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THITIWUT VONGKAMPANG | APPLIED MICROBIOLOGY | LUND UNIVERSITY

Mer de Glace is the largest glacier in France's alpine, covering 30.4 sq. km with the length of 11.5 km and the thickness of 200 m. The glacier is located in the north of Mont Blanc at an altitude of 1,913 m above sea level. In 1988, it only took three walking steps to get to the ice grotto. However, in 2019, you must walk down at least 580 steps (115 m) to reach the ice grotto. This effect is one of the signs indicating that global warming is happening and threatening the alpine ecosystem. Moreover, it is predicted that the glacier's snout may shrink by approximately 1.2 km in 2040. If we continuously release a massive amount of greenhouse gases, Mer de Glace will be gone by the end of this century or earlier than that in the worst-case scenario.

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Exploring strategies to improve volumetric hydrogen productivities of *Caldicellulosiruptor* species

Thitiwut Vongkampang

DOCTORAL DISSERTATION

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Abstract
Ongoing consumption of fossil-based fuels generates a massive amount of greenhouse gases. This may lead to global warming that is currently threatening human society and wild animal habitats. Hydrogen is an energy carrier with the highest energy content per weight compared to other all fuels and no carbon dioxide is released when combusted. Thermophilic bacteria belonging to the genus of *Caldicellulosiruptor* have the ability to produce hydrogen from an array of substrates such as poly-, oligo-, di-, and monosaccharides, including lignocellulosic material. *Caldicellulosiruptor* species have the capacity to produce hydrogen at nearly the maximum theoretical yield of 4 $mol \cdot mol^{-1}$ hexose.

In this work, pure and co-cultures of *Caldicellulosiruptor* species degraded and fermented heat-treated wheat straw. The outcome indicated that the performance of *C. kronotskyensis* is superior and it is thus promising candidate for utilizing wheat straw through consolidated bioprocessing. Therefore, the physiology of *C. kronotskyensis* was further investigated using defined media containing glucose and xylose mixtures corresponding to the sugar ratio present in wheat straw hydrolysate. Interestingly, growth of *C. kronotskyensis* did not possess a diauxic-like growth pattern during its growth on glucose and xylose mixtures like was observed with *C. saccharolyticus*. This phenomenon was determined by both the volumetric productivity profile of hydrogen (Q_{H2}) and carbon dioxide (Q_{CO2}). The maximum growth rate (μ_{max}) of *C. kronotskyensis* on xylose was 0.57 h^{-1} which is twice the μ_{max} on glucose (0.28 h^{-1}). *C. kronotskyensis* was grown on sugar mixtures i.e. xylose-cellobiose and glucose-cellobiose. The uptake of xylose and cellobiose occurred concurrently. However, for glucose and cellobiose mixtures, *C. kronotskyensis* consumed cellobiose faster than glucose. These results indicated that *C. kronotskyensis* has adapted to pentoses and oligosaccharides.

Cell immobilization and co-cultures offered a promising technique for retaining cells in the system. During this work, chitosan and rubber were used as a carrier to retain biomass, thereby improving volumetric hydrogen productivity (Q_{H2}). Chitosan exhibited the property to retain *C. saccharolyticus* and *C. owensensis* but did not improve the Q_{H2}. Acrylic fibres filled in a homemade stainless-steel cage was introduced in continuous stirred tank reactors (CSTR). Notably, the highest Q_{H2} obtained was 30 ± 0.2 $mmol \cdot L^{-1} \cdot h^{-1}$ at a dilution rate (*D*) of 0.3 h^{-1} with a pure culture of *C. kronotskyensis* with acrylic fibres and chitosan. In the co-culture of *C. kronotskyensis* and *C. owensensis* with acrylic fibres, the population dynamics indicated that *C. kronotskyensis* was the dominant species in the biofilm fraction, whereas *C. owensensis* was the dominant in the planktonic phase. Bis-(3',5')-cyclic di-guanosine-mono-phosphate (c-di-GMP) is an intracellular messenger correlated with planktonic and biofilm lifestyle. *C. owensensis* is a high producer of c-di-GMP, while *C. kronotskyensis* produced less during its fermentations. In this study, a co-culture of *C. kronotskyensis* and *C. owensensis* without carrier obtained the highest concentration of c-di-GMP at 260 ± 27.3 µM.

In conclusion, this study revealed that immobilization of *Caldicellulosiruptor* species improved the Q_{H2}. Secondly, it revealed the superior performance of *C. kronotskyensis* in relation to consolidated bioprocessing, biofilm formation and Q_{H2}. Therefore, it is recommended to carry out more research with *C. kronotskyensis* to pursue a breakthrough in cost-effective hydrogen production.

Key words *Caldicellulosiruptor kronotskyensis*, volumetric hydrogen productivity, non-diauxic, acrylic fibres, cell immobilization, chitosan, biofilm, c-di-GMP, cellobiose, xylose, consolidated bioprocessing, heat-treated wheat straw

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MADE IN SWEDEN

To my parents

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Popular science summary

The rapid melting of giant glaciers in the Arctic and Antarctica has raised the awareness of global warming. Climate change is currently impacting our society, including a decline in agriculture production due to long-term drought, insufficient water supply, and extinction of wild animals. Indeed, the cause of these effects is partly the massive emission of greenhouse gases from fossil-based fuels. To mitigate these concerns, many international organizations have issued policies focusing on renewable energy that can be substituted the use of petroleum-based fuels.

Hydrogen is the cleanest energy carrier as it does not add carbon dioxide into the atmosphere when combusted, and only water is the by-product. Besides, hydrogen has the highest energy content among all fuels. The hydrogen demand is increasing each year, and to date, most hydrogen is produced from non-renewable resources. Therefore, in this thesis, I studied a microorganism that can be used for hydrogen production via a biological process, which is eco-friendly and does not rely on fossil-based feedstocks.

Caldicellulosiruptor species are hydrogen-producing bacteria that can grow on various substrates, including lignocellulosic materials such as wheat straw. Wheat straw is considered as agricultural waste after the harvesting season. This study was initiated by screening the most promising *Caldicellulosiruptor* species utilizing wheat straw material, of which *Caldicellulosiruptor kronotskyensis* is the most interesting. Later in this study, this species was investigated for its growth pattern on different sugars and kinetic studies related to sugar transporters. Interestingly, *C. kronotskyensis* prefers to take up glucose in the form of cellobiose (two glucose molecules), which is found in various types of lignocellulosic materials. From my studies, I propose that *C. kronotskyensis* is a promising candidate for hydrogen production.

This study has also focused on the co-culture technique, which is the cultivation of two bacterial species or more in one system. The co-culture strategy has got attention due to its improved hydrogen productivity. Moreover, two carriers, chitosan, and rubber were introduced for retaining bacteria cells. However, rubber did not retain cells of *C. saccharolyticus* and *C. owensensis*, whereas chitosan displayed promising properties. Although chitosan could maintain bacterial cells in the bioreactor, it could not enhance volumetric hydrogen productivities (Q_{H2}).

To accomplish higher volumetric hydrogen productivities, I used acrylic fibres as a carrier material enclosed in a homemade stainless-steel cage and installed inside the bioreactor. By doing this way, the volumetric hydrogen productivity could be enhanced by nearly four-fold by a culture of *C. kronotskyensis* together with chitosan and acrylic fibres. Therefore, I would like to remark that immobilized strategies can be used to improve the volumetric hydrogen productivity, but the hydrogen production still requires further research and development to achieve the productivity required at an industrial scale.

Abstract

Ongoing consumption of fossil-based fuels generates a massive amount of greenhouse gases. This may lead to global warming that is currently threatening human society and wild animal habitats. Hydrogen is an energy carrier with the highest energy content per weight compared to other all fuels and no carbon dioxide is released when combusted. Thermophilic bacteria belonging to the genus of *Caldicellulosiruptor* have the ability to produce hydrogen from an array of substrates such as poly-, oligo-, di-, and monosaccharides, including lignocellulosic material. *Caldicellulosiruptor* species have the capacity to produce hydrogen at nearly the maximum theoretical yield of 4 $mol{\cdot}mol^{-1}$ hexose.

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List of publication

This thesis is based on the following publications and manuscripts, which will be referred by Roman numerals:

I. **Consolidated bioprocessing of *Caldicellulosiruptor* species utilizing wheat straw**

Vongkampang, T., Novy, V., Nubong Pride Afah, N., van Niel, EWJ. *Manuscript*

II. **Characterization of simultaneous uptake of xylose and glucose in *Caldicellulosiruptor kronotskyensis* for optimal hydrogen production**

Vongkampang, T., Sreenivas, K., Engvall, J., Grey, C., van Niel, EWJ. *Submitted, revised and under review*

III. **Chitosan flocculation associated with biofilms of *C. saccharolyticus* and *C. owensensis* enhances biomass retention in a CSTR**

Vongkampang, T., Rao, NS., Grey, C., van Niel, EWJ. *Submitted, revised and under review*

IV. **Immobilization techniques improve volumetric hydrogen productivity of *Caldicellulosiruptor* species in a modified continuous stirred tank reactor**

Vongkampang, T., Sreenivas, K., Grey, C., van Niel, EWJ. *Manuscript*

I have also contributed to the following article:

A. **Biofilm formation by designed co-cultures of *Caldicellulosiruptor* species as a means to improve hydrogen productivity**

Pawar, S.S., Vongkumpeang, T., Grey, C., and van Niel, E.W. 2015, Biotechnology for Biofuels, 8 (1), 19.

My contributions to the papers

I. I and Dr. Ed van Niel planned and designed the experiments together. I performed the fermentations in the bioreactor. I trained a master student, Neba Nubong Pride Afah, who conducted batch cultivations and metabolites analysis under my supervision. I supervised a master student, Marc-Kilian Dullin, who performed hydrophobicity experiments. I drafted the entire manuscript.

II. I conceived the idea based on the results in paper I. I and Dr. Ed van Niel planned and designed the fermentation conditions. I performed all the fermentations together with a master student, Krishnan Sreenivas, and a bachelor student, Jonathan Engvall, who conducted batch cultivations and metabolites analysis under my supervision. I conducted the kinetic calculation along with Dr. Carl Grey. I drafted the entire manuscript.

III. I generated the idea, planned and designed continuous fermentations. I prepared chitosan solution with the help of Dr. Carl Grey. I trained a master student, Nikhil Seshagiri Rao, who conducted continuous cultivations and metabolites analysis under my supervision. I drafted the entire manuscript.

IV. I and Dr. Ed van Niel planned and designed the experiments together. I trained a master student, Krishnan Sreenivas, who conducted continuous cultivations, metabolites analysis, primer design and population dynamics analysis under my supervision. I and Krishnan Sreenivas prepared C-di-GMP samples. I conducted the quantification of C-di-GMP along with Dr. Carl Grey. I drafted the entire manuscript.

Abbreviations

H_2	Hydrogen
Q_{H2}	Volumetric hydrogen productivity
ABC	ATP-binding cassette
GHs	Glycoside hydrolases
CBMs	Carbohydrate binding modules
D	Dilution rate
q_{Glu}	Glucose consumption rate
q_{Xyl}	Xylose consumption rate
K_I	Inhibition constant
$K_{I,glu}$	Inhibition constant of glucose
$K_{I,xyl}$	Inhibition constant of xylose
DD	Degree of deacetylation
Mw	Molecular weight
c-di-GMP	Bis-(3',5')-cyclic di-guanosine monophosphate
PCR	Polymerase chain reaction

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1. Introduction

"*Climate change knows no borders. It will not stop before the Pacific Islands and the whole of the international community here has to shoulder a responsibility to bring about sustainable development*."

Angela Merkel, Chancellor of Germany, G20 summit's final communique 2014.

1.1 Global warning – causes and actions

Fossil-based fuels have been used for several decades to support the requirements for the expansion of urbanization, transportation, and the industrial area for boosting up economic growth (Seelam et al., 2020). Within these energy consumptions, massive greenhouse gases (GHGs) i.e. carbon dioxide (CO_2), sulfur dioxide (SO_2) from fossil fuels and methane (CH_4) from agricultural wastes, are continuously emitted into the atmosphere (Gupta et al., 2016, Singh and Das, 2019). Therefore, one of the unavoidable impacts is the increase of the average global temperature, thereby contributing to climate change. Renewable energy resources such as biofuels offer a promising solution for dealing with such environmental problems (Singh and Das, 2019).

