	1106	12
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1242	209	30	0.9933	0.90	430	0	0	1123	13
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	0	0	0	0	0	0	0	0	0	0
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0

P, accumulative hydrogen (mL); R, hydrogen production rate; λ, lag time; VFA, volatile fatty acids; HAc, acetic acid, HPr, propionic acid, i-HBu, isobutyric acid; n-HBu, n-butyric acid; EtOH, ethanol.

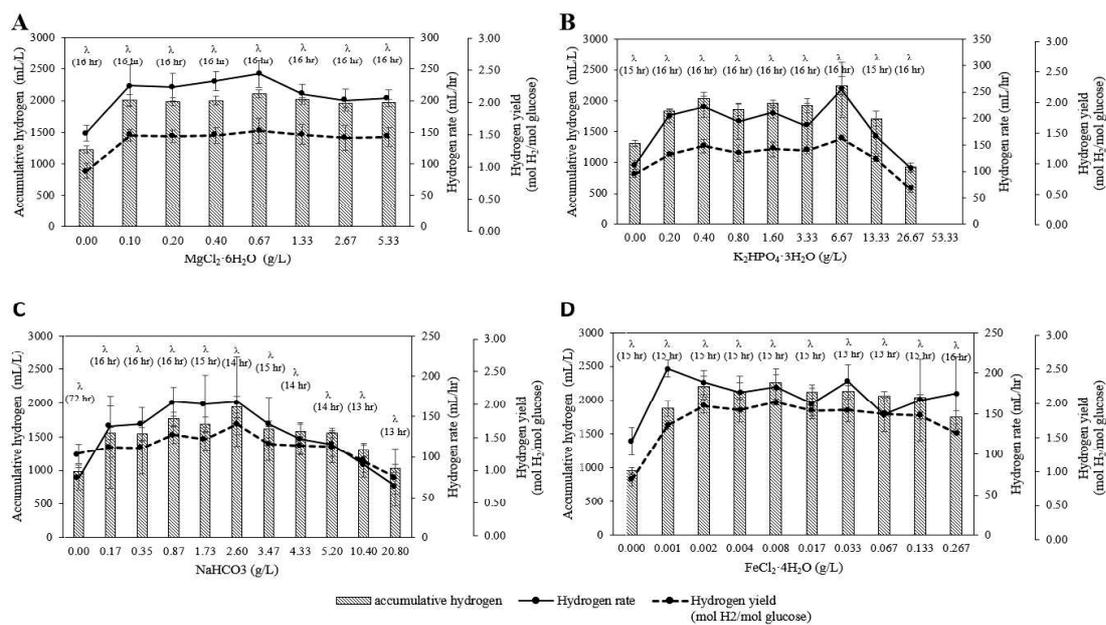


Fig 3. 5 Effect of different concentrations of (A) MgCl₂·6H₂O, (B) K₂HPO₄·3H₂O, (C) NaHCO₃, and (D) FeCl₂·4H₂O on the accumulated hydrogen, hydrogen production rate, and hydrogen yield by *C. butyricum* RAK25832.

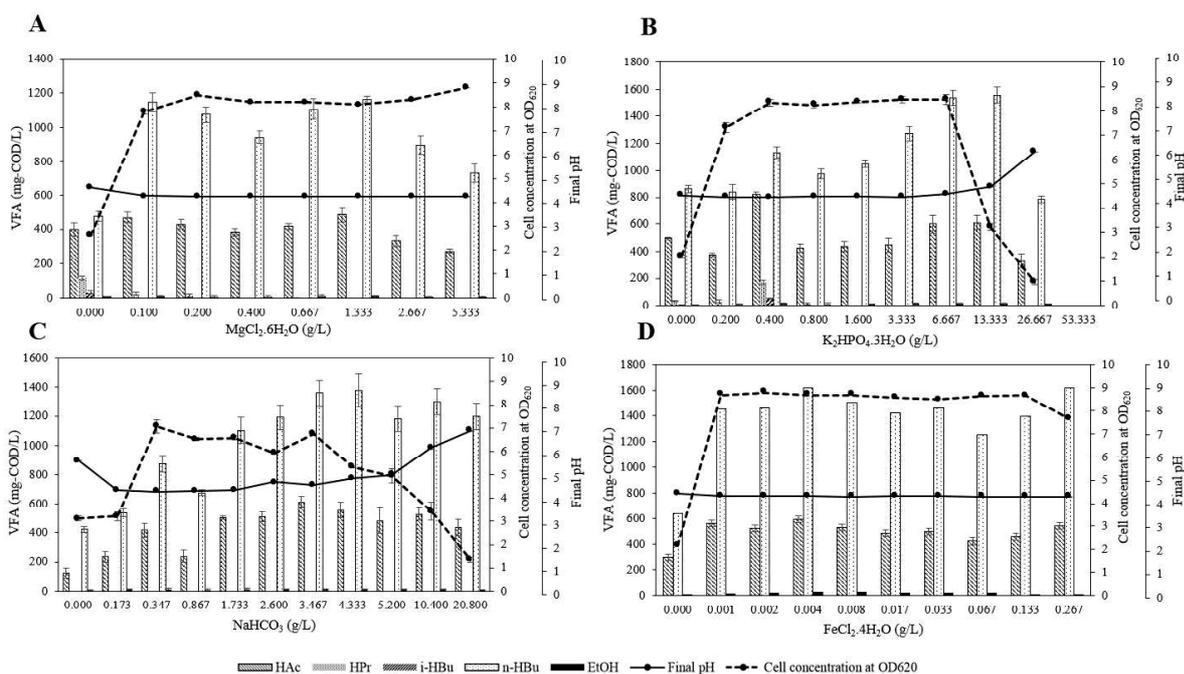


Fig 3. 6 Volatile fatty acids associated with different concentrations of (A) MgCl₂·6H₂O, (B) K₂HPO₄·3H₂O, (C) NaHCO₂, and (D) FeCl₂·4H₂O compared to the final pH and cell concentration (OD₆₂₀).

3.3.6.2 Optimizing the concentration of K₂HPO₄·3H₂O

Nine concentrations of K₂HPO₄·3H₂O (0.2–53.33 g/L) were evaluated, along with a control experiment without K₂HPO₄·3H₂O. As shown in Fig. 3.5B, increasing the K₂HPO₄·3H₂O concentration increased hydrogen production until the concentration of 6.67 g/L, showing H₂ production of 2244 mL/L, a production rate of 254 mL/hr, and a yield of 1.62 mol H₂/mol glucose. However, the K₂HPO₄·3H₂O concentration did not have any effect on the lag time (16 hr). Further increasing the concentration decreased the production to such an extent that it ceased completely at 53.33 g/L K₂HPO₄·3H₂O, indicating a toxic effect above 6.67 g/L. The K₂HPO₄·3H₂O concentration had a significant effect on the cell concentration with a proportional increase with

concentration until 6.67 g/L, and then a decrease in cell concentration at higher concentrations. The VFAs increased along with the increase in cell concentration (Fig. 3.6B).

3.3.6.3 Optimizing the NaHCO₃ concentration

The effects of NaHCO₃ concentration were evaluated in the range of 0 to 20.8 g/L. The results indicated that elimination of NaHCO₃ had a significant effect on lag time, which increased to 72 hr (Fig. 3.5C). Hydrogen production, hydrogen production rate, and hydrogen yield were all increased until 2.6 g/L NaHCO₃, at 1945 mL/L, 168 mL/hr, and 1.41 mol H₂/mol glucose, respectively. Further increase in the concentration led to a decrease in production. The NaHCO₃ concentration also had a significant effect on the cell concentration (Fig. 3.6C). Previous studies have used NaHCO₃ as a buffer in the feeding medium to neutralize the acidic products (Ai et al., 2014; Lee et al., 2004); however, NaHCO₃ has also been shown to increase the cell concentration but not hydrogen production (Chookaew et al., 2015; Xu et al., 2008). He et al., (He et al., 2004) found that 0.124% NaHCO₃ (w/v) was the optimum concentration under their experimental conditions with *C. butyricum*.

