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in Biology

Cell factory and cell-free conversion of brown seaweed into valuable compounds by metabolic engineering

Antoine Moenaert

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FACULTY OF NATURAL SCIENCES

Cell factory and cell-free conversion of brown seaweed into valuable compounds by metabolic engineering

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Dissertation submitted in partial fulfillment of a
Philosophiae Doctor degree in Biotechnology

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Abstract

In the context of fossil fuel shortage and the need for more sustainability, recent emphasis has been put on finding new biomasses suitable for biotechnological applications, such as brown seaweed. They are the most abundant seaweed in Northern Europe and contain laminarin and alginate as main carbohydrates. The aim of this study was to utilize this biomass as a feedstock for microorganisms, using metabolic engineering, both *in vivo* and *in vitro*, to improve conversion of these carbohydrates to value-added products. The anaerobic thermophilic bacteria *Thermoanaerobacterium AK17* was used as a cell factory and engineered to produce ethanol as the sole fermentation product. The resulting strain AM6 reached an ethanol yield close to the maximum theoretical yield (95%), using mannitol, glucose and oligosaccharides from seaweed hydrolysate. Further genetic manipulations could increase the productivity of this new strain, by enabling the use of alginate and by increasing the ethanol tolerance. A cell-free approach was also considered, and a cascading enzymes reaction was designed to produce KDG, a precursor for bioplastic production, from alginate and laminarin, using different thermophilic enzymes. After fine tuning various enzymatic parameters, such as pH and temperature, KDG could be produced from alginate, but further optimizations are needed to produce it also from laminarin. In this context, a gene encoding a novel laminarinase was cloned and the recombinant enzyme characterized. Overall, this study is a proof of concept for brown seaweed valorization using metabolic engineering and paves the way toward a more sustainable world.

Útdráttur

Brúnþörungar, sem vaxa við Íslandsstrendur, hafa vakið athygli og áhuga sem hráefni fyrir líftækniiðnað, sérstaklega í ljósi minnkandi aðgengis að jarðefnaeldsneyti og aukinnar þarfar fyrir sjálfbærar lausnir í umhverfismálum. Brúnþörungar eru ríkir af sykrum og hafa þann kost fram yfir landplöntur að þeir krefjast hvorki ræktanlegs lands, ferskvatns, né áburðar. Jafnframt framleiða þeir meiri lífmassa á skemmri tíma en landplöntur. Markmið þessa rannsóknarverkefnis var að nýta brúnþörunga sem hráefni fyrir örverur og ensím og framleiða með hjálp efnaskiptaverkfræði og erfðatækni verðmætar afurðir úr þörungasykrum. Verkefninu var skipt í fjóra hluta. (1) Thermoanaerobacterium islandicum AK17, loftfirrt hitakær baktería, var þróuð sem frumuverksmiðja fyrir framleiðslu á etanóli. (2) Með erfðabreytingum tókst að fella út efnaskiptaferla og beina flæði orku og kolefnis að etanólframleiðslu. Nýr, breyttur stofn, AM6, framleiddi etanól á skilvirkan hátt (95% af fræðilegu hámarki) úr mannitóli, glúkósa og fásykrum í þörungahýdrólýsati. (3) Þróuð var aðferð til að framleiða 2-Keto-3-deoxygluconate (KDG), sem er milliefni í framleiðslu ýmissa verðmætra efna, úr alginati og lamínarin sykrum með hjálp hitakærra ensíma. (4) Ofangreindir framleiðsluferlar krefjast niðurbrots viðkomandi sykra úr brúnþörungum. Í tengslum við slíka niðurbrotsferla var nýtt ensím (laminarinasi) sem brýtur niður beta-glúkön í smásykrur, tvísykrur og glúkósa, framleitt með erfðatæknilegum aðferðum og eiginleikar þess rannsakaðir. Í heild sýna niðurstöður verkefnisins að með því að beita efnaskiptaverkfræði og erfðatækni geta brúnþörungar nýst sem sjálfbært hráefni í líftækni. Þannig getur verkefnið stuðlað að innleiðingu aðferða til að draga úr notkun jarðefnaeldsneytis og þar með þróun lífhagkerfisins, og sjálfbærari framtíð.

Table of Contents

List of Figures

List of Tables

List of Publications

List of Original Papers

1. **Moenaert, A**., López-Contreras, A.M., Budde, M., Allahgholi, L., Hou, X., Bjerre, A.-B., Örlygsson, J., Karlsson, E.N., Friðjónsson, Ó.H., Hreggviðsson, G.Ó. Evaluation of *Laminaria digitata* hydrolysate for the production of bioethanol and butanol by fermentation. *Fermentation* **2023**, *9*, 59. doi: 10.3390/fermentation9010059
2. **Moenaert, A.,** Bjornsdottir, B., Haraldsson, E.B., Allahgholi, L., Zieri, A., Zangl, I., Sigurðardóttir, S., Örlygsson, J., Karlsson, E.N., Friðjónsson, Ó.H., Hreggviðsson, G.Ó. Metabolic engineering of *Thermoanaerobacterium* AK17 for increased ethanol production in seaweed hydrolysate. Biotechnol Biofuels Bioprod. 2023 Sep 11;16(1):135. doi: 10.1186/s13068-023-02388-y.
3. **Moenaert, A.,** Csavas, G., Jensen, S., Karlsson, E.N., Friðjónsson, Ó.H., Hreggviðsson, G.Ó. Development of an enzymatic reaction cascade for the production of KDG from brown seaweed carbohydrates. Manuscript.
4. Allahgholi, L., Derks, M.G.N., **Moenaert, A.**, Dong, Z., Linares-Pastén, J.A., Friðjónsson, Ó.H., Hreggviðsson, G.Ó., Karlsson, E.N. Characterization of a GH17 laminarinase, MlGH17A, from a laminarin polysaccharide utilization locus in the marine bacterium *Muricauda lutaonensis*. Manuscript submitted to Applied and Environmental Microbiology.

Contributions not included in the thesis

5. Allahgholi. L., Derks, M.G.N., Dobruchowska, J.M., Jasilionis, A., **Moenaert, A**., Jouy, L., Ara, K.Z.G., Linares-Pastén, J.A., Friðjónsson, Ó.H., Hreggviðsson, G.Ó., Karlsson, E.N. Exploring a novel β-1,3-glucanosyltransglycosylase, MlGH17B, from a marine *Muricauda lutaonensis* strain for modification of laminari-oligosaccharides.Glycobiology.2024 Jan 25:cwae007. doi:10.1093/glycob/cwae007
6. Daugbjerg Christensen, M., Allahgholid, L., Dobruchowska J.M., **Moenaert, A.**, Guðmundssona, H., Friðjónssona, Ó.H., Karlsson, E.N., Hreggviðsson, G.Ó., Freysdottir, F. Laminarins and their derivatives affect dendritic cell activation and their crosstalk with T cells. International Journal of Biological Macromolecules, 306 (1) 2025,141287, https://doi.org/10.1016/j.ijbiomac.2025.141287
7. Hreggviðsson, G. Ó., Karlsson, E.N., Tøndervik, A., Aachmann, F.L., Dobruchowska, J. M., Linares-Pastén, J., Daugbjerg-Christensen, M., **Moenaert, A**., Kristjansdottir, T., Sletta, H., Fridjonsson, O. H., & Aasen, I. M. (2020). Biocatalytic refining of polysaccharides from brown seaweeds. Sustainable Seaweed Technologies, 447–504. doi: 10.1016/B978-0-12-817943-7.00016-0.

Author's contribution to the papers

1. A. López-Contreras and A. Moenaert contributed equally to this work. AM planned and performed all the cultures for *Thermoanaerobacterium* AK17, analyzed the data and wrote the draft manuscript together with A. López-Contreras, and was responsible for editing the co-author contributions.

2. A. Moenaert planned the study and performed most of the experiments. AM supervised master students for the cloning of vectors (A. Zieri and I. Zangl) and the recombinant expressions of enzymes (E.B. Haraldsson). AM analyzed all the data, wrote the draft manuscript, and was responsible for editing the coauthor contributions.

3. A. Moenaert planned the study and performed most of the experiments. AM supervised a master student for the cloning and expression of recombinant enzymes (G. Csavas). AM analyzed all the data and wrote the manuscript (submitted) and was responsible for editing the co-author contributions.