According to the Paris Agreement in 2015, the action on reducing the emission of greenhouse gases (GHGs) is executed, aiming to keep the increase of the global temperature below 2℃ since it raised at the dawn of the pre-industrial period (1850-1900) (United Nations, 2015). For other actions, the International Energy Agency (IEA) announced the policy for using low carbon-footprint resources for the hydrogen production platforms. The future perspective for the roadmap in 2030 is aimed towards building hydrogen refilling stations for hydrogen cars and scaling up the uses of hydrogen for industrial sectors i.e. hydrogen fuel for logistics (IEA, 2020). In addition, the European Commission has issued the policy to accomplish carbon neutrality by 2050 and focused on sharing approximately 24% of global energy demand with clean hydrogen technologies (European Commission, 2020).

1.2 What is hydrogen?

Elemental hydrogen is the simplest atomic structure comprising of one proton and one electron (Morrison, 2021). For chemical properties, it is odourless, tasteless, and colourless. Moreover, it is the most abundant element in the universe and is the first element in the periodic table. Hydrogen gas (H_2) gains more interest since it does not emit any CO_2 during combustion, with only a water molecule is produced. Moreover, a gravimetric energy content (~122kJ/g) of hydrogen is almost three-fold higher than hydrocarbon fuels (Venkata Mohan and Pandey, 2019).

1.3 H_2 production - a current status

As depicted in Figure 1, the H_2 production gradually increase to supply the global market. In 2018, approximately 70 million metric tonnes of H_2 is produced annually from fossil resources (> 95%) and is predicted to be increased in forthcoming year.

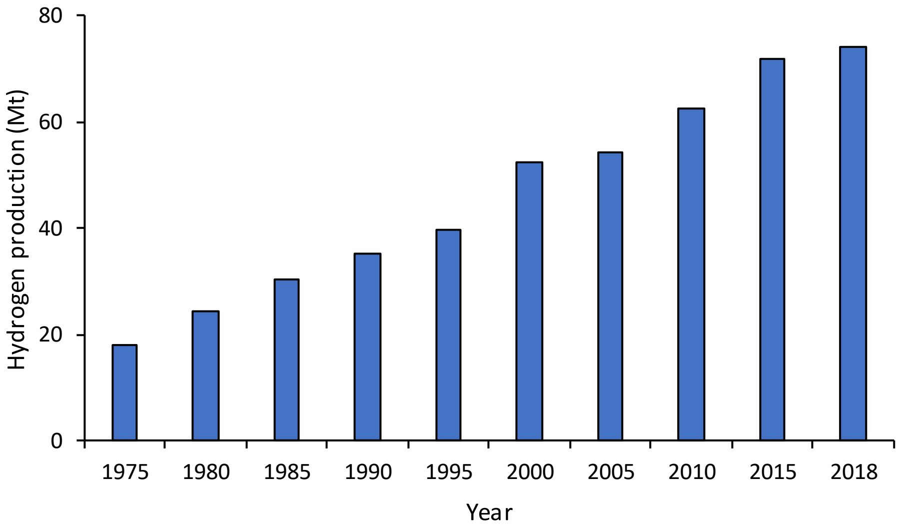

Figure 1 Estimated global demand of H_2 produced from fossil resources (> 95%) between 1975-2018[1].

[1] Modified from https://www.iea.org/reports/the-future-of-hydrogen

In general, H_2 is used in various applications, such as, methanol production, ammonia synthesis and refining industry (Mansilla et al., 2018). Currently, H_2 is mainly produced from non-renewable resources (96%) i.e. natural gas, oil, and coal, whereas only 4% of H_2 is generated by renewable resources i.e. water electrolysis, solar panel, geothermal, and biomass (Figure 2).

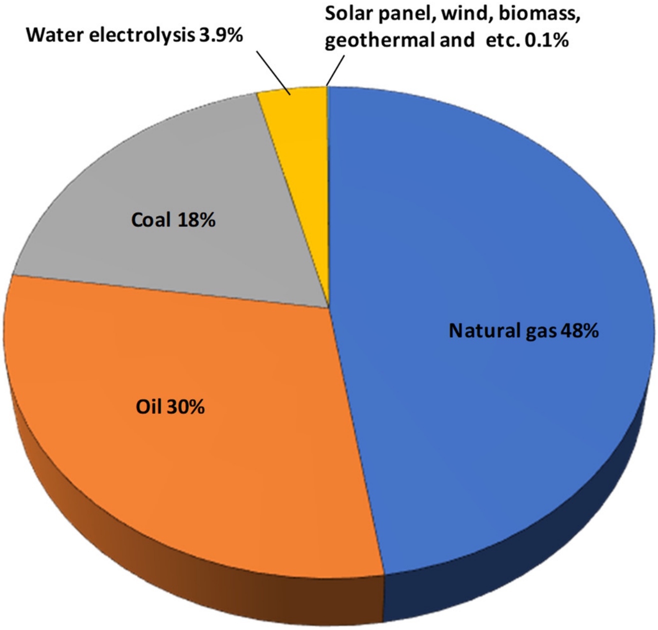

Figure 2 The current methods for H_2 production. Adapted from Nikolaidis and Poullikkas (2017).

Although H_2 production from renewable resources is eco-friendly and sustainable, the major obstacle is the price not being competitive with that of H_2 production from fossil-based resources. Estimated costs for renewable H_2 are 2.5-5.5 euros per kg, while the price of fossil-based H_2 is approximately 1.5 euros per kg (European Commission, 2020). Therefore, the alternative methods for renewable H_2 production require further research to improve the technology with the perspectives of economic feasibility.

1.4 Sustainable H_2 production – future challenge

There are several methods for sustainable H_2 production. The methods can be mainly categorized into non-biological production and biological production (Singh and Das, 2019).

1.4.1 Non-biological H_2 production

Non-biological H_2 productions use the natural existing renewable resources i.e. wind, solar or geothermal to generate H_2 through electrolytic splitting of water molecules (Seelam et al., 2020). Currently, the commercial electrolytic H_2 production performs at low temperature (20-80°C) with an efficiency of 59-70%. In contrast, the high temperature electrolysis offers better efficiency for H_2 production (>90%), but this technology requires further research and development to complete set up for large-scale commercial production (Chi and Yu, 2018).

1.4.2 Biological H_2 production

Biological methods have potential due to the processes being performed at ambient temperature and pressure, requiring lower energy input. The methods can be divided into four groups: i) direct biophotolysis, ii) indirect biophotolysis, iii) photo-fermentation and iv) dark fermentation (Seelam et al., 2020). Direct biophotolysis is a process for H_2 production by splitting water using sunlight to activate electrons in the photosystem of microalgae under anaerobic condition (Brentner et al., 2010). For indirect biophotolysis, the mechanism is mostly similar to direct biophotolysis, but differs in that is a two-step process for H_2 production. The first process is carbohydrates production, and in the second process is conversion into H_2 and other products via dark fermentation (Huesemann et al., 2010, Dalena et al., 2017). Photo-fermentation is a process that photosynthetic bacteria convert organic acids to produce H_2 using nitrogenase (Sağır and Hallenbeck, 2019). This process requires solar energy for creating a proton gradient and to drive cellular processes, including the reduction of nitrogenase through reverse electron flow. However, photo-fermentation has a lower conversion efficiency and needs large surface area for the collection of sunlight (Dalena et al., 2017, Kayfeci et al., 2019). Dark fermentation is a process occurring under anaerobic condition and in the absence of sunlight (Savla et al., 2020). Conversion of organic matter to H_2 occurs via the acetate pathway and butyrate pathway where the theoretical yield of H_2 is 4 and 2 $mol \cdot mol^{-1}$ glucose, respectively (Nikolaidis and Poullikkas, 2017). There are two types of microorganisms in dark fermentation, facultative and obligate anaerobes. These bacteria can utilize various renewable biomass such as industrial, agriculture or municipal waste, making dark fermentation more interest and eco-friendly (Singh and Das, 2019, Savla et al., 2020). However, accumulation of acid intermediates during this process is a major drawback that affects a lower yield of H_2 (Singh and Das, 2019). Because of this and technical challenges, process development of dark fermentation requires further research.

Among these H_2 production methods, the current study herein will be focused on state of the art concerning the consolidated biological H_2 production with dark fermentation. Thermophilic hydrogen producing bacteria of the genus *Caldicellulosiruptor* were investigated for their performance of utilization lignocellulosic materials i.e., pretreated wheat straw (**Paper I**). In addition, a selected *Caldicellulosiruptor* species was further investigated for its sugar uptake (**Paper II**). Co-culture of *Caldicellulosiruptor* species were cultivated with different types of carriers to obtain higher volumetric hydrogen productivity (Q_{H2}) together with the association of biofilm formation (**Paper III** and **IV**).

2. *Caldicellulosiruptor* species

Thermophilic microorganisms, known as "heat loving", of the genus *Caldicellulosiruptor* are strictly anaerobic gram-positive, rod-shaped and non-spore-forming bacteria. These bacteria grow optimally at temperatures between 70-80°C and pH ranging between 6.7 and 8.0 (Rainey et al., 1994). Importantly, this genus has gained attention due to their ability to metabolize a broad spectrum of mono-, di-, and polysaccharides, including glucose, xylose, arabinose, fructose, cellulose, pectin, mannan, xylan and starch (Rainey et al., 1994, Huang et al., 1998, Miroshnichenko et al., 2008). In addition, *Caldicellulosiruptor* species can achieve hydrogen yields near the maximum theoretical yield of 4 mol $H_2 \cdot mol^{-1}$ hexose (Thauer et al., 1977). Within their abilities, *Caldicellulosiruptor* species are considered to form a promising platform for H_2 production. The total number of members to date of the genus of *Caldicellulosiruptor* have been recently published (Table 1). Within the species, there is a genetic similarity of 93-95% and the most well-studied so far are only two species i.e. *C. saccharolyticus* and *C. bescii*.

2.1 *Caldicellulosiruptor* species

2.1.1 *C. saccharolyticus*

C. saccharolyticus was isolated from a geothermal spring in Taupo, New Zealand (Rainey et al., 1994). A completed genome sequence of *C. saccharolyticus* has been studied, including the sugar transporters and the metabolic capacities for substrate utilization. Moreover, *C. saccharolyticus* can co-ferment both C_5 and C_6 sugars simultaneously, because of the absence of carbon catabolite repression (CCR) (van de Werken et al., 2008). The sugar transporters were later accurately re-annotated in connection to genes related to degradation of plant biomass (Chowdhary et al., 2015). It is worth noting that *C. saccharolyticus* prefers C_5 sugars rather than C_6 sugars (Vanfossen et al., 2009). Recently, the growth profiles of *C. saccharolyticus* on diluted wheat straw hydrolysate and defined sugar mixtures based on the sugar ratios present in wheat straw hydrolysate revealed that *C. saccharolyticus* possesses diauxic-like pattern (Bjorkmalm et al., 2018).

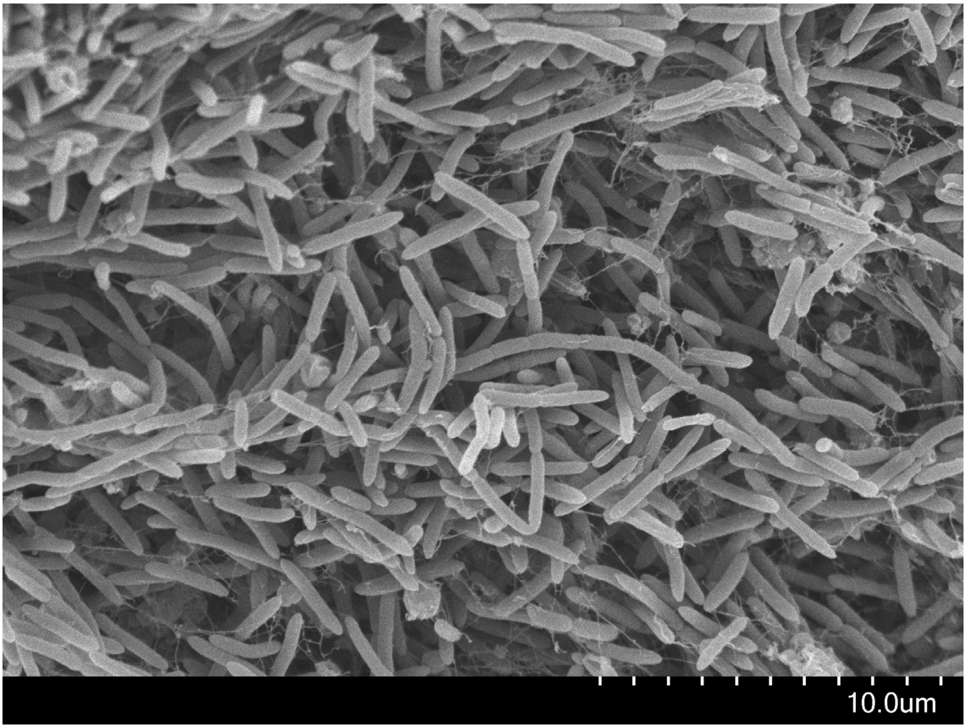

Figure 3 Scanning electron microscope image of a biofilm of *C. saccharolyticus* and *C. owensensis* (Pawar et al. 2015).