3.3.6.4 Optimizing the FeCl₂·4H₂O concentration

The effect of the FeCl₂·4H₂O concentration was studied in the range of 0 to 0.267 g/L. The results indicated that elimination of FeCl₂·4H₂O had a significant effect on hydrogen production. The hydrogen production, production rate, and yield at 0.002 g/L FeCl₂·4H₂O was 2202 mL/L, 189 mL/hr, and 1.59 mol H₂/mol glucose, respectively, with no effect at higher concentrations. The cell concentration and VFA were not affected by an increase in the FeCl₂·4H₂O concentration (Fig. 3.6D). Iron is an important nutrient for the formation of hydrogenase and other enzymes. Iron-sulfur affects protein functions primarily as an electron carrier, and is involved in pyruvate

oxidation to acetyl-CoA, CO₂, and H₂. Iron could also induce metabolic alterations and is involved in FeeS and non-FeeS protein functions in hydrogenase (Lee et al., 2001). A previous study (Alshiyab et al., 2008) in *Clostridium* showed that the H₂ yield was enhanced from 391 to 408 mL/g of glucose utilized by the addition of 25 mg/L of FeSO₄·7H₂O, but a further increase in the concentration had a negative effect. In addition, the biomass increased as the iron concentration increased, and reached the maximum at 1000 mg/L of FeSO₄·7H₂O. This result was substantiated in the present study given the increased percentage of glucose consumption observed at higher FeSO₄·7H₂O concentration.

3.3.7 Effect vitamin supplementation

Several vitamins (Table S2) were added to the medium as supplemental nutrients to determine their effect on hydrogen production. As shown in Fig. 3.7, none of the vitamins utilized by *C. butyricum* RAK25832 had a significant effect on hydrogen production or on the cell concentration. In addition, there was no effect of vitamin supplementation on VFAs (Fig. 3.8).

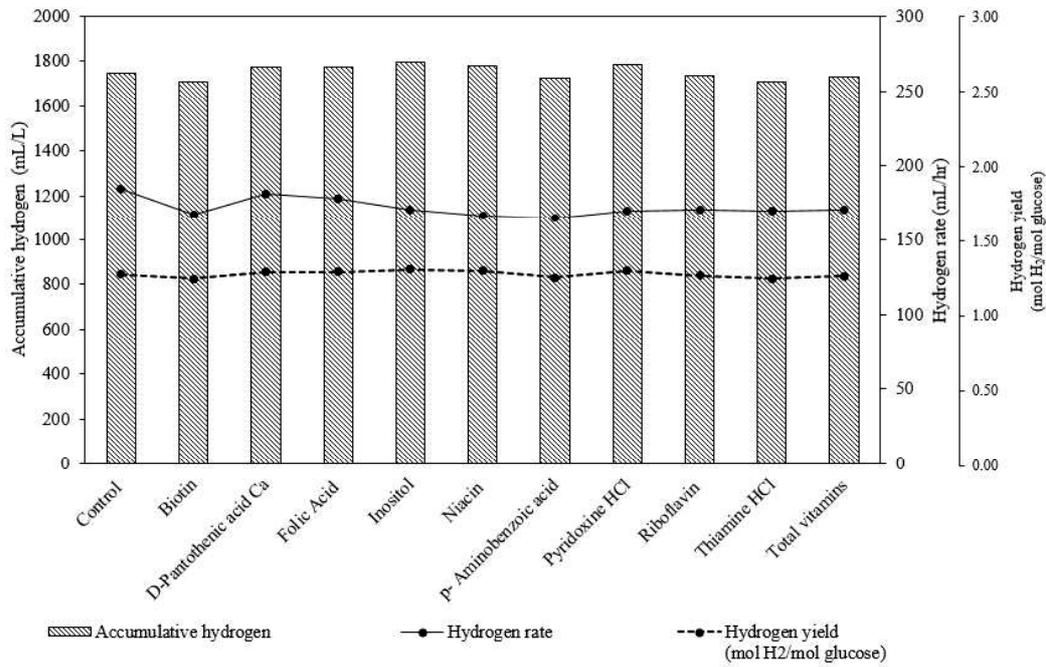


Fig 3. 7 Effect of vitamin supplementation on the cumulative hydrogen production, hydrogen production rate, and hydrogen yield by *C. butyricum* RAK25832.

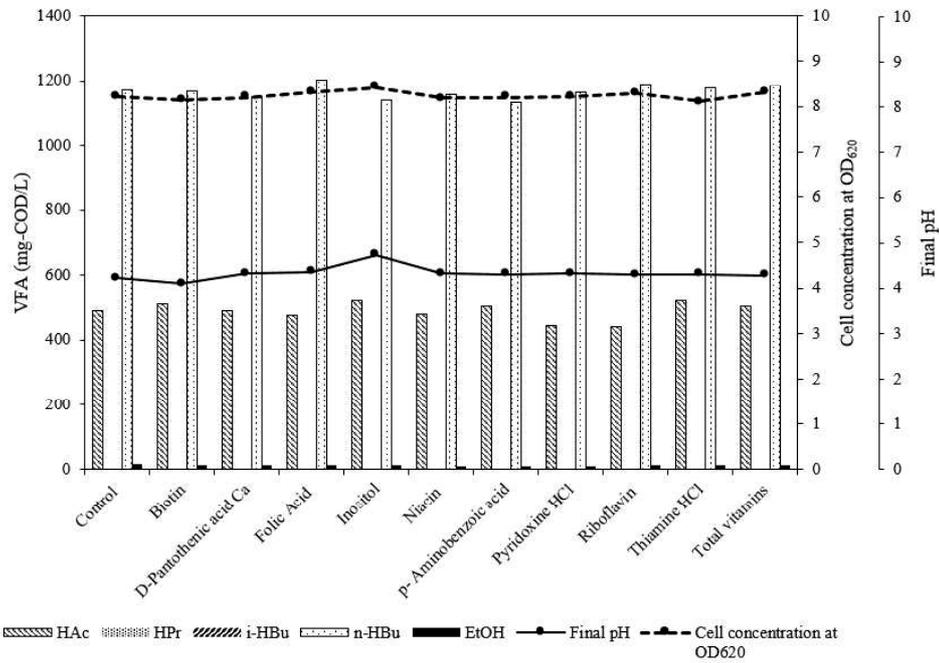


Fig 3. 8 Effect of vitamin supplementation on volatile fatty acids produced by *C. butyricum* RAK25832.

3.3.8 Hydrogen fermentation characteristics under the optimum conditions and growth kinetics.

Hydrogen production was investigated under the optimum conditions of glucose (10 g/L), casamino acids (26.67 g/L), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1 g/L), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (6.67 g/L), NaHCO_3 (2.6 g/L), and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.002 g/L). Maximum hydrogen production was achieved (3074 mL/L) at a cell concentration of $\text{OD}_{620} = 13.1$ with a lag time of 4.5 hr and R_m of 183 ml/hr ($R^2 = 0.9955$), and the hydrogen yield reached the maximum with 2.23 mol H_2 /mol glucose. The decrease in pH from 8 to 4.5 was attributed to the accumulation of acetic acid (550 mg-COD/L) and butyric acid (1102 mg-COD/L), followed by trace amounts of ethanol (52 mg-COD/L). The high acetic and butyric acid concentrations showed that the fermentation process of glucose by strain RAK25832 was of the acetate butyrate type, which is considered to be the main fermentation type of *Clostridium* species (Zhang et al., 2009).

Table 3.5 shows a comparison of the maximum hydrogen yield of strain RAK25832 to that of other strains reported in the literature. Most previous studies carried out experiments at 37 °C, although strain RAK25832 produced a good yield at 30 °C. However, the pH ranged from 5.6 to 8. Strain RAK25832 had a similar hydrogen yield to that of *Clostridium butyricum* EB6 (2.2 mol H_2 /mol glucose) (Chong et al., 2009).