4. A. Moenaert identified the gene in the genome of a *Muricauda* species. AM cloned and expressed the gene in E.*coli*, verified the activity and conducted the first characterization of the enzyme. L. Allahgholi and co-workers performed further detailed characterization, and homology modeling. LA. wrote the draft manuscript (submitted) and was responsible for editing the coauthor contribution

Abbreviations

1,2PD: 1,2-Propanediol

A/IBE: Acetone, Isopropanol, Butanol, Ethanol (biofuel products)

Adh51: Aldo-Keto Reductase enzyme from *Rhodothermus marinus*

AK17: *Thermoanaerobacterium* strain AK17

AlyRm3 / AlyRm4: Alginate Lyases from *Rhodothermus marinus*

AM6: Engineered mutant strain derived from AK17

ACK: Acetate Kinase

ATP: Adenosine Triphosphate

BK: Butyrate Kinase

CaldoCas9: Thermostable Cas9 from *Geobacillus stearothermophilus*

Cat: Chloramphenicol Acetyltransferase (resistance marker gene)

CFS: Cell-Free Systems

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DEH: 4-deoxy-L-erythro-5-hexoseulose uronic acid

DhadSs: Dihydroxyacid Dehydratase from *Sulfolobus solfataricus*

DNA: deoxyribonucleic acid

Erm: Erythromycin Resistance Marker Gene

FDCA: Furandicarboxylic acid

FOA: 5-Fluoroorotic Acid

GeoCas9: Geobacillus-derived Cas9 Protein

GH: Glycoside Hydrolase

GHGs: Greenhouse Gases

IEA: International Energy Agency

IPCC: Intergovernmental Panel on Climate Change

KDG: 2-Keto-3-deoxygluconate

Kan: Kanamycin Resistance Marker Gene

MlGH17A: Laminarinase from *Muricauda lutaonensis*

MlGH17B: Glucanotransglycosylase from *Muricauda lutaonensis*

NAD(P)+: Nicotinamide adenine dinucleotide phosphate (oxidized form)

NAD(P)H: Nicotinamide adenine dinucleotide phosphate (reduced form)

PAM: Protospacer Adjacent Motif

PEF: Polyethylene Furanoate (bioplastic)

PPF: Poly(propylene 2,5-furan dicarboxylate) (bioplastic)

Pta-ack: Phosphotransacetylase–Acetate Kinase pathway

PTA: Phosphotransacetylase

PTB: Phosphotransbutyrylase

PyrF: Orotidine 5′-phosphate decarboxylase (uracil biosynthesis gene)

WT: Wild Type

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I have reserved this section for the very end, as a way to look back on everything I have been through, to think of all the people I met along the way, and to reflect on what I have learned from them, and about myself. As I look back on these years, I can only think of the words of Bilbo Baggins: "It's a dangerous business, going out your door. You step onto the road, and if you don't keep your feet, there's no knowing where you might be swept off to." This PhD has truly been that kind of road: unpredictable, challenging, and full of unexpected turns and detours. But a road I would gladly walk again. It led me to places I never expected, taught me more than I could have imagined, and introduced me to people who turned this journey into something far greater than the destination. In the end, I realize that every step, even the uncertain ones, was worth taking.

First and foremost, I would like to express my sincere gratitude to my supervisors Professor Guðmundur Óli Hreggviðsson and Dr. Ólafur H. Friðjónsson. I would not have started this journey if they were not part of it. I cannot thank them enough for their invaluable supervision, immense knowledge, guidance, and support throughout my studies. Their advice and trust helped me find my own way through research. They gave me room to try, to fail, and to grow, not only as a scientist but also as a person. I will always be thankful for their patience, support, and the example they set as researchers and mentors. Ólafur even taught me to fly-fish, and I kept practicing until he once told me that "the student had become the master." I hope to hear that again someday, though perhaps in a more scientific context. I am also sincerely grateful to my committee member, Professor Eva Nordberg Karlsson, for her supervision, scientific sparring, and invaluable feedback. Her comments on my manuscripts may have caused some sleepless nights, but they were always relevant and made every draft stronger. I also wish to thank my administrative supervisor, Professor Snædís Huld Björnsdóttir, for her understanding, expert guidance, and help in bringing this thesis to completion.

A big part of this experience was also mentoring, training, and encouraging the next generation of scientists to thrive in the world of research. This work is therefore also dedicated to all the students I had the privilege to supervise and who took part in this or other project: Léonie, Gabriel, Eveline, Anna, Einar, Wilbert, Stefanie, Katarina, Mathilde, Eloise, Nóa Sólrún, Arnar Kári, Cheryl, Madeleine, Amandine, Margaux, and Gabriella. A special thanks to Gabriella, who completed both her bachelor and master theses with me at Matís and later joined as an employee, giving me hope that perhaps I am not such a terrible supervisor after all. I am also grateful to my collaborators in the various projects I was involved in, especially Dr. Ana M. López-Contreras for helping me publish my first paper,

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Doing a PhD is also about learning balance, between what you enjoy and what you endure, between work and life, between effort and rest. Thanks to Ólafur (again), I rediscovered one of my oldest passions: fishing. And if I never counted the hours I spent in the lab, I certainly lost track of those spent by a river or a lake. Few things compare to the peace of those moments, shared with people who became more than friends: my fishing companions. This trip, as we also call our weekends, is also dedicated to them: Olivier, Tryggvi, Philippe, and David. Without your company, I would have faced more than one burnout along the way. You reminded me that a failed experiment can always be fixed, but a missed fishing trip cannot, and that laughter, shared stories, and a bit of competition are sometimes the best form of therapy.

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And what is a journey without a companion, a partner, a person who holds your hand even when you wear latex gloves? So, last but certainly not least, I want to thank my closest collaborator and dearest friend, Laurine. She has shared the sleepless nights, the long silences, and the excitement when experiments finally worked or a paper got published. Her kindness and love brought light to the most demanding moments, and her belief in me never failed, even when myself I stopped believing. None of this would have been the same, or even possible, without her by my side.

Now that this chapter is over, and a new one is about to begin, another quote from my old friend becomes more relevant than ever: "The greatest adventure is what lies ahead. Today and tomorrow are yet to be said. The chances, the changes are all yours to make."

1 Introduction

1.1 Global energy challenges and the potential of biomass

1.1.1 Energy crisis and sustainability challenges

The world is currently facing an unprecedented energy challenge characterized by an increasing global demand for energy and a heavy reliance on fossil fuels. As the global population grows and economies expand, the demand for energy continues to rise. According to the International Energy Agency (IEA), global energy consumption is projected to increase by nearly 50% by 2050, with the majority of this growth driven by developing countries and urbanization [1]. This rising demand places intense pressure on existing energy resources and infrastructure, raising concerns about energy security and the sustainability of current energy supply chains.

Fossil fuels, including coal, oil, and natural gas, have been the primary sources of energy since the Industrial Revolution. However, these are non-renewable resources, formed over millions of years and limited in supply. The depletion of fossil fuel reserves poses a significant threat to future energy security. Additionally, the extraction and consumption of fossil fuels are unsustainable in the long term, as they contribute to environmental degradation, pollution, and climate change. Burning fossil fuels releases large quantities of carbon dioxide (CO_2) and other greenhouse gases (GHGs) into the atmosphere, which are major contributors to global warming. The Intergovernmental Panel on Climate Change (IPCC) reports that anthropogenic GHG emissions, primarily from fossil fuel combustion, are responsible for over 75% of the observed increase in global average temperatures since the mid-20th century [2]. This warming has led to more frequent and severe weather events, rising sea levels, and adverse impacts on ecosystems and biodiversity. Moreover, air pollution from fossil fuels poses significant risks to public health. These environmental and health impacts underscore the urgent need to transition from unsustainable reliance on fossil fuels to cleaner, renewable energy sources.

Given these challenges, there is a growing recognition of the need for sustainable and renewable energy solutions that can meet global energy demands without compromising environmental integrity or economic stability.

1.1.2 Renewable energy sources

Renewable energy sources, by definition, are replenished naturally and offer a sustainable alternative to fossil fuels. Solar, wind, hydropower, and geothermal energy are among the most widely used renewable energy sources (Figure 1). Solar and wind technologies have developed rapidly due to falling costs and improved efficiency. Still, both are limited by

intermittency, as solar power is only generated during daylight hours, and wind power depends on unpredictable wind patterns [3]. Hydropower, generated by harnessing the energy of flowing water, provides a more stable source of energy. Still, its use is geographically limited, and large dams can have a significant environmental impact [4]. Geothermal and tidal energy offer more stable outputs but are also geographically constrained, making them less scalable.