2.1.2 *C. owensensis*

C. owensensis was isolated from sediments in a freshwater pond in the area of Owen Lake, California, USA (Huang et al., 1998). *C. owensensis* has an ability to degrade cellulose, sharing traits in common with the other members of *Caldicellulosiruptor*. *C. owensensis* also carries an ability to form biofilm during fermentation, including that it is a natural biofilm former (Peintner et al., 2010). With this property, co-cultures of *C. owensensis* and *C. saccharolyticus* improved the volumetric hydrogen productivity (Q_{H2}) in an Up-flow Anaerobic bioreactor (Pawar et al., 2015). In addition, micrograph under electron microscopy depicted that exopolysaccharide (EPS) produced by *C. owensensis* could retain the cells of *C. saccharolyticus* in the bioreactor at a higher dilution rate (*D*) (Figure 3).

2.1.3 *C. kronotskyensis*

In the current study, one of the interesting *Caldicellulosiruptor* species is *C. kronotskyensis*. It was isolated from a thermal hot spring in Kamchatka peninsula, Russia (Miroshnichenko et al., 2008). Like the other members, *C. kronotskyensis* possesses a cellulolytic ability to utilize wide ranges of substrates. *C. kronotskyensis* gains more attention due to its tāpirin proteins on cell wall (Blumer-Schuette et al., 2015). This protein makes it possible for *C. kronotskyensis* to firmly attach to the lignocellulosic biomass. Furthermore, the previous study noted that *C. kronotskyensis* is classified as strongly cellulolytic, while *C. saccharolyticus* was categorized as moderately cellulolytic. In contrast to those two species, *C. owensensis* was considered as weakly cellulolytic species (Blumer-Schuette et al., 2015, Lee et al., 2019).

2.2 H_2 production in *Caldicellulosiruptor*

A previous study revealed that *C. saccharolyticus* produces H_2 via Embden-Meyerhof pathway (EMP) (van de Werken et al., 2008). In addition, *C. saccharolyticus* does not possess the oxidative part of the pentose phosphate pathway (PPP) and the Entner-Doudoroff pathway. *C. saccharolyticus* takes up substrates, i.e., glucose (hexose) through ATP-binding cassette (ABC) transporters (Figure 4). Glucose is oxidized to pyruvate by the glyceraldehyde-3-phosphate dehydrogenase generating NADH. Subsequently, pyruvate is converted by a pyruvate:ferredoxin oxidoreductase (POR) to acetyl coenzyme A (acetyl-CoA) (Bielen et al., 2013). The generation of H_2 is obtained by: i) conversion of NADH by Fe-Fe hydrogenase and ii) Ni-Fe hydrogenase coupled with reduced ferredoxin (Fd_{red}), which is formulated during the conversion of pyruvate to acetyl-CoA (van de Werken et al., 2008, Bielen et al., 2013, Cha et al., 2016). A recent study proposed that *C. bescii* possesses glyceraldehyde-3-phosphate (GAP) ferridoxin oxidoreductase (GOR) pathway whereby Fd_{ox} is converted to Fd_{red}, which is further oxidized by the Ech hydrogenase (Figure 4) (Scott et al., 2019).

Glycolysis of *C. saccharolyticus* produces metabolites such as acetic acid and lactic acid. High concentrations of these acids interfere with the yield of hydrogen (Y_{H2}) and more severely inhibites H_2 production (van Niel et al., 2003, Willquist and van Niel, 2010) due to their combination to the osmolarity. The latter results in cell lysis and a shift of metabolic flux (Willquist et al., 2009, Ljunggren and Zacchi, 2010). Furthermore, higher H_2 partial pressures lead to a shift in metabolic flux from aetate towards lactate and ethanol (Willquist et al., 2011).

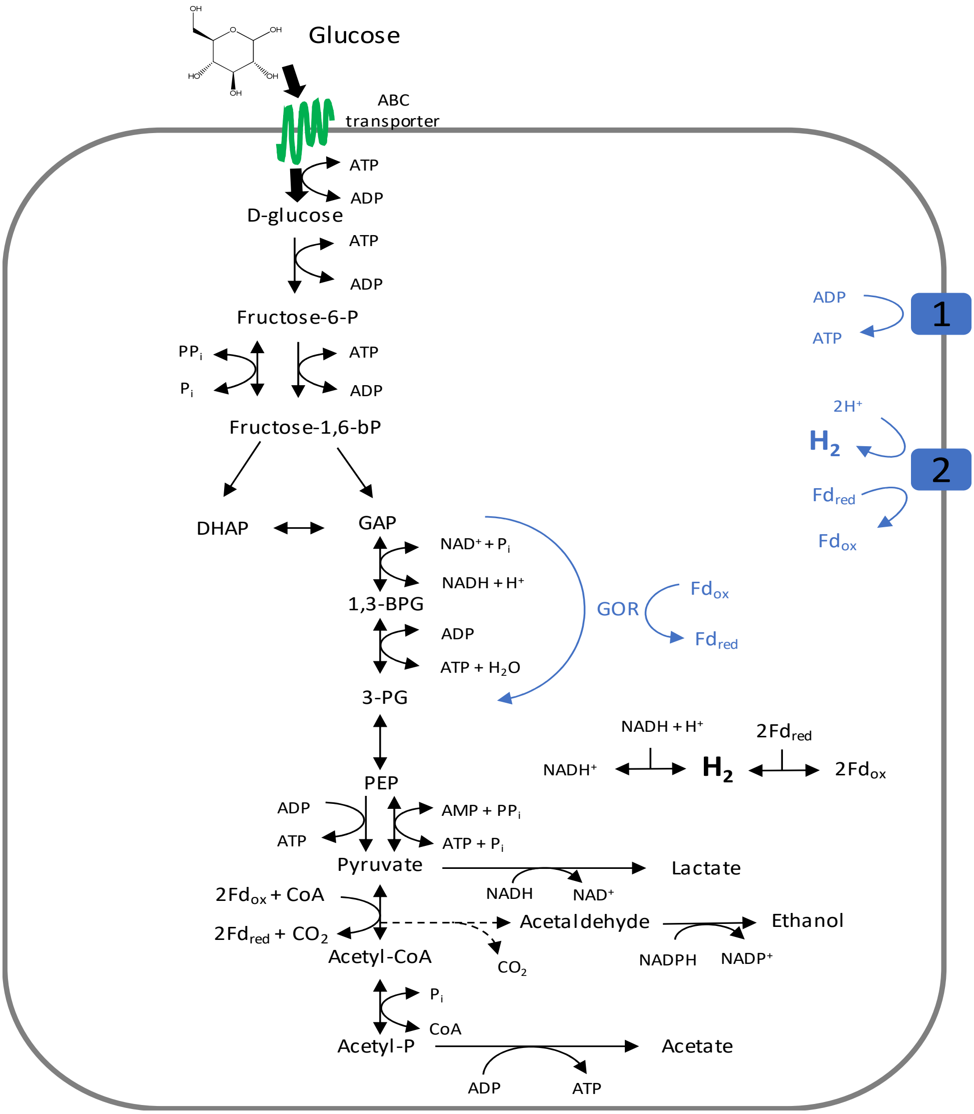

Figure 4 Schematic of the Embden-Meyerhof pathway (EMP) in *C. saccharolyticus* (Black colour). Dotted line represent that the pathway has not yet been validated. The proposed alternative GOR pathway in *C. bescii* coupling with ATP synthase (No.1) and the Ech hydrogenase (No.2) are shown in blue colour. DHAP; Dihydroxyacetone phosphate; GAP, D-glyceraldehyde 3-phosphate; 1,3-BPG, 1,3-Bisphosphoglycerate; 3-PG, 3-phosphoglycerate; PEP, Phosphoenolpyruvate; PPi, Pyrophosphate. Adapted from van der Werken et al. (2008), Bielen et al. (2013) and Scott et al. (2019)

2.3 Plant biomass degradation

Thermophilic microorganisms have a reputation for degradation of polysaccharides found in plant biomass i.e. cellulose, hemicellulose, and xylan. Many of these microorganisms can be found in the domains archaea and bacteria, for example, *P. horikoshii* (archaea), *T. maritima* (bacteria), *C. saccharolyticus* (bacteria) and *C. thermocellum* (bacteria) (Vanfossen et al., 2008, Akinosho et al., 2014). *C. thermocellum* uses cellulosomes consisting of enzyme complexes for utilizing such plant material. In contrast, thermophilic bacteria especially those belonging to *Caldicellulosiruptor* lack cellulosomes, but they can secrete extracellular enzymes to degrade plant biomass (Rainey et al., 1994, Huang et al., 1998, Miroshnichenko et al., 2008).

The genome of *C. saccharolyticus* was annotated to examine its glycolytic pathway related to monomeric and oligomeric sugar uptake via ATP-binding cassette (ABC) transporters (van de Werken et al., 2008). The genome analysis of *Caldicellulosiruptor* revealed that glycoside hydrolases (GHs) and carbohydrate binding modules (CBMs) play a crucial role in the breakdown of lignocelluloses (Blumer-Schuette et al., 2012). Interestingly, the study revealed that *C. kronotskyensis* possesses a higher number of GH domains than others *Caldicellulosiruptor* species, whereas *C. danielii* carries the highest number of CBMs (Blumer-Schuette et al., 2012, Lee et al., 2018). The most important feature of both GHs and CBMs in *Caldicellulosiruptor* involve the cellulolytic capacity for lignocellulose degradation. *C. acetigenus*, *C. hydrothermalis*, *C. kristjanssonii*, and *C. owensensis* are catagorized in the weakly cellulolytic group, whereas *C. lactoaceticus* and *C. saccharolyticus* are classified in the group of moderate cellulolytic activity. Finally, *C. bescii*, *C. changbaiensis*, C. *danielii*, *C. kronotskyensis*, *C. morganii*, *C. naganoensis*, C. obsidiansis, and *Caldicellulosiruptor* sp. *F32* are characterized as strongly cellulolytic species, carrying an important CelA cellulase enzyme composed of GH9-CBM3-CMB3-CMB3-GH48, especially for crystalline cellulose degradation (Table 1). It is interesting to note that strongly cellulolytic *Caldicellulosiruptor* species carry cellulose-degrading GH48 domains in their genome, which are absent in weakly cellulolytic species (Blumer-Schuette et al., 2010, Lee et al., 2018). For example, the absence of GH 48 domains in *C. owensensis* revealed no further growth on pretreated wheat straw, thereby the fermentation was terminated earlier than the fermentation of *C. kronotskyensis* and *C. saccharolyticus* (**Paper I**). In contrast to *C. owensensis*, the fermentation of *C. kronotskyensis* with pretreated wheat straw showed significantly the highest H_2 accumulation in both single culture and co-cultures compared with *C. bescii*, and *C. saccharolyticus* (**Paper I**). This initiated further studies with *C. kronotskyensis* involving with its physiology (**Paper II**) and improvement of Q_{H2} with immobilization techniques (Paper **IV**).