Table 3. 5 Comparison for hydrogen yield of glucose fermentation by *Clostridium butyricum*

Microorganism	Substrate	pH	Temperature (°C)	H ₂ yield mol H ₂ / mol glucose	Reference
<i>Clostridium butyricum</i> A1	Glucose (10 g/L)	6.5	37	1.9	(Jenol et al., 2013)
<i>Clostridium butyricum</i> TM-9A	Glucose (10 g/L)	8	37	2.67–3.1	(Junghare et al., 2012)
<i>Clostridium butyricum</i> EB6	Glucose (15.7 g/L)	5.6	37	2.2	(Chong et al., 2009)
<i>Clostridium butyricum</i> IAM 19002	Glucose (9 g/L)	7	37	1.04	(Junghare et al., 2012)
<i>Clostridium butyricum</i> IFO 3847	Glucose (9 g/L)	7	37	1.26	(Karube et al., 1976)
<i>Clostridium butyricum</i>	Glucose (3 g/L)	6.5	37	2.09	(Seppälä et al., 2011)
<i>Clostridium butyricum</i> DSM 10702	Glucose (10 g/L)	6.8	37	2.4–3.1	(Hu et al., 2013)
<i>Clostridium. butyricum</i> RAK25832	Glucose (10g/L)	8	30	2.23	This study

The cell growth and substrate consumption kinetics during the growth of the strain in batch-fermentative hydrogen production were investigated using the Monod model. Lineweaver-Burk plots (Fig. 3.9) fitted the experimental results fairly well with an R^2 value of 0.9741. The estimated μ_{\max} and K_s values when glucose was used as the substrate were 0.35 h^{-1} and 9.27 g/L , respectively. Based on the final fermentative products from glucose degradation were mainly butyric acids and acetic acids, at the optimum conditions a mol glucose converted to acetate direction by 24% and to butyrate direction by 76%.

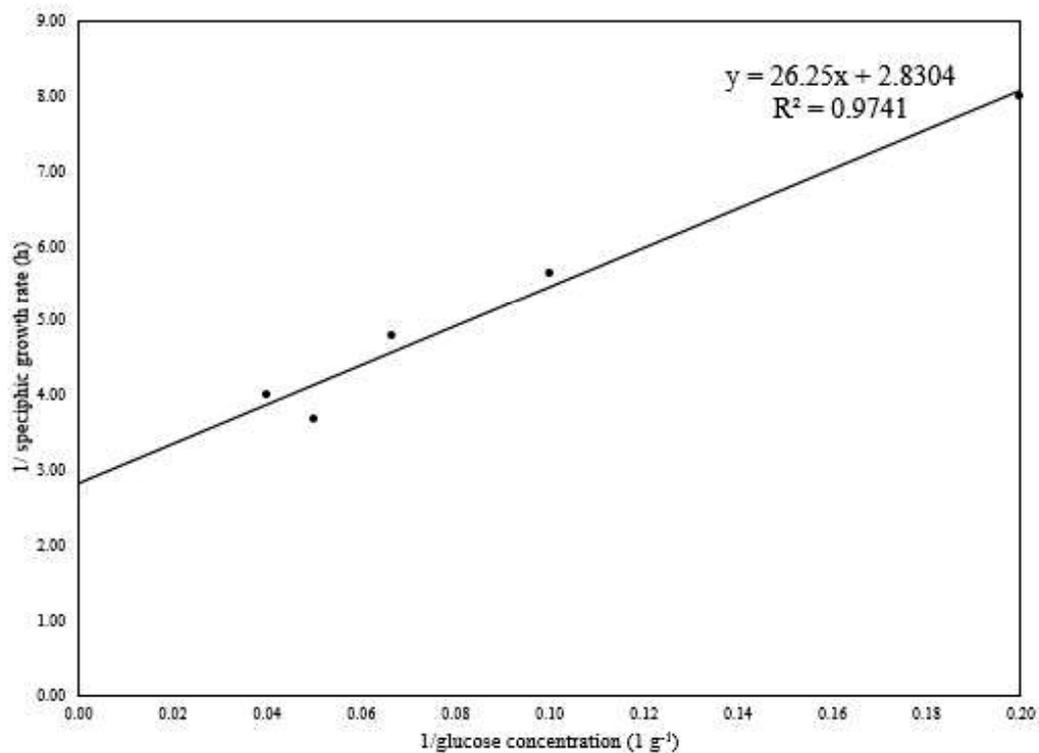


Fig 3. 9 Lineweaver-Burk plot for prediction of *C. butyricum* RAK25832 growth kinetic parameters at pH 8.0 and 30°C.

3.4 Conclusions

We here provide the first demonstration of the applicability of casamino acids as a nitrogen source for biohydrogen production using *C. butyricum* RAK25832. The most important elements found to be essential for growth and hydrogen production were MgCl₂, K₂HPO₄, NaHCO₃, and FeCl₂. Under the optimum conditions, the yield increased to 2.23 mol H₂/ mol glucose and the cumulative hydrogen production was 3,073 ml H₂/L. In future work, it will be important to determine the amino acids that will best promote hydrogen production in *C. butyricum*. Further optimization of these conditions could help to achieve sustainable biohydrogen production with a good yield as an alternative to fossil fuels.

References of chapter III

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CHAPTER IV:

Enhancement of hydrogen production by combining a blend ratio for two types of algal waste biomass and reducing casamino acids supplementation by *Clostridium butyricum* RAK25832.

4.1 Introduction

Concerns regarding fossil fuel depletion and their environmental impact from burning—particularly greenhouse gas emissions—have prompted a search for alternative renewable energy sources (Hahn-Hägerdal et al., 2006). Biofuel produced from biomass is an alternative to fossil fuels that has many benefits, including reduced environmental impact, greater energy security, and foreign exchange savings (Balat, 2010; Kan, 2009).

Dark fermentation has attracted increasing interest over the last decade for its potential to produce ‘green’ hydrogen from readily available and low-cost organic biomass (Azwar et al., 2014); a high rate and yield are important for the sustainability of commercial biohydrogen production. Fermentative hydrogen production by hydrogen-producing bacteria (HPB) requires trace amounts of essential metal ions (Wang & Wan, 2009), although higher concentrations inhibit bacterial metabolism (Liu et al., 2009). In a previous stage of this work, it was shown that hydrogen production by *Clostridium butyricum* RAK25832 required four critical minerals, namely $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, NaHCO_3 , and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, and that casamino acids (CAAs) are an important source of nitrogen for hydrogen production (Aly et al., 2017). CAAs are amino acids derived from casein acid hydrolysis and are used as a supplement in bacterial cultures to enhance growth. However, CAAs are expensive to scale up (Lev, 1977; Lindsay & Murrell, 1983; Nolan, 1971), which is a major impediment to large-scale biohydrogen production. There is, therefore, a need to develop more economically feasible strategies (Cheng et al., 2011).

One way to lower the cost of biohydrogen production is to use low-cost and renewable feedstock. Aquatic biomass—especially macro- and micro-algae—have many advantages as biofuel substrates. Firstly, algae have higher productivity than agricultural crops and do not require arable land, and therefore do not compete with human food production (Dismukes et al., 2008; Fargione et al., 2008; Sialve et al., 2009). Secondly, their low lignin content facilitates the process of energy conversion from biomass to biofuels (Jones & Mayfield, 2012; Wei et al., 2013). The use of residual biomass after lipid extraction can maximize energy production from micro-algal biomass (Brennan & Owende, 2010). *Chlorella fusca* was shown to be a good feedstock for biodiesel production (lipid content 20.4%) (unpublished data). Biofuels may be derived from oil (biodiesel) or sugar (e.g., biohydrogen and bioethanol) via sequential or independent processes (biorefinery concept).