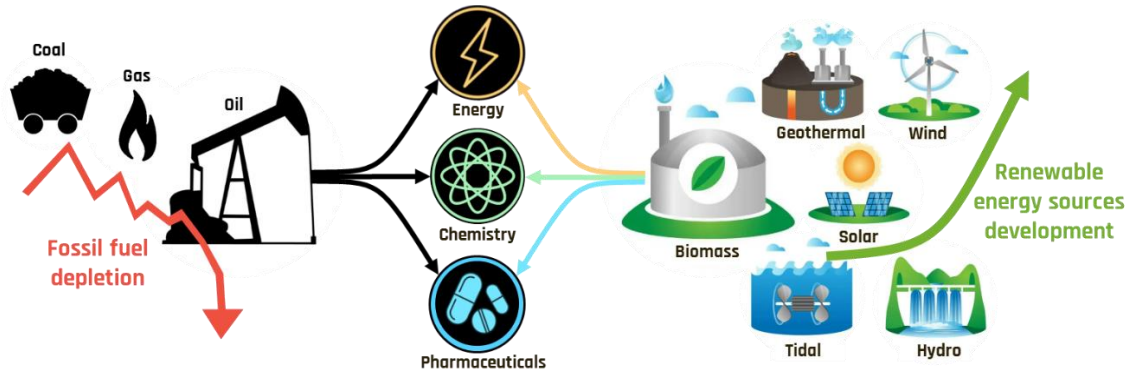

Figure 1 : Fossil fuel shortage and the development of renewable energy sources, especially using biomass

In contrast, biomass offers a more reliable and versatile renewable energy source. The energy in biomass is stored in the chemical bonds of organic molecules [5]. This stored energy can be released and harnessed in several ways. Through combustion, biomass is burned to generate heat, which can then be used for electricity production. In biochemical processes, such as fermentation, plant-based sugars are converted into biofuels, including bioethanol, which can be used as a renewable fuel for transportation. Anaerobic digestion breaks down organic waste materials to produce biogas, a renewable substitute for natural gas. Thermochemical processes, such as pyrolysis and gasification, can convert biomass into synthetic gases or bio-oils that can be further refined into fuels.

Biomass can also be considered carbon neutral when managed sustainably because the carbon dioxide emitted during energy production is balanced by the carbon absorbed by plants during their growth [6]. Among renewable options, biomass stands out for its versatility and potential to provide energy in forms beyond electricity, making it a critical component of the transition to a sustainable energy future.

1.1.3 Biomass as a renewable resource

Biomass refers to organic material, or feedstock, which can be used as a renewable energy source. In the context of biomass, feedstock is the raw material that is processed to produce energy, biofuels, or biochemicals. Depending on the generation of biomass, feedstock can range from food crops and agricultural residues to algae and waste products. Each type of feedstock varies in its availability, cost, and ease of conversion into usable energy (Figure 2).

First-generation biomass uses food-based feedstock such as corn, sugarcane, and vegetable oils. These feedstocks are rich in sugars and oils, which can be easily processed into biofuels such as bioethanol and biodiesel through fermentation or transesterification. The majority of bioethanol produced globally comes from first-generation feedstocks. In 2023, the U.S. alone produced approximately 59 billion liters of bioethanol from corn, while Brazil

produced around 31 billion liters from sugarcane [7]. While these figures demonstrate the significant role of first-generation biofuels in the global energy market, their reliance on food crops has raised concerns about food security, land use, and water consumption. It is estimated that by 2050, the world will need a 50% increase in food production compared to 2012 to satisfy the demand of an increasing population. But at the same time, the increase in land available for agriculture is only expected to rise about 5% [8]. As demand for biofuels grows, the competition between food production and energy production intensifies, and the exploration of other types of biomass becomes necessary.

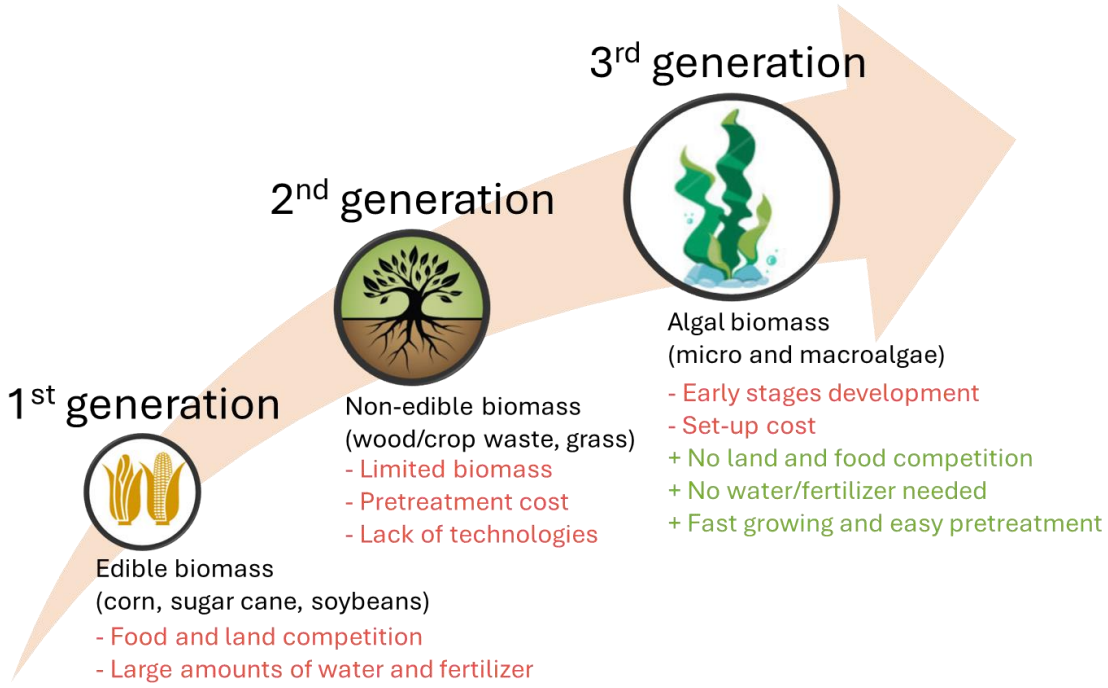

Figure 2: The three generations of biomass and their associated drawbacks and benefits.

Second-generation biomass relies on non-food feedstock, including agricultural residues (e.g., corn stover, straw), forestry waste (e.g., wood chips), and lignocellulosic materials [9]. These feedstocks, composed mainly of cellulose, hemicellulose, and lignin, do not compete with food production and are considered more sustainable. However, the production of second-generation biofuels is still in the early stages. As of 2019, only 12 lignocellulosic biorefineries were commercially operational in the world, producing only ~10 million liters of ethanol each [10]. The lower production volume is mainly due to the technical challenges of breaking down lignocellulosic materials into fermentable sugars, which require energy-intensive pretreatment and advanced enzymatic processes [11,12]. These challenges, combined with high production costs, have hindered the commercial scalability of second-generation biofuels and prompted the search for more accessible biomass.

Third-generation biomass, which includes algae, presents an even more promising and sustainable alternative. Algae can be divided into two main types: microalgae and macroalgae (seaweed). Microalgae are single-celled organisms that can grow in freshwater or saltwater environments, while macroalgae are multicellular and grow in marine environments. They both share similar properties, with an elevated productivity per area, fast growth rates, do not compete with food for land, do not require a fresh water supply, the

ability to absorb large amounts of CO_2 during growth, and potentially are easier to convert into final products than lignocellulosic biomass [13,14].

Microalgae are particularly valued for their high lipid content, which can be used to produce biodiesel [15]. They can also be used as nutraceuticals, functional food ingredients, pharmaceuticals, and for bioremediation, where they can absorb pollutants from wastewater, contributing to environmental sustainability [16–18]. However, they are commonly grown in photobioreactors, and commercial scalability of microalgae-based biofuels is still limited by high production costs, particularly for cultivation and harvesting [19]. On the other hand, macroalgae are naturally found in the ocean.

1.1.4 Seaweed as a promising biomass

Macroalgae, also known as seaweeds, are a diverse group of photosynthetic organisms found in marine environments. They are classified into different types, according to their chemical composition and pigmentation: red algae (Rhodophyta), green algae (Chlorophyta), and brown algae (Phaeophyceae) [20]. Red algae are primarily found in marine environments and are characterized by their reddish color due to the presence of pigments such as phycoerythrin and phycocyanin [21]. Green algae usually grow in freshwater and contain high amounts of chlorophyll pigment, responsible for their color, but some species are found in the sea [22]. Brown algae are the most abundant seaweeds in the world, primarily found in colder coastal waters. According to the relative abundance of the pigments fucoxanthin and chlorophyll, their color can vary from green to dark brown [23].