2.4 Role of tāpirins

Caldicellulosiruptor species carry a unique protein, so called "tāpirins" that means "to join", in Māori (Blumer-Schuette et al., 2015). Therefore, *Caldicellulosiruptor* species can bind strongly to cellulose. Interestingly, the group of strongly cellulose degrading *Caldicellulosiruptor* species (*C. bescii*, *C. kronotskyensis*, and *C. obsidiansis*) have two classes of tāpirins proteins encoded in their genome. Although *C. kristjanssonii* and *C. lactoaceticus* carry two genes for tāpirins proteins, but the sequencing alignment identified the similarity below 41% compared with tāpirins proteins of those strong cellulolytic species. As aforementioned, *C. owensensis* performed poorly for lignocellulosic degradation (**Paper I**). *C. owensensis* carries two classes of tāpirins proteins, but they are highly different comparing with tāpirins found in the strongly cellulolytic group (Blumer-Schuette et al., 2015). In addition, no degradation of avicel by *C. owensensis* has been observed (Blumer-Schuette et al., 2010). Recently, the fermentation of knockout of tāpirins genes in *C. bescii* showed no growth on microcrystalline cellulose (Lee et al., 2019). Therefore, tāpirins proteins in *Caldicellulosiruptor* play a significant role in binding on the lignocellulosic biomass for better degradation.

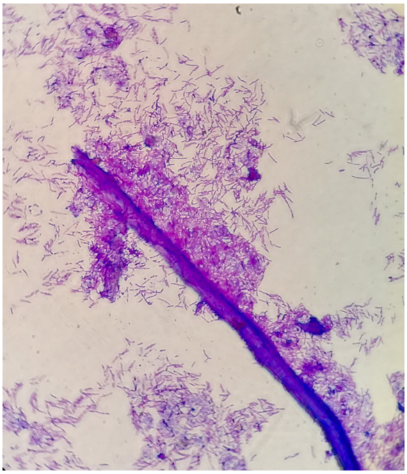

Figure 5 Micrograph of *C. kronotskyensis* on heat-treated wheat straw. All cells and pretreated wheat straw were stained with crystal violet.

2.5 Non-diauxic like growth pattern in *C. kronotskyensis*

Diauxic or bi-phasic growth describes the phenomenon where microbial culture grows on two different carbon sources, exhibiting two exponential growth phases (Chu and Barnes, 2016). This phenomenon was originally demonstrated by Monod, where *E. coli* was cultivated in medium containing glucose and lactose (Monod, 1949). In addition, there are two factors related to this phenomenon; i) regulation of enzyme(s) involved in uptake system(s) and ii) repression of uptake of the secondary substrate. Recently, growth of *C. saccharolyticus* on sugar mixtures found in wheat straw hydrolysate showed diauxic-like pattern (Bjorkmalm et al., 2018). This phenomenon depicted uptake of all pentoeses in the first exponential phase, allowing uptake of glucose. After a certain lag phase, a second growth phase started characterized by non-growth but increased consumption of glucose to the fermentation products. In contrast to *C. saccharolyticus*, the preliminary results in **Paper I** suggested that *C. kronotskyensis* might grow on sugar mixtures (glucose-xylose) differently. In a dedicated study, it was revealed that growth of *C. kronotskyensis* on medium containing sugar mixtures (glucose-xylose mixture) possessed no diauxic-like pattern (**Paper II**). The only growth phase was similar to that of the first exponential phase of *C. saccharolyticus* in which glucose was taken up at very slow rate, a rate that continued after xylose was depleted. Additional fermentation with other sugar mixtures i.e. xylose-cellobiose and cellobiose-glucose made clear that uptake of glucose in the form of monosaccharide is inferior to the uptake of disaccharide form (cellobiose).

2.6 Sugar transporters system in *Caldicellulosiruptor*

Next, a bioinformatics investigation of sugar transporters in *C. kronotskyensis* compared to *C. saccharolyticus* was conducted. ABC transporters in *Caldicellulosiruptor* can be classified into three subgroups, which are carbohydrate uptake 1 family (CUT1), carbohydrate uptake 2 family (CUT2), and di/oligopeptide uptake (Dpp/Opp) (Vanfossen et al., 2009). In the current study, carbohydrate transporters in *C. kronotskyensis* are mostly similar to the sugar transporters presented in *C. saccharolyticus* (**Paper II**). However, both the Dpp/Opp group for fructose and sucrose uptake, and CUT1 group for xyloglucan uptake were absent in *C. kronotskyensis* (Table 2). Furthermore, *C. kronotskyensis* assimilated xylose-cellobiose concurrently during the cultivation with these sugar mixtures. This indicated that the uptake of xylose and cellobiose occurred with different sugar transporters, but further studies are necessary to identify any lack of glucose transporter (**Paper II**).

Table 1 Members of the genus of *Caldicellulosiruptor*. Adapted from Byrne 2019, Lee et al. 2018 and Blumer 2020.

Species	Origin	Optimum Temperature (℃)	pH optimum	Main metabolites	GHs*	CBMs**	Cellulolytic activity	Reference
C. acetigenus [a]	Hveragerdi-Hengill geothermal area, Iceland	65-68	7.0	Lactate	66	21	Weak	Nielsen et al. (1993), Onyenwoke et al. (2006)
C. bescii [b]	Kamchatka Peninsula, Russia	78-80	7.1-7.3	Acetate and Lactate	52	22	Strong	Yang et al. (2010)
C. changbaiensis	Changbai Mountains, China	75	7.8	Acetate and Lactate	Not stated	Not stated	Strong	Bing et al. (2015)
C. danielli [c]	Waimangu, New Zealand	Not stated	Not stated	Not stated	69	53	Strong	Lee et al. (2018), Lee et al. (2015)
C. hydrothermalis	Geyser Valley, Kamchatka, Russia	75	7.0	Acetate and Lactate	62	12	Weak	Miroshnichenko et al. (2008)
C. kristjansonii	Hot spring, Iceland	78	7.0	Acetate	37	15	Weak	Bredholt et al. (1999)
C. kronotskyensis	Geyser Valley, Kamchatka, Russia	70	7.0	Lactate	77	28	Strong	Miroshnichenko et al. (2008)
C. lactoaceticus	Hveragerdi, Iceland	68	7.0	Acetate and Lactate	44	18	Moderate	Mladenovska et al. (1995)
C. morganii [d]	Rotorua, New Zealand	Not stated	Not stated	Not stated	49	45	Strong	Lee et al. (2018), Lee et al. (2015)
C. naganoensis [e]	Hot spring in Nagano Prefecture, Japan	75	8.0	Acetate	44	38	Strong	Lee et al. (2018), Taya et al. (1988)
C. Obsidiansis	Obsidian Pool, Yellowstone National Park, United States	78	6.7-7.0	Acetate	47	18	Strong	Hamilton-Brehm et al. (2010)
C. owensensis	Owens Lake, Califomia, United States	75	7.5	Acetate and Lactate	51	16	Weak	Huang et al. (1998)
C. saccharolyticus	Geothermal spring in Taupo, New Zealand	70	7.0	Acetate	59	17	Moderate	Rainey et al. (1994)
Caldicellulosiruptor sp.F32	Compost, China	75	7.0	Acetate and Lactate	45	12	Strong	Ying et al. (2013)

[a] Formerly, *Thermoanaerobium acetigenum*
[b] Formerly, *Anaerocellum thermophilum*
[c] Previously named *Caldicellulosiruptor sp. strain Wai35.B*
[d] Previously named *Caldicellulosiruptor sp. strain Rt8.B8*
[e] Previously named *Thermoanaerobacter cellulolyticus strain NA10*
*GHs, number of glycoside hydrolase families; **CBMs, number of carbohydrate-binding modules

Table 2 Comparison of ABC transporters between *C. kronotskyensis* (**Paper II**) and *C. saccharolyticus* (Vanfossen et al, 2009).

ABC transpoter (Csac_)	**Group**	**ABC transporter (Calkro_)**	**Features**
0238,0240-0242	CUT2	0382,0384-0386	Arabinose, galactose, xylose
0261-0265	Dpp/Opp	**None**	Fructose, sucrose
0427-0428,0431	CUT1	0283-0284,0287	Maltodextrin
0440-0442	CUT1	2234-2236	Galactose
0692-0694	CUT1	2010-2012	Monosaccharides
1028-1032	Dpp/Opp	0798-0802	Monosaccharides
1557-1559	CUT1	**None**	Xyloglucan
2321-2322,2324,2326	CUT1	0930,0932,0933-0934	Glucose, xylose, fructose
2412-2414	CUT1	2389-2391	Xylooligosaccharides
2417-2419	CUT1	2394-2396	Xylooligosaccharides
2491-2493	CUT1	0321-0323	Xylose, glucose, fructose
2504-2506	CUT2	0128-0130	Xylose, glucose, fructose
2514-2516	CUT1	0108-0110	Glucooligosaccharides

2.7 Stoichiometry of sugar uptake in *Caldicellulosiruptor*

There is no clear indication of the stoichiometry between glucose and xylose uptake as it was depending on the xylose/glucose concentration ratio in the medium. Indeed, the specific xylose consumption rate (q_{Xyl}) declined with decreasing xylose/glucose, while the specific glucose consumption rate (q_{Glu}) increased with increasing glucose/xylose ratios. Assuming competitive inhibition, the inhibition constants (K_I) were established: $K_{I,glu}$ was 0.01 cmol·L^{-1}, which was ten times higher than $K_{I,xyl}$ (0.001 cmol·L^{-1}) (**Paper II**). For the fermentation on xylose-cellobiose, both sugars were taken up simultaneously according to linear stoichiometry. The culture on a cellobiose-glucose mixture also had a linear stoichiometry. The sugar preference of *C. kronotskyensis* was also depicted in the maximum specific growth rate (μ_{max}, h^{-1}): xylose (0.57 h^{-1}) > cellobiose (0.30 h^{-1}) > glucose (0.28 h^{-1}) (**Paper II**). Therefore, it was proposed that *C. kronotskyensis* has adapted to glucose-uptake in the form of disaccharide (cellobiose) instead of monosaccharide (glucose). In addition, this hypothesis is supported by the study of tāpirins proteins encoding as Calkro_0844 in *C. kronotskyensis*, showing the most highly affinity binding with cello-oligosaccharides (Blumer-Schuette et al., 2015).

3. Lignocellulose – renewable resource

As discussed in Chapter 2, *Caldicellulosiruptor* species possesses the ability to grow on various substrates such as mono-, di-, and polysaccharides, including lignocellulosic biomass (Pawar and van Niel, 2014, Blumer-Schuette, 2020). Several glycoside hydrolase enzymes secreted by *Caldicellulosiruptor* species play a crucial role for degrading plant biomass. Lignocellulosic biomass is the most abundant biopolymer resource on Earth that is currently exploited for the production of valuable chemicals and renewable fuels (Abdel-Hamid et al., 2013). In fact, the term "biomass" refers to organic matter i.e. agricultural residues, agricultural crops, municipal waste, wood residues, aquatic plants, and animal waste. In general, plant biomass consists of cellulose (30-35%), hemicellulose (25-30%), and lignin (10%) (Chen, 2014). However, the percentage of each compound varies depending on the plant species (Pedersen and Meyer, 2010). This chapter will be focused on compositions of plant biomass and methods for pretreatment of lignocellulosic biomass.

3.1 Composition of lignocellulose

3.1.1 Cellulose

Cellulose $(C_6H_{10}O_5)_n$ is a β-glucan linear polymer consisting of glucose units linked via β-(1,4) glycosidic bonds where n represents the degree of polymerization (DP) ranging between 100-1,000 glucose units or more (Chen, 2014). The hydrolysis of cellulose occurs randomly at β-(1,4) glycosidic bonds, thereby oligomers are produced. Typically, cellulose formation is arranged in the form of crystalline bundles of chains, so-called microfibrils. The packing of several microfibrils together forms cellulose fibrils (McKendry, 2002, Zoghlami and Paës, 2019).

3.1.2 Hemicellulose

In contrast to cellulose, hemicellulose is a branched heteropolymer composed of various monosaccharides, i.e. pentoses (xylose and arabinose) and hexoses (glucose, rhamnose, galactose, and mannose). In addition, hemicellulose contains other organic acids such as acetic acid, ferulic acid, and 4-O-methyl glucuronic acid (Sun et al., 2003). It is worth noting that the presence of the acetyl content in hemicellulose can reduce enzyme efficiency during pretreatment (Zoghlami and Paës, 2019). In plant physiology, hemicellulose acts as a glue for cellulose fibrils, making the plant cell wall stronger and more robust. Moreover, these complex structures impede the accessibility of hydrolytic enzymes during the pretreatment process (Yousuf et al., 2020).