Macro-algal blooms have many detrimental effects. Seaweed wrack accumulates along shorelines and produces foul odors (Wilce et al., 1982), while deep seaweeds physically obstruct other coastal life (Hauxwell et al., 2001); moreover, decaying algal organic matter creates anoxic conditions that kill fish and shellfish (Diaz, 2001). *Ulva lactuca* blooms are an environmental problem in the coastal areas of Japan, especially in the summer; in the Seto Inland Sea, blooms have replaced seagrasses (Sugimoto et al., 2007). Nonetheless, *U. lactuca* is a potential substrate for hydrogen production (Park et al., 2009).

This chapter aimed to investigate whether algal waste could be used to produce hydrogen at a low cost with high yield. Yield optimization by $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, NaHCO_3 , and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ supplementation was investigated. Additionally, the applicability of biohydrogen produced by blending *C. fusca* with *U. lactuca* waste materials at various ratios to reduce the

amount of CAAs required for dark fermentation by *Clostridium butyricum* RAK25832 was assessed.

4.2 Material and methods

4.2.1 Microorganism

Fermentation was carried out using *C. butyricum* RAK25832 (Aly et al., 2017). The inoculum was cultured under anaerobic conditions in medium composed of glucose (10 g/l), CAAs (26.67 g/l), MgCl₂·6H₂O (0.1 g/l), K₂HPO₄·3H₂O (6.67 g/l), NaHCO₃ (2.6 g/l), and FeCl₂·4H₂O (0.002 g/l) (pH 8) at 30°C, and was harvested at late exponential phase. The supernatant was transferred to anaerobic bottles that were sealed with a rubber and aluminum cap and then flushed with nitrogen gas. The initial inoculum was adjusted to a final optical density at 620 nm (OD₆₂₀) of 0.1 by measuring the absorbance with a U-1800 spectrophotometer (Hitachi, Tokyo, Japan) (Pan et al., 2008). All chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

4.2.2 Algal feedstock

The micro-algal biomass *C. fusca* was obtained from the river Nile in Alexandria, Egypt and identified by Petrotech-ffn Egypt R & D Center (Mansoura, Egypt). The isolate was grown in a 10-l Magnaferm fermentor (New Brunswick Scientific Co., New Brunswick, NJ, USA) in commercial medium (1 g urea and 0.3 g potassium phosphate/10 l, pH 7) at 25°C and under a light intensity of 1,200 lux (540 Meter; Testo, Sparta, NJ, USA) measured at the surface of the photobioreactor, with aeration at a rate of 10 l/min. For harvesting, the biomass was allowed to settle by gravity and then centrifuged at 4000 rpm for 10 min (Beckman Model; TJ-6, USA) after removing most of the liquid phase. The wet biomass was oil extracted by the method of Bligh and Dyer (Bligh & Dyer, 1959), yielding 510 mg/l dry weight biomass with a lipid content of 20.4%. The

algal pellet remaining after extraction was washed three times with clean water, dried overnight (12 h) at 70°C, and stored at -20°C until use as a substrate in the fermentation reaction. The macro-alga *U. lactuca* was harvested during the summer (May to July) at low tide on Wajiro beach, Fukuoka prefecture, Japan. The biomass was cleaned with tap water, dried at 70°C, passed through a 200- μ sieve, and stored at -20°C.

The chemical composition of algal biomass was determined, with measurements performed in duplicate (Table 1). Total solid (TS) and volatile solid (VS) in each type of algal biomass were determined according to Standard Methods 2540 G (APHA, 2005). Moisture content was determined by drying the sample in an oven at 105°C until it reached a constant weight. Total ash was determined by incineration at 550°C in a muffle furnace. The average moisture and ash contents were used to calculate VS content. Protein content was estimated with the Lowry protein assay (Berges et al., 1993), and total lipid content was determined according to Bligh and Dyer method (Bligh & Dyer, 1959). Carbon, hydrogen, and nitrogen content was determined with a Micro Corder JM 10 element analyzer (J-Science Lab Co., Kyoto, Japan). Amino acid content was determined with an LC-500/V2 amino acid analyzer (Shimadzu, Kyoto, Japan) with lithium citrate (pH 2.98) as the buffer solution.

4.2.3 Biohydrogen production

Twelve sets of experiments were carried out to establish the optimal substrate blend ratio that would enhance hydrogen yield (HY). *U. lactuca* and *C. fusca* (6 g VS each) were mixed in a 225-ml serum bottle at various ratios. The working volume was adjusted to 100 ml after adding MgCl₂·6H₂O (0.1 g/l), K₂HPO₄·3H₂O (6.67 g/l), NaHCO₃ (2.6 g/l), and FeCl₂·4H₂O (0.002 g/l). The 12 *C. fusca*: *U. lactuca* ratios by g VS were 6 : 0, 0 : 6, 3 : 3, 4 : 2, 4.5 : 1.5, 4 : 8, 1.2,

5 : 1, 2 : 4, 1.5 : 4.5, 1.2 : 4.8, 1 : 5, and (3 : 3) (Table 4.2). Groups 1 and 2 (6 : 0 and 0 : 6 ratios, respectively) represented the controls for each feedstock, while group 12 (3 : 3 ratio) served as a control without mineral supplementation. The experiment was performed in triplicate and the results are expressed as average \pm standard deviation. Cultures were placed in a 120-rpm shaking water bath at 30°C for 48 h, with the initial pH adjusted to 8.00 with 1 M NaOH and 1 M HCl, and were sterilized in an autoclave at 121°C for 15 min (2 atm) before starting the fermentative process. The initial inoculum was obtained at an OD₆₂₀ of 0.1 in the final exponential phase. Bottles were sealed with rubber stoppers and purged with N₂ to maintain anaerobic conditions.

Biogas volume was measured every 6 h by releasing the pressure in the bottle using wetted glass syringes (50–100 ml) (Owen et al., 1979). The composition of the liquid phase was determined at the end of the fermentation process. HY (ml H₂/g VS alga) was calculated by dividing the total volume of hydrogen produced by the amount of algal biomass used as fermentation substrate in terms of its VS content.

The most favorable algal blend ratio obtained was optimized by including the following minerals to enhance final yield: FeCl₂·4H₂O (0, 0.001, 0.002, and 0.004 g/l), NaHCO₃ (0, 0.87, 1.73, 2.6, and 3.47 g/l), MgCl₂·6H₂O (0, 0.1, 0.2, and 0.4 g/l), and K₂HPO₄·3H₂O (0, 1.6, 3.33, and 6.67) g/l. The potential for reducing CAA supplementation (392-00655; Wako Chemical Industries) was evaluated by mixing algal waste at specific ratios with CAA (3.33, 6.66, 13.33, and 26.67 g/l).

4.2.4 Analysis of chemical composition

The biogas was collected directly from the headspace of the serum bottles using a gas-tight syringe. The gaseous phase was analyzed by gas chromatography (GC-8APT; Shimadzu) with a thermal conductivity detector and a Porapak Q stainless-steel column packed with an activated

carbon 60/80 column (1.5 m × 3.0 mm internal diameter). Argon was used as the carrier gas at a flow rate of 6.5 ml/min. Operation temperatures for the injector, column, and detector were 50°C, 60°C, and 50°C, respectively. Volatile fatty acid (VFA) concentration in the liquid phase was determined by gas chromatography with a flame ionization detector (FID) and 8-foot glass column packed with 10% polyethylene glycol-20M and 2% H₃PO₄ (80/100 mesh). The temperatures of the injection port, FID detector, and oven were 250°C, 140°C, and 140°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 20 ml/min. Ethanol concentration was determined by high-performance liquid chromatography (LC_10AD equipped with a Shim pack SPR-PB column; Shimadzu); the oven temperature was 80°C, and deionized water was used as the mobile phase at a flow rate of 0.6 ml/min with a refraction index detector. Ammonia level was detected at the end of the fermentation process according to 4500-NH₃ standard methods (APHA, 2005). The analytical methods and kinetic modeling were did as mentioned before in chapter III.