The chemical composition of macroalgae varies depending on the species, but typically includes structural carbohydrates (agar, alginate, carrageenan, fucoidan), storage carbohydrates (laminarin, mannitol, floridean starch), along with proteins, pigments, lipids, vitamins, and minerals [24]. Seaweeds grow rapidly in marine environments, requiring no arable land or freshwater, making them an environmentally sustainable option [25]. Their productivity can reach values up to 13.0 kg m^{-2} $year^{-1}$ on dry weight basis for some species. They are considered to have at least two times more biomass productivity than the highest obtained by any land plant, in which sugar cane is considered to be one of the most productive, with up to 6–8 kg m^{-2} $year^{-1}$ [26]. Seaweed carbohydrates can be processed into biofuels through biochemical conversion, such as fermentation, offering a promising solution to the growing demand for sustainable energy [27]. In addition, these carbohydrates could also be used to produce polymers and bulk chemicals which are traditionally produced from 1st generation biomass, like poly-lactic acid (PLA), or polyhydroxyalkanoates (PHAs) and building blocks including succinic acid and other metabolites with potential as bulk chemicals for the chemical industry [28]. These new building blocks could then be transformed to produce high value derivatives, like new fibers, solvents, materials, with new properties [29]. While still underdeveloped, ongoing research aims to unlock the potential of seaweed as a viable third-generation biofuel and bioproducts feedstock.

1.2 Brown seaweed biomass for sustainable biotechnological applications

1.2.1 Brown seaweed composition and key targets for biorefinery

Commonly harvested brown seaweed species include *Laminaria digitata*, *Saccharina latissima*, and *Ascophyllum nodosum*, each valued for their high carbohydrate content and ability to thrive in cold, nutrient-rich waters. Iceland, with its extensive coastline and largely unused brown seaweed resources, represents a significant opportunity for developing and scaling up a seaweed-based biorefinery. The composition of brown seaweed varies significantly depending on species, environmental conditions, and seasonality, with changes in temperature, light, and nutrient availability causing fluctuations in biochemical composition [30]. In general, brown seaweeds are composed of carbohydrates (40-60%), ash and minerals (20-40%), proteins (4-20%), lipids (1-6%), and polyphenols (3-5%) [31]. The high content of carbohydrates makes them an attractive alternative biomass, while the ash content, which reflects the inorganic mineral composition, can impact the efficiency of conversion processes in biofuel production, as it represents non-combustible material [32]. Carbohydrates in brown seaweed consist of three main biochemicals: alginate, laminarin, and mannitol (3). Other carbohydrates can be present in brown seaweed, like fucoidans and cellulose. But they are not described here as they will not be of interest in this thesis.

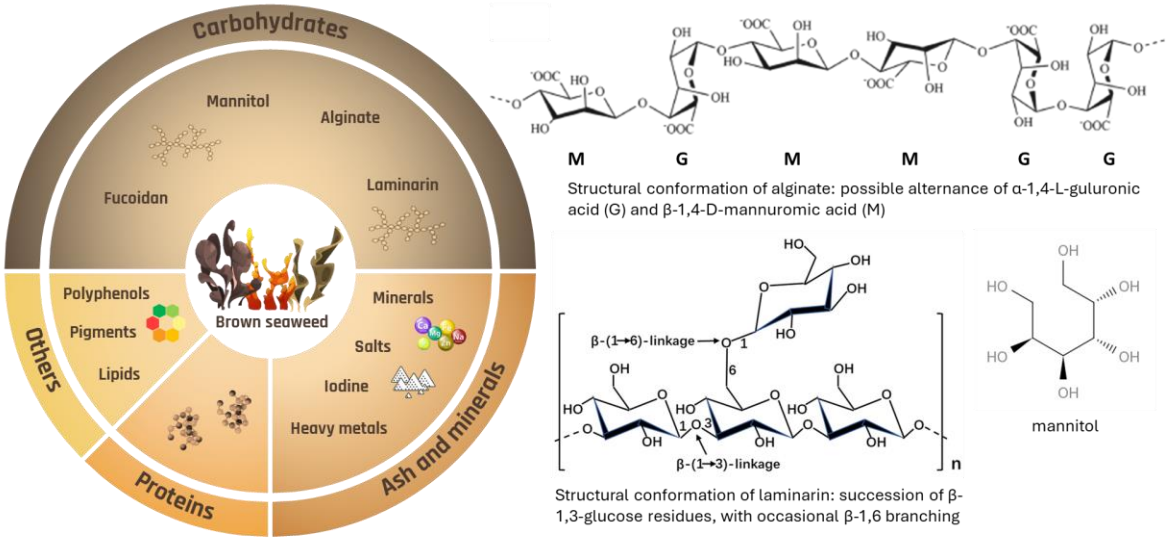

Figure 3: Brown macroalgae composition and structural conformation of the main carbohydrates.

Alginate is the predominant polysaccharide component found in the cell walls of brown macroalgae, accounting for 20-40% of their dry weight [33]. Alginate is a linear polysaccharide composed of two conformational isomer monomers: β-d-mannuronic acid (M) and α-l-guluronic acid (G) connected through 1,4-glycosidic linkages [34]. These monomers can be arranged in a homogeneous manner (as poly-M or poly-G) or in a heterogeneous manner, as randomly arranged M and G units. The relative proportions of M and G, as well as their arrangement within the polymer chain, vary according to numerous factors, including the algae species, growth conditions, season, and part of the algae [35].

The molecular weight of alginate can vary between 10 to 600 kDa [36]. Alginate is primarily used as a texturizing ingredient (for thickening and gelling), an emulsifier, and a fat substitute in the food industry. It is also used in the pharmaceutical industry, as a drug delivery system or for biosensing and wound healing [37].

Laminarin is the major storage carbohydrate of brown macroalgae. It is commonly found in the fronds of seaweed and can account for 5-35% of their dry weight [38]. Laminarin is a β-glucan, mainly composed of β-1,3-glucose residues, with occasional β-1,6 linkages [39]. There are two types of laminarin chains, according to the last residue at the reducing end. If the residue is mannitol, the chain is called an M-chain, and if the residue is a glucose, the chain is called a G-chain [40]. The ratio of β-1,3- and β-1,6-glycosidic bonds in the polysaccharide depends on the type of algae [41]. The molecular weight of laminarin is about 5 kDa, with a degree of polymerization between 20 and 25 [42]. Laminarin has been reported to have potential pharmacological properties such as antioxidant, anti-tumor, anti-coagulant, anticancer, immunomodulatory, anti-obesity, anti-diabetic, and anti-inflammatory [43].

Mannitol is produced during photosynthesis and can be found on its own as a free compound, or at the reducing end of laminarin M-chains, as described above, in a range of 5–25% of dry weight [44]. It is a sugar alcohol derived from D-mannose, which has a lower oxidation level compared to glucose, and can be directly fermented for biofuel production [45]. Mannitol is also utilized as a food additive and sweetener, and has been used to treat acute stroke and as a protective and therapeutic agent during neurological or renal failure [46].

Although these different carbohydrates can represent a significant portion of the seaweed biomass, they must be pre-treated and pre-processed to maximize the extraction and conversion of these key targets before they can be effectively utilized for biorefinery applications.

1.2.2 Seaweed preprocessing and pretreatment

Preprocessing and pretreatment involve a series of steps conducted post-harvesting and before utilizing seaweed biomass. They play a crucial role in the extraction of carbohydrates and serve two significant purposes: disrupting the cell wall of the macroalgae to facilitate the efficient release of carbohydrates into the extraction solvent and preventing the co-extraction of interfering bioactive compounds with similar solubility to carbohydrates [47].

Preprocessing prepares the biomass for further treatment and typically involves several steps. First, a washing step is performed, where the seaweed is generally washed to remove salt, sand, and other impurities. This step is essential because residual salts and minerals in seaweed can increase the ash content, which may later inhibit microbial fermentation processes. Then, there is a drying step, where the seaweed is usually dried to reduce its moisture content and prevent microbial degradation during storage. Drying methods can vary, including air drying, freeze-drying, or solar drying. Reducing moisture content to below 10% is recommended to ensure stability and prevent decomposition and fooling during long-term storage. Alternatively, instead of the energy-intensive drying step, other methods have been developed to reduce the cost of seaweed preservation, such as quick freezing and/or acidification [48,49]. Finally, a milling step is performed, where the seaweed is milled into fine particles to increase the surface area for subsequent enzymatic or chemical treatments. The particle size can influence the efficiency of pretreatment steps, as smaller particles provide more contact surface for chemical or enzymatic reactions. The

preprocessed seaweed biomass can then undergo several treatments according to the final target product/utilization (Figure 4). This can involve either the separation of major components or, as in **Paper 1**, the use of enzymes, such as commercial enzyme mixtures, to produce fermentable substrates.

Alginate is conventionally extracted through a process that involves acid pre-treatment followed by alkaline extraction. During acid treatment, alginate is converted into its insoluble form, alginic acid, by lowering the pH using mild acids, such as HCl. This step helps remove non-target compounds such as polyphenols and other polysaccharides. Next, the alginic acid is subjected to alkaline treatment, typically with sodium carbonate (Na_2CO_3) or sodium hydroxide (NaOH), which transforms it into water-soluble sodium alginate. After extraction, the alginate is precipitated using either alcohol or calcium chloride, depending on the desired alginate form [37].