3.1.3 Lignin

Lignin is a very complex phenolic polymer constituting the third most after hemicellulose. The derivative lignin can be divided into three units i.e. *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Chen, 2014, Zoghlami and Paës, 2019). Lignin plays an important role in plant biomass by enhancing rigidity to the plant structure and protecting (hemi-)cellulose from degradation by microorganisms. In general, lignin is part of bark and wood, and it is enclosed water transport tubes inside tree stem (Neutelings, 2011).

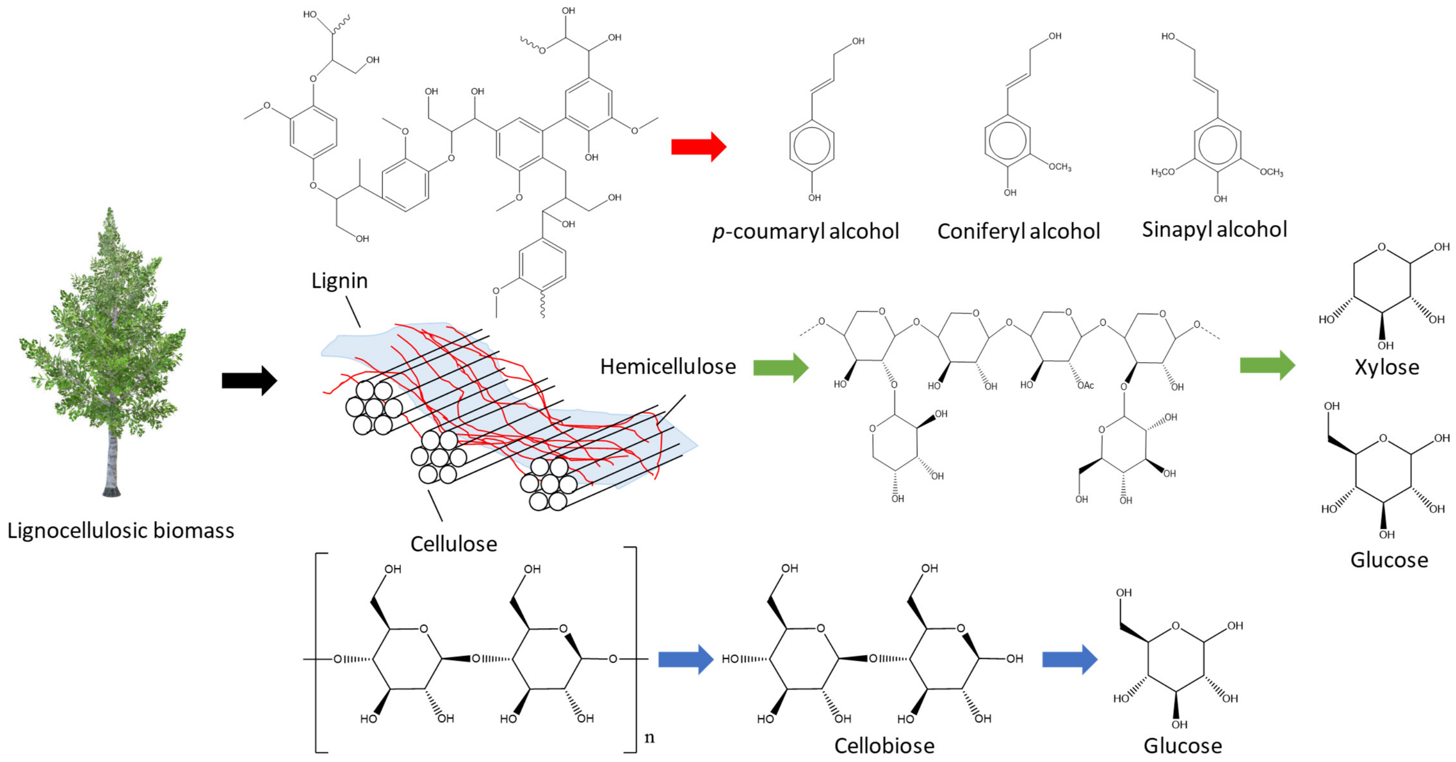

Figure 6 The components of lignocellulosic material.

3.2 Methods for pretreatment of lignocellulose

One of the factors related to the recalcitrance of lignocellulosic biomass is the strongly interconnection between (hemi-)cellulose and lignin, making it difficult to separate. Therefore, pretreatment techniques are required for reducing its complexity i.e. utilizing the polymers to monomers. The pretreatment methods can be classified into physical, chemical, physio-chemical and biological treatments (Kumar and Sharma, 2017).

3.2.1 Physical pretreatment

Mechanical milling (grinding) method is one of the conventional methods to reduce the size (comminution) of biomass. There are different types of physical pretreatment i.e. hammer milling, grinding and chipping (Behera et al., 2014). The chipping technique can reduce the particle size to 10-30 mm, whereas grinding method can reduce the biomass size to approximately 0.2 mm using high shear force during the process (Kumar and Sharma, 2017). The purpose of the grinding method is to increase the surface area for enzyme accessibility. This method has been used as the primary step for treating wheat straw in **Paper I**.

Mechanical extrusion is a common method for pretreatment of lignocellulosic biomass. This method is performed under high temperature and high shearing forces to disrupt the structure of the cellulose matrix. This method offers the advantages of providing shorter biomass fibres and increase surface area for enzyme hydrolysis. However, this method requires high energy and is not applicable for industrial scale (Kumar and Sharma, 2017).

The other methods such as microwave irradiation, ultrasound, and pulsed-electric field are also used for pretreatment of biomass. For microwave technique, it has various advantages i.e. easy operation, low energy intensive, less inhibitors, and require short time during operation. Nonetheless, this method is set up for lab scale and requires further development for industrial scale (Kumar and Sharma, 2017, Mota et al., 2018). For ultrasound, it uses sonication that create cavitation bubbles to break the structure of cellulose and hemicellulose, resulting in more surface area for cellulose degrading enzymes. In contrast to ultrasound method, pulse-electric field (PEF) applies a sudden high voltage (5-20 Kv/cm) during a short retention time. This pretreatment results that the pores are created, allowing cellulose degrading enzymes to enter into the biomass structure (Kumar and Sharma, 2017).

3.2.2 Chemical pretreatment

Both acid and alkali pretreatment are a conventional method for extraction fermentable sugars from biomass materials. For acid pretreatment, the most common acids are sulfuric acid (H_2SO_4), oxalic acid, maleic acid, hydrochloric acid (HCl), and acetic acid (CH_3COOH) (Kumar and Sharma, 2017, Amin et al., 2017). In addition, acid pretreatment is also widely used at industrial scale. The use of dilute acids can achieve higher reducing sugars. However, inhibitor compounds can be generated during the acid pretreatment, for example, furfural and hydroxymethylfurfural (HMF), resulting in inhibited growth (Amin et al., 2017). In general, acid pretreatment is performed either at high temperature for short duration or at low temperature for long duration (Behera et al., 2014). In **Paper I**, wheat straw material was impregnation with 2% acetic acid overnight and was pretreated by steam explosion at 190℃ for 10 min.

On the other hand, alkali pretreatment is typically carried out with sodium hydroxide (NaOH), potassium hydroxide (KOH) or calcium hydroxide ($Ca(OH)_2$), thereby degrading the link between carbohydrate fraction and lignin. Alkali reagents affect the reduction of cellulose crystallization, including the degree of polymerization in the structure of lignocellulosic biomass (Behera et al., 2014, Kumar and Sharma, 2017). However, this pretreatment is suitable for lignocellulosic biomass containing low lignin content (Amin et al., 2017). $Ca(OH)_2$ can neutralize the acetyl groups releasing from hemicellulose (Behera et al., 2014).

Ionic liquids (ILs) have gained interest due to its various advantages i.e. it performs best at low temperature (<100 ℃), it possesses high chemical stability, high polarities, non-flammability, and negligible vapor pressure (Behera et al., 2014, Kumar and Sharma, 2017). The most effective imidazolium salts such as 1-allyl-3-methylimidazonium chloride (AMIMCl) and 1-butyl-3-methylimidazonium chloride (BMIMCl) have been used for separation of cellulose (Behera et al., 2014). The previous studies highlighted that ILs can improve the solubilization of cellulose and hemicellulose (Dadi et al., 2006, Kuo and Lee, 2009). Although ILs have benefits, challenges remain such as high cost of ILs, the process for recycling of ILs, and their inhibiting property require further studies (Kumar and Sharma, 2017).

3.2.3 Physico-chemical pretreatment

Steam explosion (STEX) is a widely used method for pretreatment of various biomass feedstocks (Galbe and Wallberg, 2019). STEX is operated under high pressure steam (0.7-4.8 MPa) at a temperature ranging between 160-260℃ for a certain retention time, a few seconds to minutes (Behera et al., 2014). Typically, the lignocellulosic material is impregnated with either diluted-acid or mild-alkali prior to the treatment with STEX. STEX is a combination process of mechanical force (de-pressurise) and hydrolysis of hemicellulose into xylose (pentose) and glucose (hexose) by the acetic acid found in hemicellulose, so called "autohydrolysis" (Kumar and Sharma, 2017). Thus, this combined process can enhance the sugar content during enzymatic hydrolysis.

Sulfite pretreatment to overcome recalcitrance of lignocellulosics (SPORL) is a popular method using sulfite for treating lignocellulosic biomass. SPORL consists of two steps: i) the treatment of biomass with a solution of sulfite salt such as calcium sulfite ($CaSO_3$), or sodium sulfite (Na_2SO_3), or magnesium sulfite (MgS) and ii) reducing biomass particle size by using disk milling (Galbe and Wallberg, 2019). The previous study showed that SPORL generates less HMF and furfural, and increases overall product yields (Zhu et al., 2009).

Finally, the ammonia fibre explosion method (AFEX) is a pretreatment of lignocellulosic biomass soaking with liquid ammonia at high temperature (60-90℃) and pressure for 30-90 min. The following step is an immediately drop in pressure, causing destruction of the biomass fibres. This step is similar to the one in STEX, but liquid ammonia is used instead of water (Behera et al., 2014). AFEX has gained attention as it does not generate inhibitors and gives high sugar yields. Moreover, liquid ammonia can be recovered and recycled after AFEX to minimize the cost of pretreatment (Kumar and Sharma, 2017).

3.2.4 Biological pretreatment

Biological pretreatment is a process associated with the use of microorganisms that is capable to degrading cellulose, hemicellulose, lignin and derivatives thereof. It has got attention due to its advantages i.e. low energy intensive, eco-friendly and no toxic product formation (Behera et al., 2014, Galbe and Wallberg, 2019). The white-rot fungi such as *Phanerochaete chrysosporium*, *Ceriporiopsis subvermispora*, and *Pycnoporus cinnarbarinus* are capable of degrading lignin due to that each species can produce peroxidase and laccases enzyme (Couto et al., 1998, Geng and Li, 2002, Abdel-Hamid et al., 2013). These basidiomycetes possess the two important extracellular enzymes involved in lignocellulosic degradation; i) hydrolytic enzymes (xylanase and cellulase) for utilizing polysaccharides present in plant biomass and ii) a unique set of ligninolytic enzymes for degrading phenolic compounds derived from lignin (Peralta et al., 2017). In addition to fungi, *Pseudomonas putida* KT2440 (gram-negative) has its ability to depolymerize lignin-related compounds (Ravi et al., 2017). Moreover, *P. putida* NX-1 can convert lignin-derived aromatics into polyhydroxyalkanoate (PHA) by using its dye-decolorizing peroxidase (Xu et al., 2021). Besides, thermophilic bacteria have also gained interest due to their cellulolytic enzymes. *Clostridium thermocellum* is a gram-positive anaerobic bacterium with a capacity for utilizing insoluble cellulose into biofuels such as ethanol. Thus, *C. thermocellum* is widely recognized as a promising candidate for consolidated bioprocessing. Nevertheless, *C. thermocellum* lacks the ability to take up pentoses (C_5) (Blumer-Schuette et al., 2014). In contrast to *C. thermocellum*, *Thermotoga maritima* is a strict anaerobe that grows on both hexoses (C_6) and pentoses (C_5). The fermentative products of *T. maritima* with carbohydrates as carbon sources are acetate, H_2, and CO_2 (Vanfossen et al., 2008). As discussed in Chapter 2, the genus *Caldicellulosiruptor* are capable to use a broad spectrum of substrates, thereby this genus has become a promising candidate for consolidated bioprocessing (Vanfossen et al., 2008).