4.3 Results and discussion

4.3.1 Determination of *C. fusca* and *U. lactuca* ratio for optimal HY

Twelve co-fermentation groups with different blend ratios of *C. fusca* and *U. lactuca* waste biomass were evaluated to determine the ratio for maximal HY (Fig. 4.1). The duration of the fermentation reaction was 48 h. HY was in the range of 22–90 ml H₂/g VS. The highest yields were for groups 6 and 7; however, ammonia concentration was also higher for group 7 (Table 4.2). The control groups had low yield: for group 1, the yield was only 53 ml H₂/g VS, likely due to an insufficient amount of free amino acids (nitrogen source) for hydrogen production by HPB. This is supported by a previous study reporting that high C/N ratios in some algae led to VFA accumulation and depleted the nitrogen available for fermentative bacteria (Xia et al., 2016). The low ammonia concentration in group 1 also indicated that there was no surplus nitrogen in the form of free amino acids. The HY for group 2 was even lower (22 ml H₂/g VS), whereas ammonia concentration was high (164 mg/l), reflecting an abundance of nitrogen in excess of bacterial needs. Low C/N ratios in some algal species can lead to a high ammonia concentration, which inhibits fermentation (Allen et al., 2013). Accordingly, blending the two types of waste biomass increased HY, since nitrogen depletion by one type of waste could be compensated by the other. A comparison of groups 3 and 12 revealed that mineral supplementation is important for hydrogen production. Mineral depletion resulted in a delay in lag time by 50%, leading to a lag time of 20 h, and the overall yield decreased 20% (35 ml H₂/g VS). The main VFAs produced were acetic acid and butyric acid (Table 2), as expected for *C. butyricum*; this resulted in acidification of the culture. The *C. fusca* : *U. lactuca* ratio of group 6 (4: 1), which had an HY of 86 ml H₂/g VS and lag time of 2 h, was selected for further experiments.

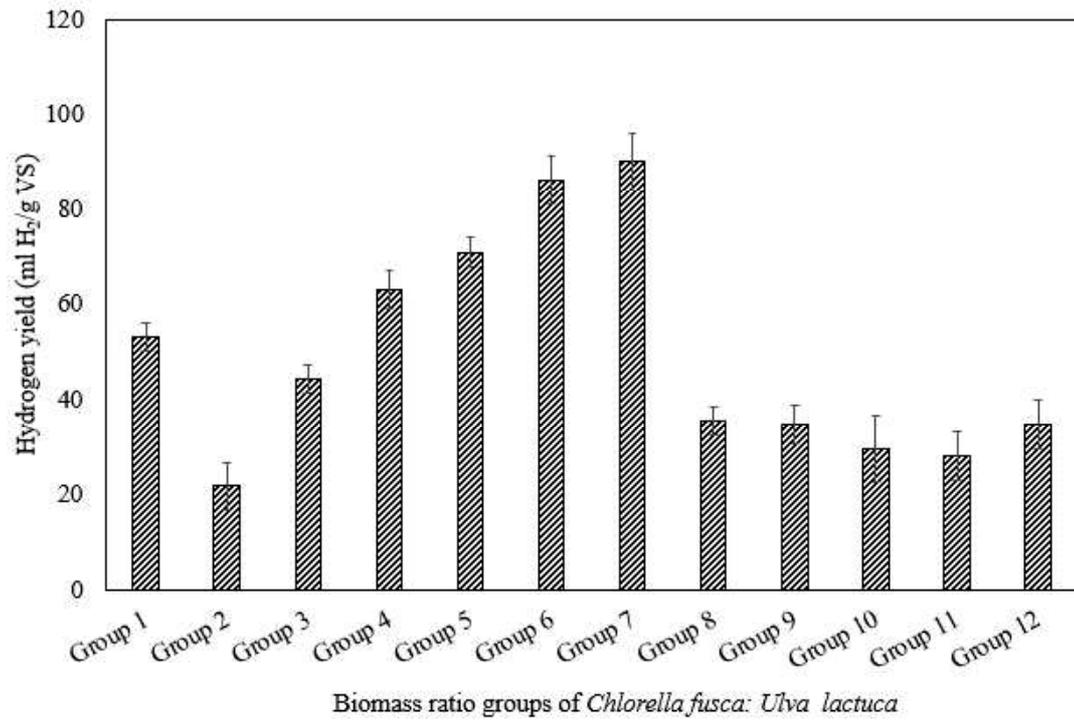


Fig. 4. 1 Effect of different *C. fusca* and *U. lactuca* biomass blend ratios on HY by *C. butyricum* RAK25832

Table 4. 1 Characteristics of macro -algal biomass (*U. lactuca*) and micro algae (*C. fusca*)

	Algal biomass	
	<i>C. fusca</i>	<i>U. lactuca</i>
TS (wwt %)	93.34	95.44
VS (wwt %)	94.60	82.18
Moisture (wwt %)	6.66	4.56
Ash (fixed solids; % TS)	5.40	17.82
C (%T)	47.48	35.36
H (%T)	6.83	5.20
N (%T)	8.76	3.95
O (%T)	31.53	37.67
C/N ratio	5.42	8.95
Proteins (% VS)	42.52	22.58
Lipids (% VS)	20.40	1.00

TS, total solid; VS, volatile solid.

Table 4. 2 Kinetics parameters of hydrogen production by *Clostridium butyricum* RAK25832

using different ratio of waste biomass micro-algae (*C. fusca*) and macro-algal biomass (*U. lactuca*).

Group	Biomass Ratio		Kinetics parameters estimated by the modified Gompertz model						VFA (mg-COD/L)				
	<i>C.fusca</i>	<i>U.lactuca</i>	Final pH	NH ₄ -N (mg/L)	P (ml)/100ml	Hydrogen rate R _m	λ (hr)	R ²	HAc	HPr	I-HBu	N-HBu	EtOH
1	1:0	6:0	4.51	8.00	281	16	15	0.9502	223	0	0	725	10
2	0:1	0:6	4.33	164.67	102	6	6	0.9858	122	0	0	677	6
3	1:1	3:3	4.23	134.06	222	20	10	0.9652	220	0	0	698	15
4	2:1	4:2	4.33	159.16	327	22	9	0.9826	340	0	0	822	22
5	3:1	4.5:1.5	4.33	74.07	365	20	2	0.9788	350	0	0	835	23
6	4:1	4.8:1.2	4.25	19.00	446	37	2	0.9988	450	0	0	1135	25
7	5:1	5:1	4.13	25.56	468	39	2	0.9455	460	0	0	1132	23
8	1:2	2:4	4.22	120.59	174	9	3	0.9669	166	0	0	689	10
9	1:3	1.5:4.5	4.25	132.10	169	8	3	0.9563	161	0	0	683	11
10	1:4	1.2:4.8	4.25	135.28	143	7	2	0.9345	158	0	0	679	10
11	1:5	1:5	4.32	140.80	135	7	1	0.9340	152	0	0	677	8
12	(1:1)	(3:3)	4.43	78.97	174	10	20	0.9819	165	0	0	690	8

EtOH, ethanol; HAc, acetic acid; HPr, propionic acid; I-HBu, isobutyric acid; NH₄-N,

ammonium nitrogen content; N-HBu, butyric acid; R², Correlation coefficient; VFA, volatile

fatty acid.

4.3.2 Yield optimization with mineral supplementation

As mentioned earlier, only four minerals are essential for hydrogen production by *C. butyricum* RAK25832. The optimal concentrations of these minerals were determined as follows: $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1 g/l), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (6.67 g/l), NaHCO_3 (2.6 g/l), and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.002 g/l).

4.3.2.1 Optimizing $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ concentration

Iron is the most important nutrient for hydrogen gas production as it is utilized for the formation of hydrogenase and other enzymes (Lee et al., 2001; Oztekin et al., 2008). $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ was tested at concentrations of 0, 0.001, 0.002, and 0.004 g/l. Eliminating $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ decreased HY by 55%. Increasing the concentration above 0.002 g/l had no effect, whereas a concentration of 0.002 g/l had a yield 86 ml H_2 /g VS and a rate 38 ml H_2 /h. On the other hand, lag time was not greatly affected by $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ concentration (Fig. 4.2A), while VFA was unaffected by increases in $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ concentration beyond 0.002 g/l (Fig. 4.3A).