Laminarin is typically extracted from brown seaweed through a series of steps involving acid extraction followed by membrane ultrafiltration. The process begins with the seaweed being combined with a mild acid, such as 0.1 M HCl, to solubilize the laminarin. After the seaweed has been subjected to acid treatment, ethanol precipitation, or membrane ultrafiltration can be used to further purify the laminarin extract. The membrane filtration step helps separate laminarin from other compounds, such as mannitol and proteins, based on molecular size [50].

Mannitol is usually subjected to hot water extraction (around 70°C). As mannitol is highly soluble in water, it readily dissolves in the solution. Then the liquid extract (containing mannitol) is separated from the solid residue through filtration or centrifugation. The filtrate, which contains mannitol along with other soluble sugars, is then further purified by ethanol precipitation and/or membrane filtration [51].

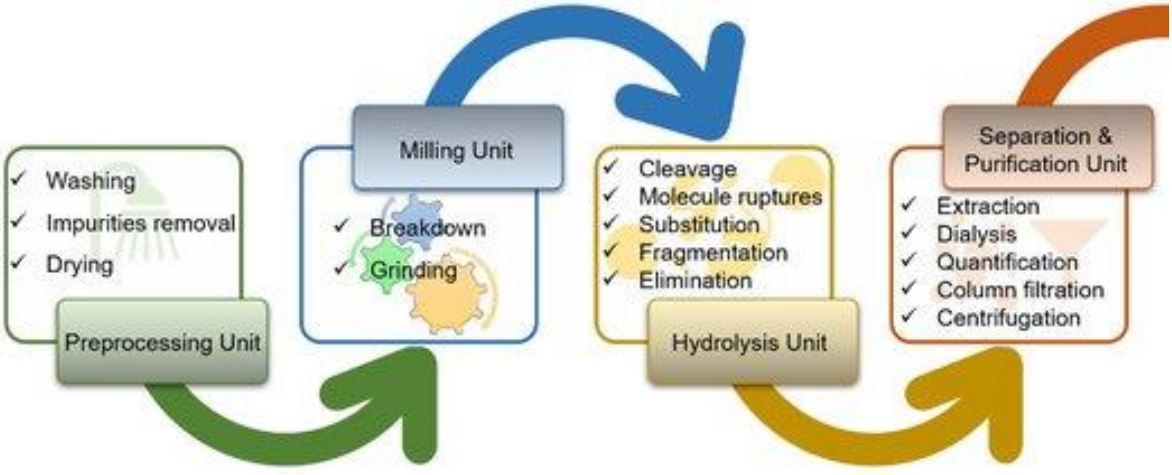

Figure 4: Example of brown seaweed preprocessing and pretreatment cascade. Adapted from the figure 4 found in Bilal et al. [52].

In the context of biofuel production, chemical or thermal treatment is not particularly suitable for subsequent fermentation, as these methods often lead to the formation of inhibitory byproducts, such as furfural and hydroxymethylfurfural (HMF), which can hinder microbial activity in fermentation [53]. Instead, enzymatic hydrolysis is favored for breaking down seaweed polysaccharides. Laminarinases are used to hydrolyze laminarin into glucose,

as seen in the newly described enzyme in **Paper 4**, while alginate lyases target the alginate structure to release fermentable sugars. Additionally, mixtures of hydrolytic enzymes, such as Viscozyme® and Celluclast®, are commercially available to enhance the efficiency of polysaccharide breakdown. These enzymatic approaches can be integrated into Separate Hydrolysis and Fermentation (SHF), where hydrolysis is completed before fermentation, or Simultaneous Saccharification and Fermentation (SSF), where hydrolysis and fermentation occur during the same step, streamlining the process and reducing time while maintaining efficiency.

1.2.3 Challenges and Future Prospects in Seaweed-Based Biofuel Production

While seaweed holds great promise as a renewable biomass for biorefinery applications, several significant challenges must be addressed to fully realize its potential. One of the primary issues is the variability in seaweed composition. The carbohydrate, protein, and mineral content of seaweed can vary significantly depending on the species, geographic location, and seasonal factors, making it challenging to standardize processing conditions and optimize the efficiency of biofuel production.

Another key challenge is the presence of inhibitory compounds in seaweed biomass. Recalcitrant compounds, such as ash, polyphenols, and heavy metals, are often co-extracted during the processing stages and can severely inhibit microbial fermentation, reducing biofuel yields [54]. In particular, the formation of byproducts such as furfural and 5-HMF during thermal or chemical hydrolysis can act as inhibitors, further complicating the fermentation process [53].

Consequently, the major limitation in current biorefinery approaches is the lack of versatile microbial strains capable of fermenting the wide range of polysaccharides found in seaweed, while also being tolerant to various inhibitory compounds. To overcome this, microbial strains must be robust and capable of fermenting seaweed carbohydrates, such as laminarin and mannitol, while tolerating high salinity and osmotic pressure. Developing or engineering highly adaptable microbial strains, such as *Thermoanaerobacterium* strain AK17, described in **Papers 1 and 2**, with broad substrate ranges and stress tolerance, could lead to microbial cell factories designed to efficiently process all seaweed-derived carbohydrates.

Furthermore, most current processes focus on extracting specific components from seaweed. This approach limits the overall utility of biomass. A more holistic approach that optimizes the use of the major seaweed carbohydrates could improve process efficiency and reduce waste and by-products [55]. Such approach could involve the development of *in vitro* cell-free synthesis systems, as the one proposed in **Paper 3**, which bypasses the limitations of microbial fermentation, and offers another promising solution. By using enzymatic systems to convert seaweed carbohydrates directly into value-added chemicals, it would be possible to extract the maximum value from the entire biomass, thereby creating a more efficient and sustainable seaweed biorefinery (see 1.4).

1.3 Microbial Cell Factories for Biofuel Production from Seaweed

1.3.1 Microbial cell factory and metabolic engineering

Microbial cell factory is an approach that considers microbial cells as a production facility in which raw materials are converted into specific products, such as biofuels, chemicals, or pharmaceuticals. By manipulating the metabolic pathways of these microorganisms, also known as metabolic engineering, they can be tailored to process a wide variety of substrates and produce specific target molecules [56].

Metabolic engineering involves altering the metabolism of an organism by modifying, adding, or deleting specific genes to enhance the efficiency of converting substrates, such as carbohydrates, into biofuel. Similarly, synthetic biology goes even further by constructing entirely new pathways or re-engineering existing ones to allow microbes to perform tasks they wouldn't naturally do, such as breaking down complex biomaterials or producing molecules that can be harmful or even toxic for them [57].

Several factors must be considered when selecting a host strain for industrial bioproduction. Ideally, the selection should be based on the metabolic characteristics of the organisms, including the substrate utilization range required for the feedstock to be used and the synthetic capabilities or potential of the organism. The latter is based on the present catabolic or anabolic pathways and the available precursor pool in the organism.

In the context of biofuel production from seaweed biomass, this translates to identifying strains that naturally utilize seaweed carbohydrates and produce biofuel. And if not possible, then engineering a strain which efficiently produces biofuel to make it use seaweed carbohydrates, or the opposite, by focusing on strains which utilize the seaweed efficiently and make them produce biofuel.

1.3.2 Current cell factories for biofuel production from seaweed and their limitation

Microbial cell factories for biofuel production have primarily focused on well-established, conventional organisms such as *Saccharomyces cerevisiae*, *Escherichia coli*, and *Clostridium* species [58]. These organisms are commonly engineered for ethanol and butanol production. While they are highly efficient with first-generation feedstocks, such as glucose, they face limitations when applied to second- and third-generation biomass, including seaweed.

Saccharomyces cerevisiae is the most widely used organism for ethanol production, and its fermentation processes are well established. It can achieve 90–95% of the maximum theoretical ethanol yield under optimal conditions, making it highly efficient when fermenting glucose. However, its limitation lies in its narrow substrate utilization range, as it cannot naturally ferment more complex carbohydrates, such as mannitol, laminarin, or alginate, which are found in seaweed. Recently, the strain *S. cerevisiae* AM1 has been engineered to utilize both mannitol and alginate, but success has been limited, with lower ethanol yields (32%) and efficiencies compared to first-generation biomass [59].

Escherichia coli, known for its ease of genetic manipulation, has also been engineered to ferment a broader range of substrates, including mannitol. For example, the E. coli KO11 strain was able to ferment *S. japonica* hydrolysates with an ethanol yield of 0.4 g/g sugars and a productivity of 0.22 g/L/h, demonstrating its potential for seaweed utilization [60]. However, despite its ability to be engineered for complex sugar utilization, *E. coli* tends to have lower ethanol yields and productivities compared to *S. cerevisiae*, and its tolerance to high ethanol concentrations remains a significant bottleneck.