Although the biological pretreatment gains more attention with its advantages, the substrate conversion rate is slow to be applied at industrial scale, when compared with chemical and physio-chemical pretreatment. Therefore, the biological pretreatment requires further research and development for accomplishing application at large scale.

4. Strategies to enhance biofilm formation

One of the major drawbacks of *Caldicellulosiruptor* cultures is their low cell density, which affects the road to obtain appropriately high Q_{H2} for large-scale production (Willquist et al., 2010). As discussed in Chapter 2, *C. owensensis* possesses characteristics of a biofilm producer (Peintner et al., 2010). Therefore, co-culture techniques were established based on *C. saccharolyticus* promoting the growth of *C. kristjanssonii* (Zeidan et al., 2010), and biofilm formation in *C. owensensis* (Pawar et al., 2015). In this way, co-cultures improved both biomass retention and Q_{H2}. A maximum Q_{H2} could reach of approximately 20 $mmol{\cdot}L^{-1}{\cdot}h^{-1}$ with granular sludge as a carrier (Pawar et al., 2015). Therefore, the combination of immobilization techniques and biofilm formation were demonstrated to be a promising strategy to enhance Q_{H2}. In the current study, different types of carriers used for immobilizing co-cultures of *Caldicellulosiruptor* species were investigated.

4.1 Chitosan

Chitosan is a biodegradable polymer composed of arbitrarily distributed β-1,4 linked D-glucosamine and *N*-acetyl-D-glucosamine subunits (Berger et al., 2004). This biopolymer is produced by partial de-acetylation of chitin (Figure 7), which is the second most abundant natural polymer in the world. Chitin is synthesized by arthropods as the component of exoskeleton structures, and moreover, in the cell wall of both fungi and yeast. There are many applications of chitosan such as food additives, biopharmaceuticals, waste-water treatment, and in agriculture (Rinaudo, 2006).

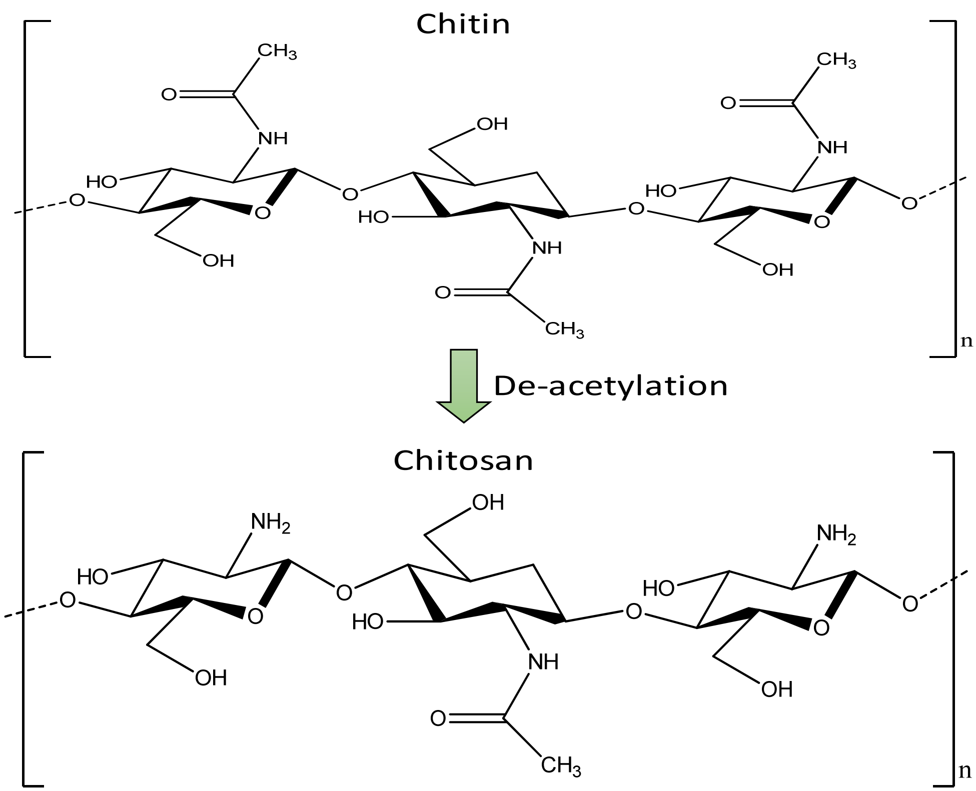

Figure 7 De-acetylation of chitin to chitosan.

4.1.1 Chemical properties and characterization of chitosan

Chitosan is often characterized by its molecular weight (Mw) and degree of de-acetylation (DD) (Islam et al., 2017). The Mw is responsible for its viscosity, water-uptake ability and biodegradability. DD represents the conversion of glucosamine to N-acetylglucosamine, which relates to its solubility. Depending on randomly distribution of the acetyl group during the treatment of chitin, commercially available chitosan is specified based on broad ranges of both Mw and DD (Szymańska and Winnicka, 2015).

4.1.2 Self-flocculation

As aforementioned of the solubility related to its DD, chitosan can dissolve in dilute acids such as 0.1 M acetic acid (Rinaudo, 2006) or 0.1 M HCl (Rehn et al., 2012 and **Paper III**). It is the protonation of the amine group in acid solution when the pH is lower than the pKa of chitosan, that makes it soluble in the aqueous phase. On the other hand, self-aggregation of chitosan occurs during deprotonation at the amine group (Figure 8), so-called "*sweep flocculation*", in a solution where pH is higher than its pKa. Interestingly, one of the natural features of chitosan is the reversibility of flocculation (Kumirska et al., 2011). The term *flocculation* means a reversible reaction, in contrast to the term *coagulation* which referred to an irreversible reaction (Rehn, 2013). Previously, chitosan was successfully used as an immobilizer to flocculate the genetically modified *E. coli* cells for enzyme expression (Rehn et al., 2012). In addition, chitosan has a high cell loading capacity of 3.2 g cells·g^{-1} chitosan (Rehn et al., 2012) and up to 100 g cells·g^{-1} chitosan (Rehn et al., 2013). In **Paper III**, chitosan was employed for flocculation of planktonic cells of the co-cultures of *C. saccharolyticus* and *C. owensensis* in continuous mode. Furthermore, the combination of chitosan and acrylic fibres was also used for flocculation of single culture of *C. owensensis* and *C. kronotskyensis*, and the co-culture of these two species in the chemostat (**Paper IV**).

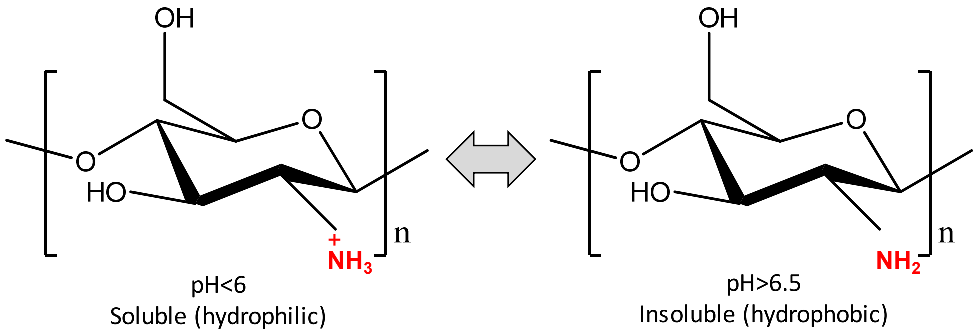

Figure 8 Schematic representations of chitosan structure and its versatility.

4.1.3 Antimicrobial properties

Chitosan can potentially be used for cell immobilization, but its track record of inherent antimicrobial activity has been of concern (Liu et al., 2004). The previous studies revealed that chitosan inhibits growth of gram-negative bacteria (Wang, 1992, Helander et al., 2001) and gram-positive bacteria (Costa et al., 2012, Zhang et al., 2013). The antimicrobial mechanism basically depends on two factors, Mw and DD of chitosan (Moratti and Cabral, 2017). It is clearly seen that the lower Mw chitosan can penetrate through the bacterial cell wall, thereby disrupting the synthesis of DNA and RNA (Goy R. C., 2009). At higher Mw chitosan, it can prevent the transportation of nutrients and ion into bacterial cells (Eaton et al., 2008). As aforementioned, its antimicrobial properties can be reduced through using suitable dosages of medium molecular weight chitosan for immobilization. This has been established for *Caldicellulosiruptor* species by determining their growth indirectly via hydrogen production (**Paper III**). The current study found that a safe concentration of chitosan for flocculating of *Caldicellulosiruptor* species was between 0.01 $g{\cdot}L^{-1}$ and 0.001 $g{\cdot}L^{-1}$, whereas the inhibitory effect was noted at the concentration of 0.1 $g{\cdot}L^{-1}$ (**Paper III**). This corresponds well with former studies reporting that the concentration of chitosan ranging from 0.1-1 $g{\cdot}L^{-1}$ display antimicrobial properties (Moratti and Cabral, 2017). In addition, the inhibition phenomenon can be elucidated by the deprotonation of the amine group (H_3N^+) that can subsequently chelate metal ions and thus bacteria cannot access essential metals for their growth. Nonetheless, increasing the pH beyond its pKa (pH $\geq$ 7) can reduce the antimicrobial property of chitosan (Goy R. C., 2009, Kumirska et al., 2011, Moratti and Cabral, 2017 and **paper III**). Interestingly, according to the study by Rehn et al. 2013, lower amount of chitosan displayed a higher capacity of immobilizing *E. coli* cells compared to other immobilizers such as Ca-alginate beads and titanium (IV) oxide.

4.1.4 Utilization of chitosan

Although chitosan is similar to the structure of cellulose, the difference is that chitosan has the amine group, which can be protonated or deprotonated depending on the pH range ((Rehn, 2013, Kumirska et al., 2011) and **Paper III**). Pure culture of *C. saccharolyticus* and *C. owensensis* or co-culture of these two species could not utilize chitosan when they were grown on a medium containing chitosan as a sole carbon source (**Paper III**). Bioinformatics analysis indicated that no gene encoding for chitosanase enzyme in the *Caldicellulosiruptor* 's genome (**Paper III**). Chitosanase is capable of hydrolysing chitosan at the β-1,4 glycosidic bond (Kaczmarek et al., 2019), which is produced by other gram-positive bacteria such as *Bacillus* species and *Streptomyces* species (Yorinaga et al., 2017). Moreover, cellulase and chitosanase also belong to the glycoside hydrolase family 8 (GH-8) (Adachi et al., 2004), but differ only in 4 amino acids residues.

4.2 Acrylic fibres

Commercial acrylic fibres are synthetic polymers produced from linear polymers of polyacrylonitrile (PAN) $(C_3H_3N)_n$ (Grishanov, 2011). The use of acrylic fibres for immobilization of *Caldicellulosiruptor* species was made possible due to that chitosan could retain biomass in the CSTR. Chitosan alone could not improve the volumetric hydrogen productivity (Q_{H2}) to a desired level (**Paper III**). To increase the surface areas for biofilms formation, acrylic fibres enclosed in a homemade stainless-steel cage was placed in a CSTR (Figure 9). Interestingly, the maximum Q_{H2} significantly increased from 8.4 mmol·L^{-1}·h^{-1} (with chitosan in **Paper III**) to 30 ± 0.2 mmol·L^{-1}·h^{-1} (with acrylic fibres and chitosan in **Paper IV**).