4.3.2.2 Optimizing NaHCO_3 concentration

NaHCO_3 concentrations in the range of 0–3.47 g/l were tested (Fig. 4.2B). Complete elimination increased lag time to 21 h and decreased yield to 41% (51 ml H_2 /g VS). The optimal concentration was 1.73 g/l, which yielded 98 ml H_2 /g VS, increased the rate to 39 ml H_2 /h, and reduced lag time to 2 h. Further increases in the concentration decreased hydrogen production. VFA production increased with concentration up to 1.73 g/l, with a slight decrease observed at higher concentrations (Fig. 4.3B). NaHCO_3 is commonly used as a buffer in the culture medium to neutralize acidic reaction by-products (Ai et al., 2014; Lee et al., 2004). A NaHCO_3 concentration of 0.124% (w/v) was previously reported as the optimal concentration for *C. butyricum* cultures (He et al., 2004).

4.3.2.3 Optimizing MgCl₂·6H₂O concentration

The effect of MgCl₂·6H₂O was examined at concentrations of 0, 0.1, 0.2, and 0.4 g/l (Fig. 4.2C). In the absence of MgCl₂·6H₂O, the yield decreased by 28%, whereas concentrations above 0.1 g/l did not increase yield beyond 87 ml H₂/g VS with a rate 37 ml H₂/h. VFA production caused the culture to become acidified (Fig. 4.3C). In addition, VFA was not affected by increasing MgCl₂·6H₂O concentration. Microorganisms mainly require Mg²⁺ for ribosome activity, as a cell membrane and cell wall constituent, and as an enzyme cofactor. Thus, a decrease in Mg²⁺ concentration reduced cell growth and ultimately, hydrogen production (Lin & Lay, 2005; Wang et al., 2007).

4.3.2.4 Optimizing K₂HPO₄·3H₂O concentration

The presence of P can improve microbial functions, including enzymatic activity and cell growth (Jo et al., 2008; Lin & Lay, 2004). The effect of K₂HPO₄·3H₂O was examined at concentrations of 0, 1.6, 3.33, and 6.67 g/l (Fig. 4.2D). Eliminating K₂HPO₄·3H₂O decreased HY by 25%. The optimal concentration was 6.67 g/l, with a yield 87 ml H₂/g VS and a rate of 39 ml H₂/h. HY and rates are reported to be low in dark fermentations due to VFA accumulation (Liu & Shen, 2004; Yokoi et al., 2001), which was observed at the end of the fermentation (Fig. 3D). VFA levels and lag time were unaffected by increasing concentrations of K₂HPO₄·3H₂O.

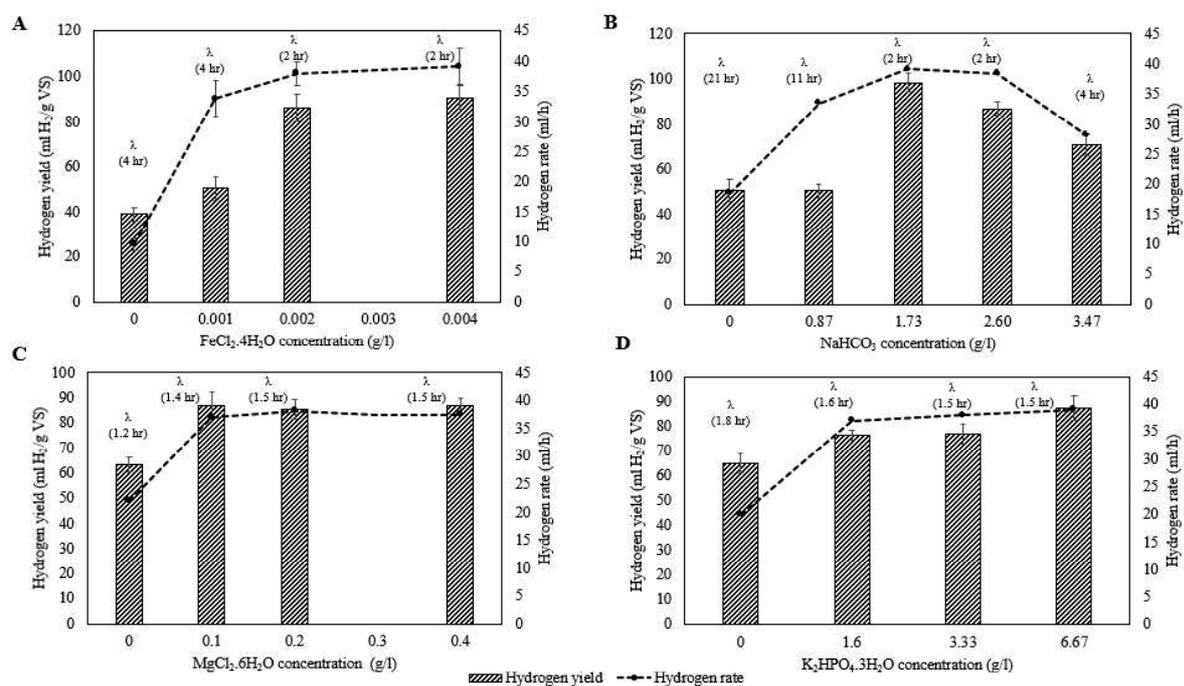


Fig. 4. 2 Effect of different concentrations of (A) $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, (B) NaHCO_3 , (C) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and (D) $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ with algal waste biomass ratio of *C. fusca*: *U. lactuca* (4:1) on the hydrogen yield and hydrogen production rate by *C. butyricum* RAK25832.

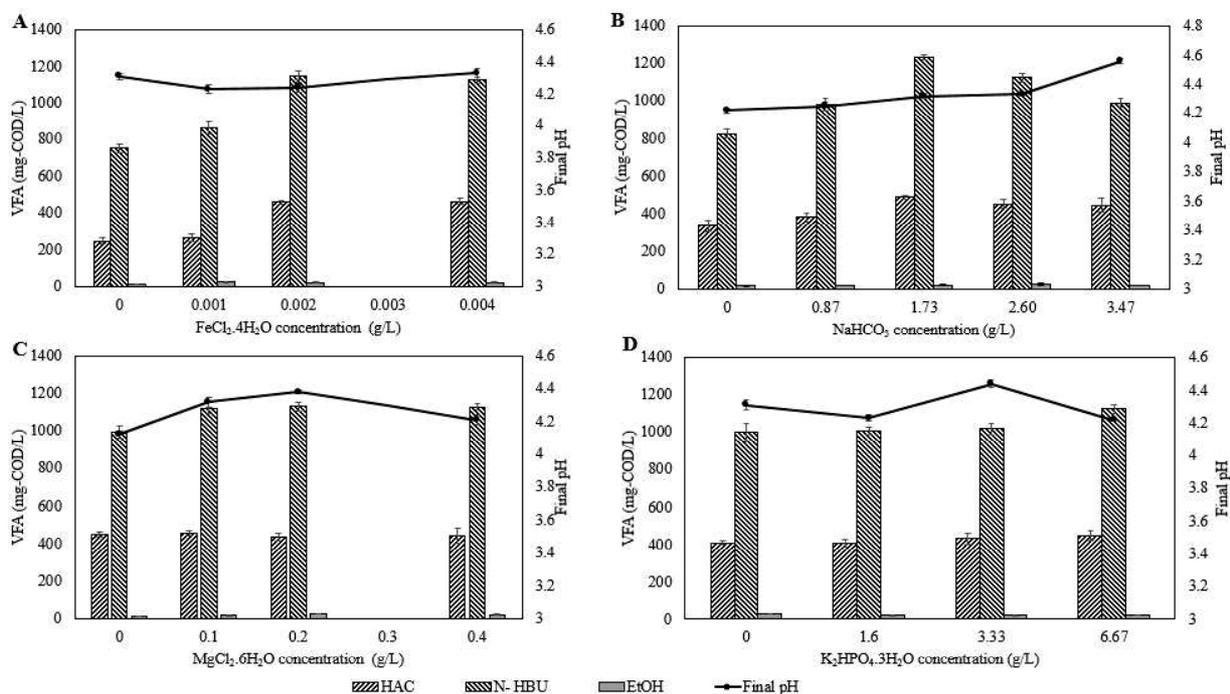


Fig. 4.3 Volatile fatty acids associated with different concentrations of (A) $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, (B) NaHCO_3 , (C) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and (D) $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ with algal waste biomass ratio of *C. fusca*: *U. lactuca* (4:1) compared to the final pH.