Clostridium species are another important group of organisms used for acetone-butanol-ethanol (ABE) fermentation, with *Clostridium beijerinckii* being a notable example. Clostridia have a broad substrate utilization range, including C5 and C6 sugars and sugar alcohols like mannitol, making them promising candidates for seaweed biorefineries [61]. *C. beijerinckii* DSM 6422 achieved a butanol yield of 0.42 g/g-consumed-substrates from seaweed hydrolysate with a productivity of 0.12 g/L/h, which is in the low range from an industrial perspective [62]. In addition, another study highlighted the need for extra purification steps (desalting and removal of hydrophobic compounds), which would increase the overall cost and lower the economic feasibility [63].

In recent years, efforts have turned toward identifying non-conventional microorganisms capable of efficiently utilizing seaweed carbohydrates. For instance, *Wickerhamomyces anomalus* M15, a marine yeast that tolerates high salt concentrations, has shown high ethanol yields from synthetic seaweed medium [64]. However, it only consumed glucose and hypothetically a small fraction of mannitol, but was mostly inhibited by the mannitol, which limits its effectiveness when working with brown seaweed feedstocks.

Other promising organisms include thermoanaerobes, which can ferment a broader range of sugars derived from seaweed. *Defluviitalea phaphyphila* Alg1, a thermophilic organism, has demonstrated the ability to ferment brown macroalgae with an ethanol yield of 0.45 g/g-consumed-substrates, but at low productivity levels of 0.13 g/L/h [65].

The limitations of current microbial cell factories highlight the need for the development of new strains or the optimization of existing non-conventional strains for the utilization of seaweed biomass. These new strains should have a broad substrate range, high yield, and productivity, and be robust enough to withstand the harsh conditions required for large-scale biofuel production from seaweed.

1.3.3 *Thermoanaerobacterium* strain AK17 as a candidate for bioethanol production from seaweed

Thermoanaerobacterium strain AK17 is a thermophilic anaerobe isolated from a geothermal hot spring in Iceland [66]. In previous studies, AK17 demonstrated the ability to grow on a variety of C6 (glucose, fructose, galactose, mannose) and C5 monosaccharides (xylose, ribose, arabinose), and disaccharides (sucrose, lactose), and also the ability to grow on polysaccharides (like xylan, starch, pectin) [67]. The fermentation products of AK17 are a mixture of ethanol, acetate, lactate, and hydrogen, with an ethanol yield reaching up to 75% [68].

One of the key advantages of AK17 is its ability to grow at elevated temperatures (60°C), which provides several benefits for industrial applications. High-temperature fermentation reduces the risk of contamination by mesophilic organisms, lowers cooling costs, and

enhances the solubility of polysaccharides, making it easier to process high-concentration seaweed hydrolysates. Additionally, these high temperatures enable the more efficient recovery of volatile products, such as ethanol, through distillation or gas stripping, thereby helping to alleviate product inhibition and extend the fermentation phase. AK17 is also aerotolerant and salt-tolerant (up to 2%), which would ease the fermentation process.

The versatility of AK17 in utilizing mixed carbon sources, capable of fermenting both simple and complex sugars to ethanol at a good yield, combined with its thermophilic nature, makes it a valuable candidate strain for use as a microbial cell factory in seaweed-based biofuel production. However, its ethanol yields and productivity, while promising, would benefit from further optimization by metabolic engineering to fully exploit its potential in industrial biorefineries.

1.3.4 Genetic engineering of *Thermoanaerobacterium* strains for bioethanol production

Thermoanaerobacterium species have emerged as robust microbial platforms for biotechnological applications due to their natural thermophilic properties, extensive substrate utilization range, and ability to produce valuable biofuels and biochemicals. These organisms thrive at elevated temperatures (50–70°C), enhancing enzymatic activity while reducing contamination risks, making them ideal candidates for industrial processes. Recent advances in genetic engineering have significantly enhanced the metabolic potential of *Thermoanaerobacterium* species, enabling higher yields and broader substrate utilization. A key engineering strategy involves the deletion of genes encoding enzymes in competing metabolic pathways to redirect metabolic flux toward desired products like ethanol. For instance, in *Thermoanaerobacterium saccharolyticum*, the deletion of lactate dehydrogenase and acetate kinase genes redirected carbon flux entirely toward ethanol production, achieving a titer of 37 g/L [69]. Similarly, in T. *aotearoense* SCUT27, the deletion of the lactate dehydrogenase gene coupled with the deletion of the *pfl* (pyruvate formate-lyase) gene increased the ethanol yield by 50% while increasing the ethanol tolerance up to 4% (v/v) [70]. These modifications not only simplify downstream product separation but also demonstrate the versatility of gene deletions in tailoring metabolic pathways for industrial purposes, as also shown in **Paper 2**.

In addition to gene deletions, heterologous gene expression can also expand the substrate range and production capabilities of *Thermoanaerobacterium* species. For instance, the introduction of the urease from *Clostridium thermocellum* in T. *saccharolyticum* allowed the assimilation of nitrogen from the low-cost urea (instead of the more expensive ammonium salts) and led to an increase in ethanol titer of 54g/L [71]. But until now, heterologous expression has usually been performed in the other way, taking genes from *Thermoanaerobacterium* species and expressing them in other hosts, such as *Clostridium thermocellum* [72–74] and *Caldicellulosiruptor bescii* [75,76]. One of the main reasons for this was the lack of easy and quick genetic tools available for other species, but not for *Thermoanaerobacterium*.

Table 1 : Genetic engineering tools available for *Thermoanaerobacterium* and development of CRISPR/Cas systems in thermophilic bacteria

Selection marker genes

Selection marker gene	Selective drug	Working concentrations (ug/mL)	Selection type	References
kan	Kanamycin	50-200	Positive	[77]
erm	Erythromycin	5-10	Positive	[69,78]
cat	Chloramphenicol	40-80	Positive	[75]
pyrF	5-fluoroorotic acid (5-FOA)	100	Negative	[79,80]
Pta-ack	Haloacetates	23	Negative	[79]

CRISPR/Cas systems

Species	Cas proteins	CRISPR/Cas system	References
Clostridium thermocellum	GeoCas9	Type II	[81]
Thermoanaerobacter ethanolicus	GeoCas9	Type II	[82]
Thermus thermophilus	CaldoCas9	Type II	[83]
Clostridium thermocellum	Multiple Cas proteins	Type I-B	[81]
Parageobacillus thermoglucosidasius	Multiple Cas proteins	Type I-B	[84]
Hungateiclostridium thermocellum DSM 1313	Thermostable dCas9	CRISPRi	[85]
Thermoanaerobacterium aotearoense SCUT27	Endogenous system	Type I-B	[86]

During the past decade, efforts have been made to remediate this shortcoming, and several genetic tools have been developed such as antibiotic selection, marker recycling systems, and plasmid-based expression systems (Table 1). More recently, advanced genome editing technologies such as CRISPR/Cas-based systems have emerged, and some have been tailored to thermophilic organisms. Despite these successes, the application of CRISPR systems in *Thermoanaerobacterium* remains relatively new and underdeveloped compared to mesophilic organisms, with only one system developed so far, in *T. aotearoense* SCUT27 in 2022 [86]. These advancements collectively lay the groundwork for more sophisticated engineering of *Thermoanaerobacterium* strains in the future.

To date, most research on *Thermoanaerobacterium* has been focused on utilizing second-generation biomass, such as lignocellulosic feedstocks, for biofuel production. No studies have explored the potential of this genus to process macroalgae as a substrate. By integrating the advanced engineering techniques and metabolic insights developed for *Thermoanaerobacterium* on lignocellulosic biomass together with the intrinsic characteristics of the AK17 strain, this study represents an innovative effort to produce biofuels from seaweed carbohydrates using a *Thermoanaerobacterium* strain (**Papers 1 and 2**).

1.4 Cell-free synthesis of value-added chemicals from seaweed carbohydrates

1.4.1 Overview of Cell-Free Synthesis

Cell-free synthesis (CFS), or system, represents a cutting-edge biotechnological approach where biochemical reactions are performed without the need for living cells. Instead, these systems rely on isolated enzymes or crude extracts from cells to catalyse reactions *in vitro*. This approach allows for highly controlled conditions and has several advantages over traditional cell-based synthesis [87]. One of the primary benefits of CFS is the ability to bypass the complex regulatory networks and metabolic bottlenecks that are inherent in living cells. Without the need to maintain cellular viability, it is possible to achieve higher productivity by optimizing the concentrations of enzymes, substrates, and cofactors [88]. Furthermore, CFS enables the synthesis of complex molecules or chemicals that might be toxic to cells, offering a solution for the production of high-value compounds [89].