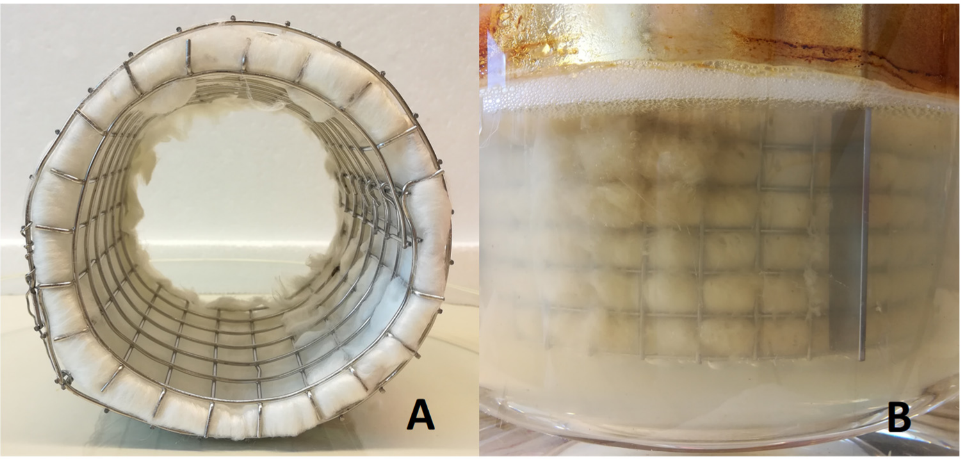

Figure 9 Installation of acrylic fibres. (A) acrylic fibres equipped with a homemade stainless-steel cage (B) installation of a homemade stainless-steel cage in the CSTR.

4.3 Rubber

In general, butyl rubber, or poly(isobutylene-isoprene), is a copolymer consisting of isobutylene (~96-99%) and isoprene (~1-4%) (Semegen, 2003, Lambla, 1989). Butyl rubber has a potential to be used as a carrier supporting biofilms formation in serum flasks experiment (Figure 10, A). These rubber pieces were fixed inside a homemade stainless-steel cage to increase the surface area for biofilm formation in a CSTR (Figure 10, B). Co-culture of *C. saccharolyticus* and *C. owensensis* were carried out with butyl rubber pieces. At lower *D*'s between 0.05 h^{-1} to 0.2 h^{-1}, biofilm formation was observed on the wall of bioreactor and butyl rubber pieces. Nonetheless, washing out started when increasing the *D* beyond 0.2 h^{-1}. Therefore, rubber did not retain biomass of the co-culture of *Caldicellulosiruptor* species as expected, whereas the presence of chitosan could retain biomass through all *D*s (from 0.05 h^{-1} to 0.9 h^{-1}) (**Paper I**).

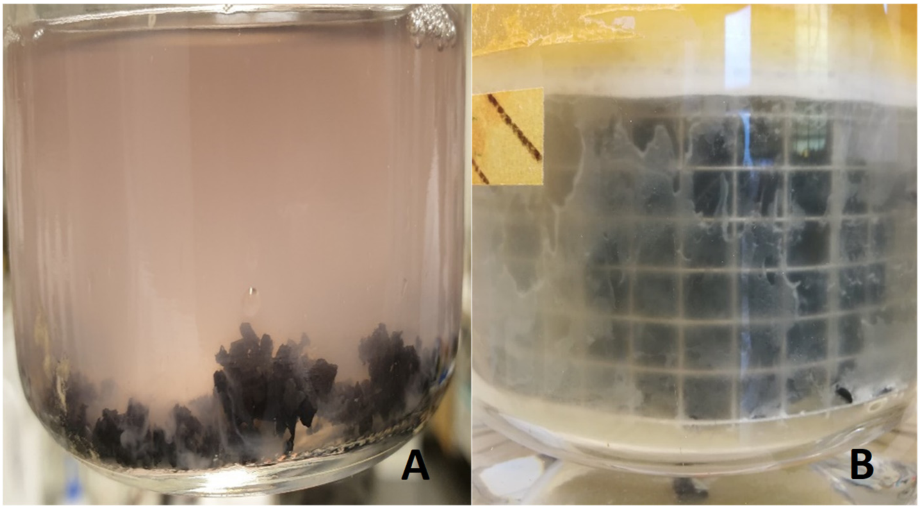

Figure 10 (A) Biofilm formation on rubber as a carrier material. (B) Biofilm formation of a co-culture of *C. saccharolyticus* and *C. owensensis* with butyl rubber as a carrier material in a stainless-steel cage in a CSTR.

4.4 Hydrophobicity

Bacterial adherence to hydrocarbon (BATH), later known as “microbial adherence to hydrocarbon (MATH)”, is a simple method to determine the hydrophobicity of bacterial cell walls and materials (Rosenberg, 1984, Rosenberg, 2006). The current study indicated that there is no significant difference in the MATH assay between *C. saccharolyticus* and *C. owensensis* (**Paper I**). In addition, both *Caldicellulosiruptor* species mostly ended up in the hydrophobic phase, indicating their hydrophobic nature (**Paper I**). For chitosan hydrophobicity, the experiments were divided into different pH ranges such as pH 6, 7, 7.5 and, 8. After addition of hexadecane, flocculated chitosan particles moved toward hydrophobic phase (Figure 11). Therefore, the results indicated that flocculated chitosan is hydrophobic, whereas dissolved chitosan is hydrophilic.

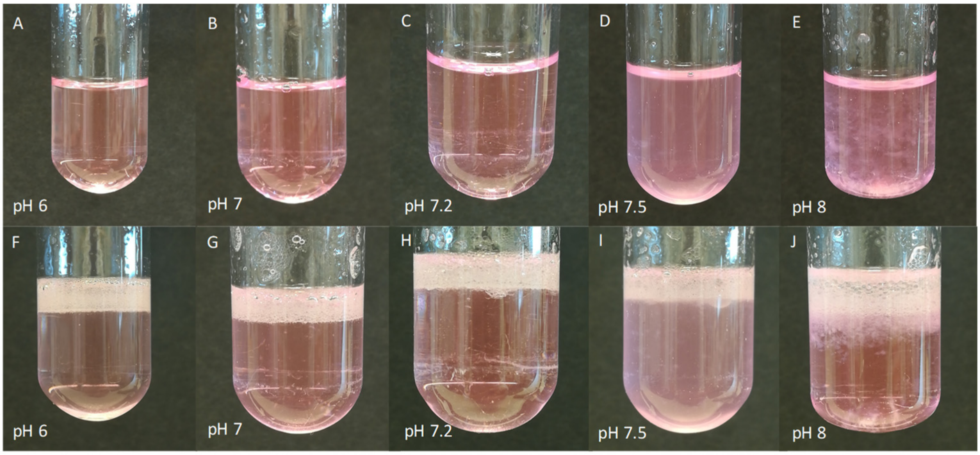

Figure 11 (A) BATH assay for hydrophobicity of chitosan at different pH in a modified DSM-640 medium. (A-E) Chitosan flocculation at various pH before the addition of hexadecane. (F-J) Chitosan flocculation after the addition of hexadecane.

5. Regulation of c-di-GMP for biofilm formation

As described in Chapter 2, *C. owensensis* has a reputation as biofilm former (Peintner et al., 2010). *C. owensensis*'s biofilms could be used for biomass retention during co-cultures of *Caldicellulosiruptor* species in continuous mode, aimed to improve Q_{H2} (Pawar et al., 2015). In addition, the level of bis-(3',5')-cyclic di-guanosine monophosphate (c-di-GMP) in *Caldicellulosiruptor* is involved in the regulatory of biofilm formation (Pawar et al., 2015). In the current work (**Paper IV**), co-cultures of *C. kronotskyensis* and *C. owensensis* were performed with (a combination of) two different types of carriers i.e. chitosan and acrylic fibres, to evaluate biofilm formation, and include quantitative analysis of c-di-GMP produced during the fermentations.

5.1 Biofilm

Biofilms are complex communities of microorganisms established on surfaces with self-produced matrices which consist of extracellular polymeric substances (EPS): exopolysaccharides, proteins, and nucleic acids (Abee et al., 2011, Valentini and Filloux, 2016). The important roles of biofilms are: i) physical shield for survival from the defence system from the host (pathogenic biofilms) and ii) protect their population in harsh environments i.e. pH changes, UV radiation, temperature and osmotic pressure (Bogino et al., 2013).

For the growth of *Listeria monocytogenes* in static condition, biofilms form a single layer of cells, thereby displaying no significant difference cell morphologies in biofilms and of planktonic cells. In contrast, cell morphology in biofilms grown under chemostat condition are remarkably spherical shaped microcolonies (Abee et al., 2011). Under chemostat conditions, *Staphylococcus aureus* biofilms formed a dense layer together with various matrices. Similar to biofilms of co-cultures of *C. saccharolyticus* and *C. owensensis* that has been obtained in a previous study (Pawar et al., 2015) (Figure 3).

5.2 c-di-GMP

Bis-(3',5')-cyclic di-guanosine monophosphate (c-di-GMP) is a second messenger that regulates the alteration between the motile and sessile lifestyle in many bacteria. Therefore, c-di-GMP plays a crucial role as a mediating molecule to promote biofilm formation (Massie et al., 2012, Valentini and Filloux, 2016, Purcell and Tamayo, 2016). The regulation of c-di-GMP was firstly observed in *Acetobacter xylinum* as a model study for bacterial cellulose synthesis (Ross et al., 1991). The latter study revealed that two molecules of guanosine triphosphate (GTP) are used for the synthesis of a molecule of c-di-GMP under the control of diaguanylate cyclase (DGC) encoding in the GGDEF domain. On the other hand, c-di-GMP is degraded by phosphodiesterase (PDE) enzyme into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) and guanosine-mono-phosphate (GMP) (Figure 12).

C. saccharolyticus and *C. owensensis* possess both diguanylate cyclase (DGC) and phosphodiesterases (PDE) (Pawar et al., 2015). And the same was found for *C. kronotskyensis* (Zurawski et al., 2015). In addition, the genome of *C. kronotskyensis* possesses four loci that relate to the Che-type signal transduction pathway. The Che-type system is related to both DGC and PDE, which are located at loci I and II. Moreover, locus IV is related to flagellum or pilus biosynthesis. It is worth noting that these genes in the Che-type system are highly upregulated when *Caldicellulosiruptor* species grow on cellulose (Zurawski et al., 2015, Khan et al., 2020).

In the current study, the highest c-di-GMP (260 ± 27.3 μM) was obtained during the co-culture of *C. kronotskyensis* and *C. owensensis* without a carrier (control study). Besides, the second most prominent c-di-GMP level was in the pure culture of *C. owensensis* with chitosan (172 μM) (**Paper IV**). In contrast to those two cases, pure culture of *C. kronotskyensis* with and without carrier could not reach c-di-GMP levels beyond 50 μM. This phenomenon could be due to: i) biofilm formation is not a phenotype of *C. kronotskyensis* and ii) *C. kronotskyensis* possesses μ_{max}'s that are higher than *C. owensensis* (**Paper II**). For the pure culture of *C. owensensis* in the presence of acrylic fibres, the c-di-GMP levels increased from below 50 μM to levels ranging between 80-150 μM. A similar pattern had been seen during a co-culture of *C. kronotskyensis* and *C. owensensis* in the presence of acrylic fibres (**Paper IV**).