4.3.3 Effect of combining CAAs with algal biomass on HY

4.3.3.1 CAAs combined with a blend of *C. fusca* and *U. lactuca*

The effect of combining CAAs with algal biomass (group 6, *C. fusca* : *U. lactuca* ratio of 4 : 1) as a nitrogen source were examined on hydrogen production by *C. butyricum* RAK25832. Cultures were also supplemented with optimal concentrations of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1 g/l), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (6.67 g/l), NaHCO_3 (1.73 g/l), and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.002 g/l) (Fig. 4.4). CAAs concentrations were 0, 1.67, 3.33, 6.67, 13.33, and 26.67 g/l. It was found that CAAs had a significant effect on hydrogen production: a concentration of 3.33 g/l increased the yield from 107 to 117 ml H_2 /g VS, possibly by providing HPB with the remaining amino acids required for

fermentation. Acetic acid and butyric acid were the main VFAs produced by fermentation (Fig. 4.5), resulting in acidification of the culture.

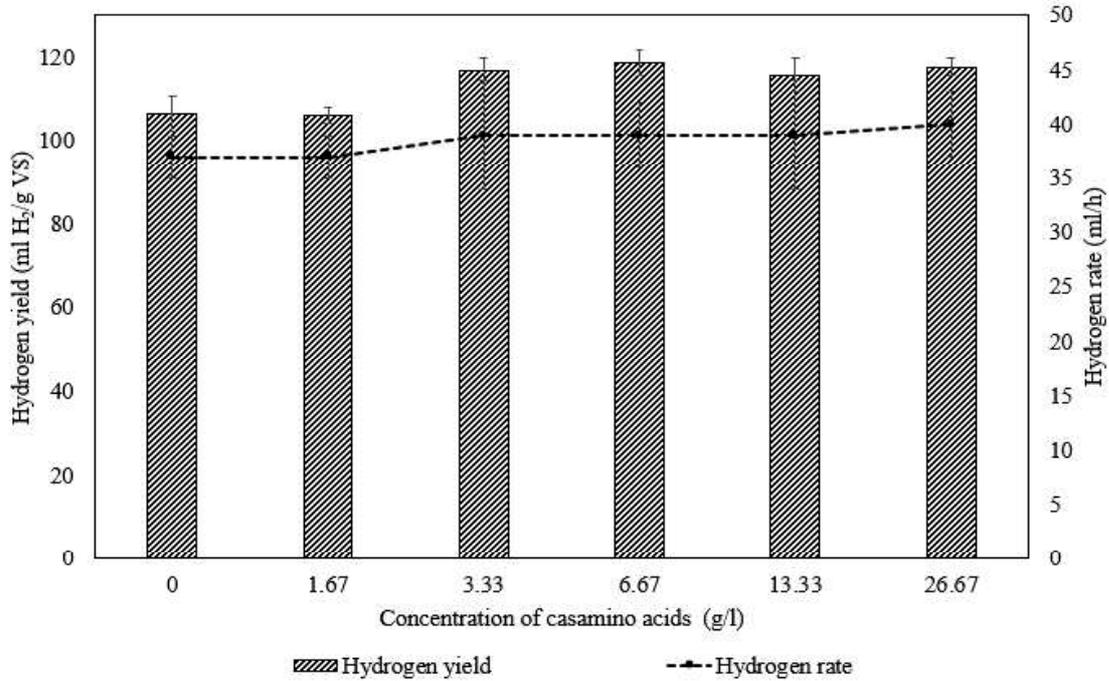


Fig. 4. 4 Effect of different concentrations of CAAs combined with *C. fusca* and *U. lactuca* biomass at a ratio of 4 : 1 on HY and hydrogen production rate by *C. butyricum* RAK25832

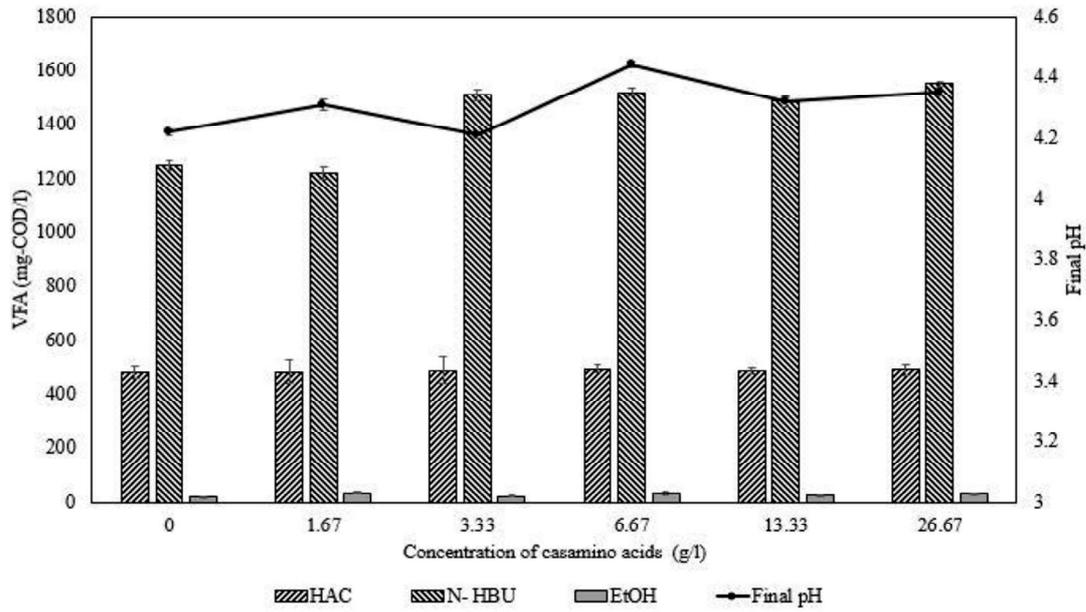


Fig. 4. 5 Volatile fatty acids associated with different casamino acids concentration combined with algal waste biomass ratio of *C. fusca*: *U. lactuca* (4:1) compared to the final pH

4.3.3.2 CAAs combined with a single type of algal biomass

A maximum HY of 111 ml H₂/g VS—representing a 50% increase—was achieved with a CAAs concentration of 13.33 g/l in the presence of *C. fusca* (Fig. 4.6A). It was speculated that a similarly high yield could be achieved by including waste algae biomass and thereby decreasing the amount of CAAs required. A CAAs concentration of 6.67 g/l was required to obtain a maximum yield of 94 ml H₂/g VS in the presence of *U. lactuca* (Fig. 4.6B), which was higher than the concentration required when using a mixture of the two types of algal biomass.

The fact that a lower amount of CAAs was required in the presence of *U. lactuca* as compared with that in the presence of *C. fusca* may be related to the higher free amino acids concentration in the former (Table 4. 3). The amino acid requirements of HPB were met by using the algal blend biomass, although CAAs compensated for the low levels of some amino acids. Thus, blending the two types of algal waste biomass could reduce the amount of CAAs required for fermentation and thereby lower the cost of hydrogen production by dark fermentation.