Cell-free protein synthesis (CFPS) is one of the most well-developed areas of cell-free systems, with applications in pharmaceuticals, proteomics, and synthetic biology [90]. CFPS allows for rapid protein production and has been adapted to create therapeutic proteins and antibodies, bypassing the limitations of cell-based expression systems, such as slow growth or the need for large-scale bioreactors [91]. However, the use of CFS is not limited to protein synthesis. They are being increasingly applied in other fields, including biofuel production and the synthesis of bioplastics. For instance, synthetic enzymatic pathways have been developed *in vitro* to convert complex biomass substrates into high-value chemicals [87].

Despite their advantages, CFS face challenges that limit their upscaling and potential industrial applications. These challenges include the cost of enzymes, their stability over prolonged reaction times, and the need for efficient regeneration of essential cofactors such as NADH and ATP. It can also require the discovery and characterization of new enzymes, such as the newly described enzymes in **Papers 3 and 4**. Addressing these bottlenecks through advancements in enzyme engineering and cofactor recycling is critical for the broader application of CFS [90]. Nonetheless, recent advancements in enzyme engineering have opened up new opportunities for their industrial application.

1.4.2 Current Applications of Cell-Free Systems

Cell-free systems (CFS) offer significant advantages for the production of a wide range of bio-based products, from fuels and platform chemicals to therapeutics (Table 2).

One notable application is in the production of fuels. For example, ethanol is produced from glucose using a six-enzyme cascade, achieving a 57% yield with a production rate of 1.5 mM/h and a concentration of 29 mM [92]. Similarly, n-butanol, a promising biofuel, is synthesized from glucose via a 16-enzyme system, with a yield of 82% and a production titer of 3.5 mM [93]. Other applications also include the production of hydrogen from both xylose and glucose present in the hydrolysate of corn stover [94].

CFS also plays a significant role in the synthesis of platform chemicals, which serve as precursors for various industrial processes. For example, glucaric acid, commonly used to

produce biodegradable polymers, is produced from sucrose through a seven-enzyme cascade, yielding 75%, with a production rate of 0.6 mM/h [95]. L-lactate, used in the production of bioplastics (polylactic acid), is produced from glucose via a five-enzyme cascade, achieving a 90% yield [96]. Other platform chemicals produced using CFS include malate, pyruvate, L-alanine, and more [97–99]. In this thesis, CFS is developed for the production of the platform chemical 2-Keto-3-deoxygluconate (KDG), via a five-enzyme cascade (**Paper 3**).

Table 2: Cell-free enzyme pathways reported for the production of biofuels or high-value chemicals from different substrates

Product	*Substrate*	*Enzymes Involved*	*Yield (% mol/mol or g/g)*	*Production Titer (mM or g/L)*	*Productivity*	*Ref.*
Fuel						
Ethanol	glucose	6 enzymes	57%	29mM	1.5mM/h	[92]
Hydrogen	corn Stover	14 enzymes	100%	N/A	2.3 mM/h	[94]
n-Butanol	glucose	16 enzymes	82%	3.5 mM	0.5mM/h	[93]
Isobutanol	glucose	8 enzymes	53%	10 mM	0.5mM/h	[92]
Platform chemicals						
Malate	starch	12 enzymes	95%	53mM	1.5mM/h	[97]
L-alanine	glucose	5 enzymes	95%	23mM	1.9mM/h	[99]
Pyruvate	chitin	12 enzymes	42%	2mM	0.4mM/h	[98]
Cystein	glucose	11 enzymes	50%	10mM	0.8mM/h	[100]
L-Lactate	glucose	5 enzymes	90%	50 mM	6mM/h	[96]
Glucaric acid	sucrose	7 enzymes	75%	35 mM	0.6mM/h	[95]
Therapeutics, cosmetics, food industry						
Monoclonal antibodies	NTPs, amino acids, ATP	cell extract	N/A	0.5 g/L	N/A	[91]
Limonene	glucose	27 enzymes	88%	92mM	0.5mM/h	[101]
Vitamin B12	hydrogenobyrate	36 enzymes	9%	4.3µM	0.3µM/h	[102]
D-Allulose	sucrose	5 enzymes	70%	N/A	1g/l/h	[103]
Myo-Inositol	glucose	3 enzymes	90%	45mM	1.9mM/h	[104]

Beyond fuels and platform chemicals, CFS is also used in the production of therapeutic proteins and high-value biomolecules. For instance, monoclonal antibodies can be synthesized using cell-free extracts, with a production titer of 0.5 g/L, demonstrating the potential of CFS in biopharmaceutical manufacturing [91]. Another example is the production of vitamin B12 from hydrogenobyrate, using a complex system of 36 enzymes, reaching a 9% yield [102]. Other examples include the production of limonene, used in flavors and fragrances, and the production of myo-inositol, which has both pharmaceutical and cosmetic applications [101,104].

Although these examples highlight the flexibility and efficiency of CFS, many other products can be obtained through similar enzymatic cascades. However, these systems now typically rely on glucose as the primary substrate, derived from first-generation biomass, which may limit their overall sustainability. To mitigate the environmental impact of CFS, future efforts could focus on utilizing seaweed carbohydrates as alternative substrates for producing platform chemicals and other high-value products.

1.4.3 Genes and enzyme candidates for conversion of seaweed biomass

Currently, there is a limited number of commercially available enzyme candidates for bioconversions with brown seaweeds as starting material. To alleviate this lack of commercial enzymes targeted for the material, in-house cloning and production are used to obtain candidates from marine resources, both for the hydrolysis of the seaweed polymers and for the production of the desired platform chemical (KDG). Access to enzymes can be assured, either by cloning genes from genome sequences of marine species deposited in databases or by isolating genomic/metagenomic DNA from relevant environments. In this thesis, both commercially available enzyme mixes (initially developed for terrestrial material, as in **Paper 1**), and in-house produced enzymes (**Papers 3 and 4**) have been used (Table 3) to obtain relevant biocatalysts for both CFS, and genes for modification of microbial pathways.

1.4.4 Production of KDG from Seaweed Polysaccharides

2-Keto-3-deoxygluconate (KDG) is a valuable precursor in the chemical industry, particularly for the potential synthesis of bio-based products like furanics, such as FDCA (furandicarboxylic acid), which is a key component for producing bioplastics (e.g., PEF and PPF) [105,106]. FDCA has significant market potential, recognized as one of the top 12 biobased chemicals by the US Department of Energy [107]. KDG is also used in pharmaceuticals, serving as a precursor for the synthesis of 2'-deoxynucleoside compounds, with applications in antiviral and anticancer treatments (patent EP1491549, 2004). Furthermore, KDG can be oxidized into 5-keto-4-deoxyglucarate (DOHA), a precursor for α-ketoglutarate, which has applications in dietary supplements and the medical industry [108,109]. This makes KDG a highly versatile and promising compound for industrial bioprocessing applications.

When designing enzymatic cascades for KDG production, the aim was to mimic the pathways found in naturally occurring thermophilic organisms that degrade brown seaweed. For instance, *Rhodothermus marinus*, a thermophilic marine organism, possesses an alginate degradation pathway, in which KDG is as a key intermediate before entering glycolysis through the Entner-Doudoroff pathway [110]. Alginate is depolymerized by alginate lyases to unsaturated monouronates, which are spontaneously converted to 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) and further catalyzed to 2-keto-3-deoxygluconate (KDG) by an aldose reductase, using NAD(P)H as cofactor.

Table 3: List of enzyme candidates for the conversion of brown seaweed carbohydrates mentioned in this thesis.