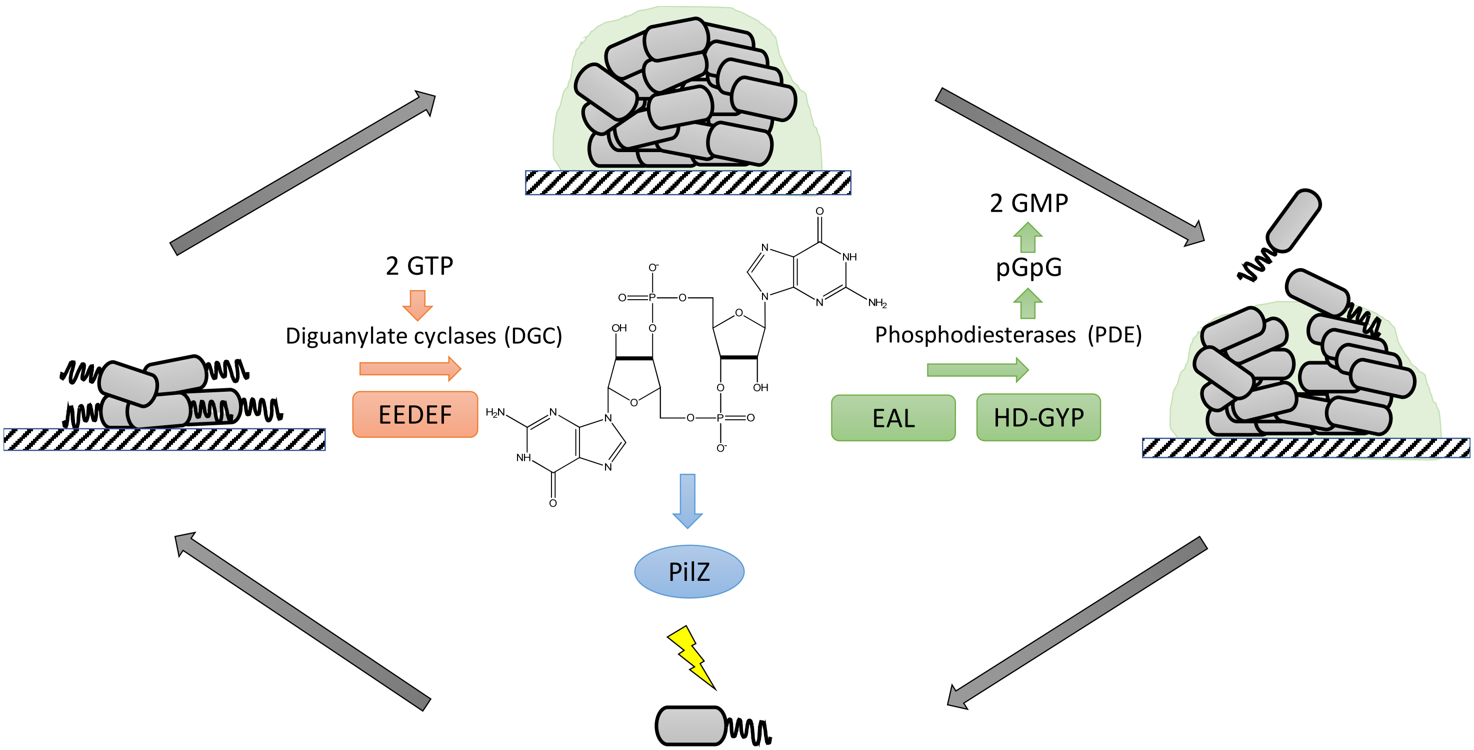

Figure 12 Regulatory of biofilm formation in *Caldicellulosiruptor* species. Adapted from Hengge, 2016, Valentini and Filloux, 2016 and Zurawski et al., 2015.

5.3 Population dynamics

As stated in Chapter 2, the genetic similarity among *Caldicellulosiruptor* species is very high. The completed genomes of *Caldicellulosiruptor* species were aligned using Mauve (Darling et al., 2004) in order to illustrate the dissimilar regions, which consequently used for the design of the specific primers for each species with Primer 3 (Koressaar and Remm, 2007). These specific primers (Table 3) were examined for their cross-reactivity with polymerase chain reaction (PCR) before being used for the quantitative population with real-time PCR (data not shown).

Table 3 Specific primers used for quantitative population dynamics.

Species	Annealing temperature	Primers	Amplicon size (base pairs)
C. kronotskyensis	61°C	5`– CAGGAGATGGAACGTGGATT – 3` 5`– CCATGGAGCAGTCCCACTAT – 3`	224
C. owensensis	61°C	5`– GGCAAGTGGGAAGAAGATGA – 3` 5`– CTCCGCAAGACTTGAACACA – 3`	190
C. saccharolyticus	53.5°C	5`– TATTATGGGGATTGGGACGA – 3` 5`– CTGGCGCACCAAAGATAAAT – 3`	207

In the presence of rubber, the distributions of *C. saccharolyticus* and *C. owensensis* were equally through all the *D*s. Nonetheless, in the presence of chitosan, *C. owensensis* was the dominant species, whereas *C. saccharolyticus* was the major species in a continuous culture without chitosan (**Paper I**). For the co-cultures of *C. kronotskyensis* and *C. owensensis*, both planktonic and biofilm samples were quantified using real-time PCR to observe species distribution (**Paper IV**). For planktonic samples, *C. kronotskyensis* was the dominant species in the co-culture without carriers (control study), whereas *C. owensensis* was the dominant species in the presence of acrylic fibres, and in the combined chitosan and acrylic fibres. However, the population dynamics of both species fluctuated only in the presence of chitosan.

Furthermore, the population analysis on biofilm samples revealed that *C. owensensis* was the dominant species in the control study (without any carriers). Nonetheless, *C. kronotskyensis* was the dominant species in the presence of acrylic fibres and combined chitosan and acrylic fibres. Only when chitosan was used for immobilization, the population of *C. kronotskyensis* gradually decreased, while *C. owensensis* relatively increased through all dilution rates (**Paper IV**).

6. Improvement of Q_{H2}

As stated in Chapter 4, chitosan has the potential to be used for biomass retention, but the Q_{H2} did not satisfy the desired level. The next idea clearly indicated that acrylic fibres would be looking further for immobilization. However, it is impossible to add acrylic fibres in a CSTR together with the operation of impellers. Therefore, acrylic fibres equipped with a homemade stainless-steel cage was used in a bioreactor. The combination of this solid immobilizer and chitosan could improve Q_{H2} (**Paper IV**). This chapter will summarize the maximum Q_{H2} accomplished in this study in comparison with the Q_{H2} values taken from literature (Table 4).

The Q_{H2} of the co-culture of *C. saccharolyticus* and *C. owensensis* with chitosan was 8.4 $mmol \cdot L^{-1} \cdot h^{-1}$ (**Paper I**). However, the Q_{H2} values obtained from previous studies were 45.8 $mmol \cdot L^{-1} \cdot h^{-1}$ (Koskinen et al., 2008) and 20 $mmol \cdot L^{-1} \cdot h^{-1}$ (Pawar et al., 2015). Regarding the results in **Paper IV**, the maximum Q_{H2} was 30 ± 0.2 $mmol \cdot L^{-1} \cdot h^{-1}$, obtained with a single culture of *C. kronotskyensis* cultivated with both acrylic fibres and chitosan. Besides, the co-culture of *C. kronotskyensis* and *C. owensensis* with acrylic fibres showed the second-best Q_{H2} at the level of 26.4 ± 1.9 $mmol \cdot L^{-1} \cdot h^{-1}$, which is similar to a single culture of *C. kronotskyensis* with acrylic fibres (25.4 ± 0.6 $mmol \cdot L^{-1} \cdot h^{-1}$). Interestingly, the population dynamics analysis revealed that the dominant species was *C. kronotskyensis*, thereby it can be assumed that *C. kronotskyensis* influenced mostly on the Q_{H2}. Furthermore, the third highest Q_{H2} (23 $mmol \cdot L^{-1} \cdot h^{-1}$) was observed when co-culture of *C. kronotskyensis* and *C. owensensis* were cultivated with both acrylic fibres and chitosan. The population dynamics results indicated that *C. owensensis* was the dominant species in the planktonic phase, whereas *C. kronotskyensis* was the dominant species on acrylic fibres.

Table 4 Volumetric hydrogen productivity (Q_{H2}) by *Caldicellulosiruptor*. (G: glucose, X: xylose, and A: arabinose)

Organism	Substrate	Sugar concentration ($g \cdot L^{-1}$)	Reactor type	Fermentation mode	Carrier	Hydrogen productivity ($mmol \cdot L^{-1} \cdot h^{-1}$)	Reference
C. saccharolyticus	Sugar	G: 4.4	CSTR	Continuous	Not stated	12.4	de Vrije et al. (2007)
C. saccharolyticus	Sugar	G: 5.4	Trickle bed reactor	Continuous	Not stated	22	Groenestijn et al. (2009)
C. saccharolyticus	Sugar	G: 5 X: 5	CSTR	Continuous	Not stated	11.6	Zeidan et al. (2010)
C. saccharolyticus	Wheat straw hydrolysate	G: 6.7 X 3.7 A: 0.4	CSTR	Continuous $D = 0.05\ h^{-1}$	Not stated	8.7	Pawar et al. (2013)
C. saccharolyticus	Wheat straw hydrolysate	G: 6.7 X: 3.7 A: 0.4	CSTR	Continuous $D = 0.15\ h^{-1}$	Not stated	8.8	Pawar et al. (2013)
C. saccharolyticus ***C. owensensis***	Sugar	G: 10	UA	Continuous $D = 1.25\ h^{-1}$	granular sludge	20	Pawar et al. (2015)
C. saccharolyticus ***C. owensensis***	Sugar	G: 10	CSTR	Continuous $D = 0.1\ h^{-1}$	K-1 carrier	8	Pawar et al. (2015)
C. saccharolyticus	Wheat straw hydrolysate	G: 18.3 X: 8.2 A: 0.6	UASB	Continuous	granular sludge	6.7	Byrne et al. (2018)
C. saccharolyticus	Wheat straw hydrolysate	G: 14.5 X: 6.3 A: 0.4	UASB	Continuous	granular sludge	4.2	Byrne et al. (2018)
C. saccharolyticus ***C. owensensis***	Sugar	G: 10	CSTR	Continuous $D = 0.8\ h^{-1}$	Chitosan	8.4	**Paper III**
C. kronotskyensis	Sugar	G: 7.3 X: 3.4	CSTR	Continuous $D = 0.3h^{-1}$	acrylic fibre	25.4 ± 0.6	**Paper IV**
C. kronotskyensis ***C. owensensis***	Sugar	G: 7.3 X: 3.4	CSTR	Continuous $D = 0.3\ h^{-1}$	acrylic fibre	26.4 ± 1.9	**Paper IV**
C. kronotskyensis	Sugar	G: 7.3 X: 3.4	CSTR	Continuous $D = 0.3h^{-1}$	Chitosan and acrylic fibre	30 ± 0.2	**Paper IV**

7. Conclusions

The main conclusions of this thesis are:

- The highest Q_{H2}, at a level of 30 ± 0.2 mmol·L^{-1}·h^{-1} (*D* of 0.3 h^{-1}), was observed during pure cultures of *C. kronotskyensis* immobilized on acrylic fibres and chitosan, which was observed the yield of hydrogen (Y_{H2}) of 2.95 ± 0.1 mol H_2 ·mol^{-1} sugar (**Paper IV**).

- The population dynamics indicated that *C. kronotskyensis* was the dominant species in biofilm fraction during co-culture of *C. kronotskyensis* and *C. owensensis* with the presence of acrylic fibres and combined acrylic fibres and chitosan (**Paper IV**).

- The combination of acrylic fibres and chitosan facilitate biofilm formation, thereby increasing the Q_{H2} for pure culture and co-culture (**Paper IV**).

- The highest amount of c-di-GMP was 260 ± 27.3 µM (*D* of 0.3 h^{-1}) that was obtained from the co-culture of *C. kronotskyensis* and *C. owensensis* without a carrier. The population dynamics indicated that *C. owensensis* was the dominant species, and thus it produced higher c-di-GMP than *C. kronotskyensis* (**Paper IV**).

- Chitosan and biofilm formation could retain biomass during co-culture of *C. saccharolyticus* and *C. owensensis* in a chemostat. The maximum Q_{H2} was 8.4 mmol·L^{-1}·h^{-1} at a *D* of 0.8 h^{-1} in the presence of chitosan as a carrier material (**Paper III**).

- *Caldicellulosiruptor* species could not utilize chitosan as a carbon source. In additon, growth of *Caldicellulosiruptor* species was inhibited by chitosan concentration beyond 0.1 g·L^{-1} (**Paper III**).

- *C. owensensis* was the dominant species in the fermentation using a safe concentration of chitosan of 0.001 g·L^{-1}, whereas *C. saccharolyticus* was the dominant species in the absent of chitosan (**Paper III**).

- *C. kronotskyensis* is a promising candidate for hydrogen production through consolidated bioprocessing (**Paper I**).

- Co-culture of *C. saccharolyticus* and *C. owensensis* have a potential to improve volumetric hydrogen productivity (Q_{H2}) from 9.4 mmol·L^{-1}·h^{-1} to 11.1 mmol·L^{-1}·h^{-1} (**Paper I**).

- *C. kronotskyensis* did not possess a diauxic-like growth pattern when it was cultivated on glucose and xylose mixtures (**Paper I** and **Paper II**).

- Like other *Caldicellulosiruptor*, *C. kronotskyensis* prefers pentoses rather hexoses, but it takes up glucose in the form of disaccharides (cellobiose) (**Paper II**).

- *C. kronotskyensis* has the best performance that can be replaced *C. saccharolyticus* as a promising candidate for hydrogen production (**Paper I**, **II** and **IV**).

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