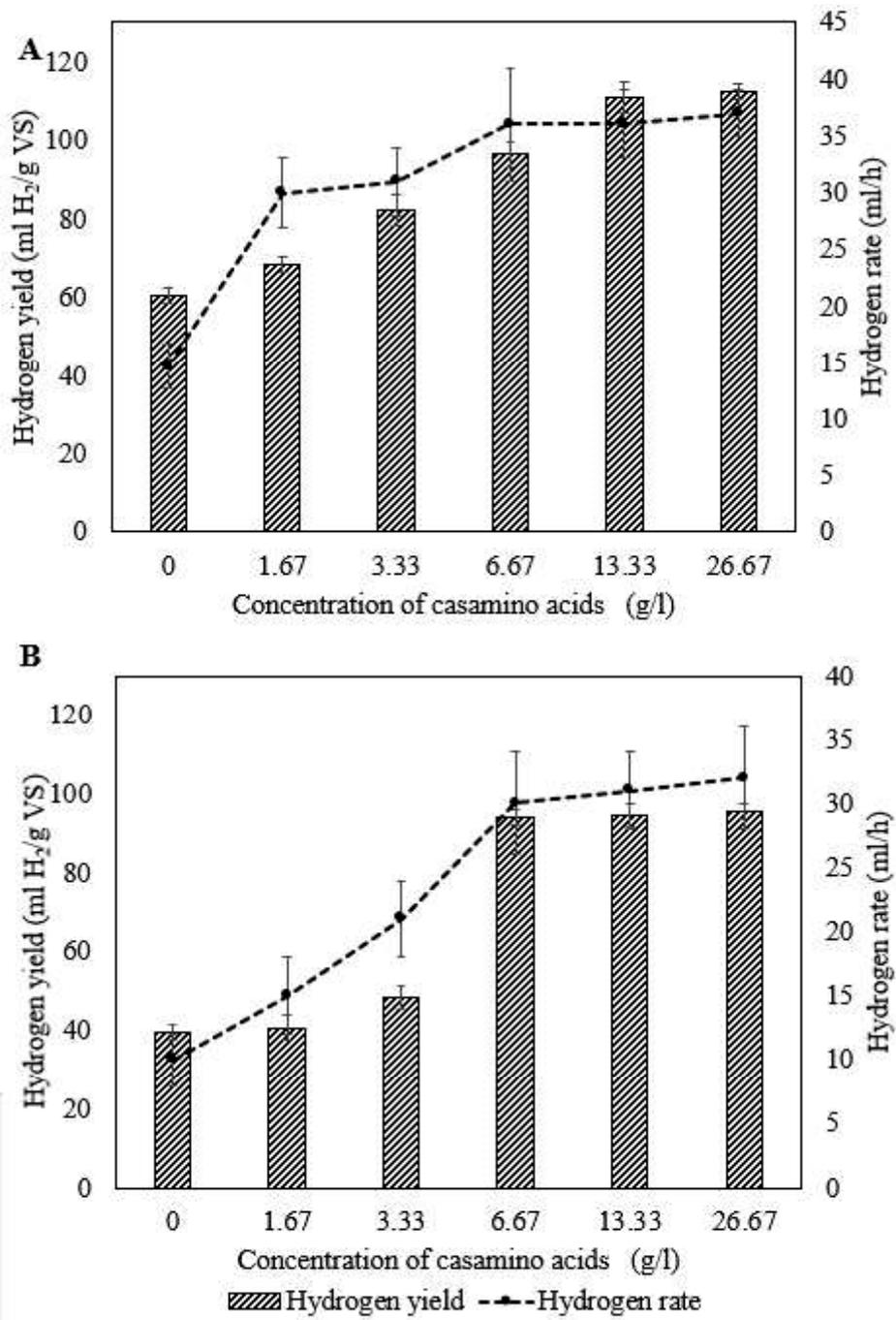


Fig. 4. 6 Effect of different concentrations of CAAs combined with A) *C. fusca* and B) *U. lactuca* on HY and hydrogen production rate by *C. butyricum* RAK25832

Table 4. 3 Analysis of amino acid composition in *C. fusca* and *U. lactuca*

Amino acid	<i>C. fusca</i> (mg/g)	<i>U. lactuca</i> (mg/g)
Asp	0.196	0.199
Thr	0.490	0.199
Ser	0.294	0.299
Asn	0.294	0.249
Glu	0.735	1.643
Gln	0.147	1.444
Gly	0.245	0.598
Ala	2.645	0.747
Cit	0.049	0.149
Val	0.294	0.498
Ile	0.147	0.349
Leu	0.245	0.349
Tyr	0.147	0.249
Phe	0.196	0.199
His	0.098	0.149
Lys	1.029	0.598
Arg	0.441	1.145
Pro	0.931	0.647

4.4 Conclusion

The present study investigated the possibility of reducing the requirement for CAAs—an effective but costly nitrogen source—in biohydrogen fermentation by *C. butyricum* RAK25832-mediated dark fermentation via the introduction of an algal waste biomass blend. A *C. fusca*: *U. lactuca* ratio of 4 : 1 reduced the amount of CAAs required to 3.33 g/l. The deficiency in metal ions in algal waste was partly mitigated by mineral supplementation. Based on these results, the use of more than one nitrogen source to meet the nitrogen requirements of dark fermentation by *C. butyricum* RAK25832 is recommended for maximal HY.

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CHAPTER V:

Conclusions

Overall data results conclusion

In this research it conduct a basic thinking for biohydrogen future production in effective way and low cost. Data obtained during this research showed:

- *C. butyricum* RAK2583 showed good hydrogen production with various carbon sources.
- *C. butyricum* RAK2583 prefer glucose, fructose, xylose, sucrose, and lactose sugars.
- Casamino acids is suitable as a nitrogen source for microbial hydrogen production.
- The best hydrogen production was obtained with 26.67 g/L casamino acid with a cumulative hydrogen production, production rate, and yield of 2505 mL H₂/L, 160 mL/h, and 1.81 mol H₂/mol glucose, respectively.
- The fermentation medium for enhanced hydrogen production was studied and found that optimal medium composition was MgCl₂.6H₂O (0.1 g/L), K₂HPO₄.3H₂O (6.67 g/L), NaHCO₃ (2.6 g/L), and FeCl₂.4H₂O (0.002 g/L).
- Vitamin supplementation of the medium showed no significant effect on hydrogen production
- Under the optimized conditions, cumulative hydrogen production reached 3074 mL H₂/L. This is the first study to demonstrate the use of casamino acids as a nitrogen source by *C. butyricum*.
- Using blended algal waste could reduce the supplement of casamino acids as they are rich with amino acids.

- Algal waste poor in mineral elements and they required supplementation with FeCl_2 , NaHCO_3 , MgCl_2 , and K_2HPO_4 .
- Blended feedstocks from different sources will reduce additional supplements of nutrients, resulting in cost reduction and effective energy production.

Supplementary materials

Table S 1. Nutrient composition.

Nutrient	Final concentration (g/L)
NH ₄ Cl	1
NaCl	0.1
MgCl ₂ ·6H ₂ O	0.1
CaCl ₂ ·2H ₂ O	0.05
K ₂ HPO ₄ ·3H ₂ O	0.4
NaHCO ₃	2.6
FeCl ₂ ·4H ₂ O	0.002
H ₃ BO ₃	0.00005
ZnCl ₂	0.00005
CuCl ₂ ·2H ₂ O	0.000038
MnCl ₂ ·4H ₂ O	0.00005
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.00005
AlCl ₃	0.00005
CoCl ₂ ·6H ₂ O	0.00005
NiCl ₂ ·6H ₂ O	0.000092
Ethylenediaminetetraacetate	0.0005
Concentrated HCl	0.001 mL
Na ₂ SeO ₃ ·5H ₂ O	0.0001
Yeast extract	0.1

Table S 2. Composition of the supplemented vitamins.

Components	Provider	weight/L (μg)
Biotin	Wako	2
d-Pantothenic acid Ca	Wako	400
Folic acid	Wako	2
Inositol	Katayama	2000
Niacin	Wako	400
p-Aminobenzoic acid	Katayama	200
Pyridoxine HCl	Wako	400
Riboflavin	Sigma	200
Thiamine HCl	Wako	400