Enzyme designation	*Activity or enzyme-type*	*Production*	*Described use in thesis section*
Celluclast	cellulase	Commercial (Novonesis)	Section 1.2.2
Viscozyme	multi-enzyme complex including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase from Aspergillus	Commercial (Novonesis)	Section 1.2.2
NS81016	mixture of beta-glucanases and beta-glucosidases	Commercial (Novonesis)	Paper 1
AlyRm3	alginate lyase from *Rhodothermus marinus*	*E. coli* (in-house)	Section 1.4.5 and paper 3
AlyRm4	reducing end cleaving exo-lyase from *R. marinus*	*E. coli* (in-house)	Section 1.4.5 and paper 3
Adh51	aldo-keto-reductase from *R. marinus*	*E. coli* (in-house) AK17 (in-house)	Section 1.4.5 and paper 3 Section 3.5.1
GDH16	glucose dehydrogenase from *Thermobacculum* sp. MAT1686	*E. coli* (in-house)	Section 1.4.5 and paper 3
DhadSs	dihydroxyacid dehydratase from *Sulfobus solfataricus*	*E. coli* (in-house)	Section 1.4.5 and paper 3
Amo176	glucanase from metagenomic DNA extracted from Icelandic hotspring (Vonarskarð). Belongs to GH16 family, with endo-activity.	*E. coli* (in-house)	Section 1.4.5 and 3.5.1
Amo939	laminarinase from *Rhodothermus marinus*. Belongs to GH16 family, with endo-activity	*E. coli* (in-house)	Section 1.4.5 and 3.5.1
Cel136	pustulanase from metagenomic DNA extracted from environmental sample. Belongs to GH30 family, with exo-activity	*E. coli* (in-house)	Section 1.4.5 and 3.5.1
MlGH17A	laminarinase from *Muricauda lutaonensis*	*E. coli* (in-house)	Section 1.4.5 and paper 4
MlGH17B	laminarin tranglycosylase from *Muricauda lutaonensis*	*E. coli* (in-house)	Paper 5
Amo78	glucanase from R. marinus. Belongs to the GH3 family, with an exo-activity	*E. coli* (in-house) AK17 (in-house)	Section 3.5.1

However, the challenge with using alginate alone is the imbalance in cofactor regeneration (NAD(P)+/NAD(P)H), which is crucial for maintaining the efficiency of the enzymatic reactions. To address this, an additional branch to the enzymatic cascade was constructed, with the 2-steps conversion of glucose into KDG. First, the glucose would be oxidized to gluconate, with the regeneration of the cofactor. And the gluconate could then be converted to KDG by a dehydratase. In addition, the glucose used as substrate in this second branch could be sourced from the laminarin, with the addition of laminarinases.

Thus, by combining the degradation of alginate and laminarin, this CFS was designed for the production of KDG from the two main polysaccharides found in brown seaweed,

maximizing the utilization of seaweed biomass and ensuring a balanced cofactor system, and theoretically enhancing both efficiency and sustainability (Figure 5).

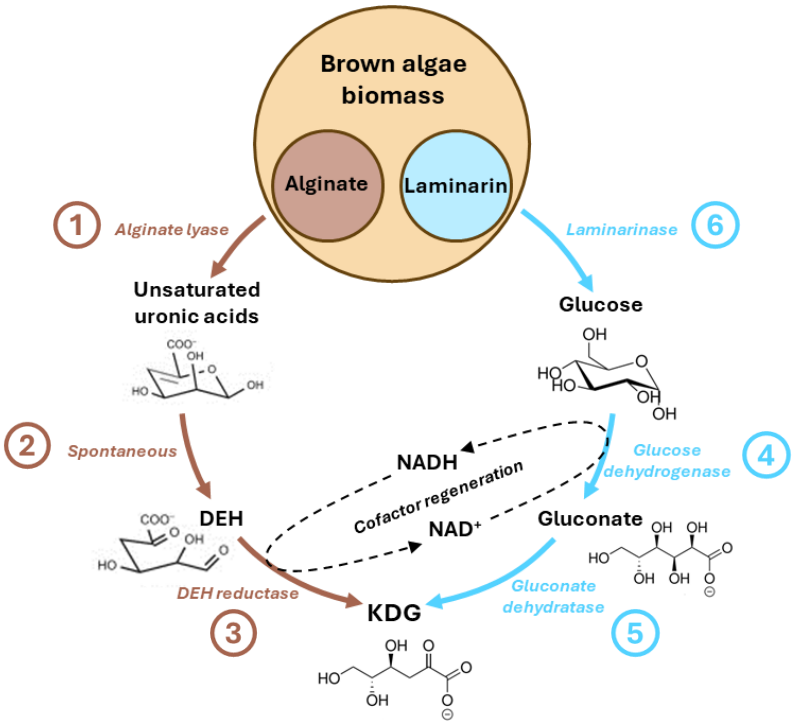

Figure 5: Schematic representation of the designed synthetic pathway for the production of KDG from seaweed carbohydrate

1.4.5 Choice of enzymes for the synthetic pathway

In this study, the enzymes chosen for the CFS were either previously described as having relevant activities or were annotated to have specific activities, as determined by the MATIS database or the literature (Table 3). Each step of the CFS is described below, and their respective numbers refer to the Figure 5:

1. Two alginate lyases, AlyRm3 and AlyRm4, are used for the depolymerization of alginate to monouronates. These two enzymes originate from *R. marinus* and have been previously described [111]. AlyRm3 is an endo-type lyase that cleaves the alginate polysaccharide chains into oligosaccharides of different lengths (di-, tri-, and tetrasaccharides). AlyRm4 is an exo-type lyase that cleaves the alginate from the reducing end, resulting in the production of unsaturated uronic acids.

2. Unsaturated uronic acids are spontaneously converted to 4-deoxy-L-erythro-5-hexosulose (DEH). It has been shown that in aqueous solution, DEH is unstable and can be further hydrated in a spontaneous process to form the cyclic hemiacetal stereoisomers. It was suggested that they could be the precursor of non-enzymatically generated organic acids [59].

3. DEH is reduced to KDG via a DEH-reductase. Within the alginate operon in *R. marinus* MAT378, there is an aldo-keto reductase called Adh51, supposedly reducing DEH to KDG,

together with other genes encoding enzymes of the Entner-Doudoroff pathway. The Adh51 is responsible for the reduction step, implying the use of a cofactor, namely NADH/NAD+ (**Paper 3**).

4. The glucose dehydrogenase GDH16 was chosen for the oxidation of glucose to gluconic acid via glucose lactone (**Paper 3**), concomitantly regenerating NADH from NAD+. The encoding gene originated from *Thermobacculum* sp. MAT1686, a thermophilic strain isolated by MATIS. It was shown to be stable at 50°C and to have affinity towards glucose (data not shown).

5. DhadSs is a dihydroxyacid dehydratase from *Sulfolobus solfataricus*, which was shown to be capable of converting gluconic acid to KDG [112]. It was chosen as the best candidate as it is the only thermostable dehydratase. Since then, a new dehydratase has been described from *Caulobacter crescentus*, which is not thermostable but highly active [113].

6. Laminarin could be digested by β-glucanases such as the laminarinase MlGH17A (**Paper 4**), or other more thermostable variants available at MATIS, and which, combined with β-glucosidases (e.g. those from *R. marinus* [114]) can hydrolyze laminarin completely into glucose. But as laminarin was hard and expensive to obtain, and laminarin-degrading enzymes were more commonly available than the other enzymes in the pathway, the CFS was only tested with alginate and glucose, as a proof of concept.

2 Hypotheses and Objectives

The overall aim of this project was to develop two systems —one cell-based and one cell-free —to produce valuable compounds from seaweed biomass. Four main hypotheses were constructed, two regarding the use of *Thermoanaerobacterium* strain AK17 as a cell factory (H1-H2), and two for the production of KDG from seaweed carbohydrate in a cell-free synthesis (H3-H4). They are all listed below.

H1 *Thermoanaerobacterium* strain AK17 can be effectively utilized for ethanol production from brown seaweed carbohydrates, increasing its suitability as a microbial platform for biofuel production.

H2 Deleting specific metabolic pathways responsible for by-product formation in *Thermoanaerobacterium* strain AK17 could significantly redirect the metabolic flux towards ethanol production, thereby increasing yield and productivity.

H3 A cell-free enzymatic cascade can be developed to convert alginate and laminarin from brown seaweed into 2-keto-3-deoxygluconate (KDG) by integrating specific enzymes.

H4 Effective recycling of the cofactor NADH can be achieved using another enzymatic cascade to convert glucose into KDG by integrating specific enzymes.

The following objectives were used to assess the hypotheses and guide the work:

O1 Assess the fermentation capabilities of *Thermoanaerobacterium* strain AK17 for ethanol production from brown seaweed carbohydrates.

O2 Genetically engineer *Thermoanaerobacterium* strain AK17 to enhance ethanol production by deleting specific metabolic pathways.

O3 Develop and optimize a cell-free enzymatic cascade for converting alginate into KDG

O4 Develop and optimize a cell-free enzymatic cascade for converting laminarin/glucose into KDG, ensuring continuous cofactor regeneration

O5 Integrate and evaluate the combined enzymatic cascades for the simultaneous production of KDG from alginate and glucose

3 Summary of the work

3.1 Evaluation of *Laminaria digitata* hydrolysate for the production of the liquid biofuels ethanol and butanol by fermentation (paper 1)

This study evaluated the potential of *Laminaria digitata* hydrolysate, derived from enzymatically processed brown seaweed, as a substrate for biofuel production. Ethanol fermentation was conducted using *Saccharomyces cerevisiae* and *Thermoanaerobacterium* strain AK17, highlighting the novel ability of AK17 to utilize oligo-laminarin. Additionally, the hydrolysate was tested for the production of acetone, isopropanol, butanol, and ethanol (A/IBE) using *Clostridium* strains. The results demonstrated the feasibility of using brown seaweed as a sustainable feedstock for producing valuable biofuels, providing insights into optimizing microbial fermentation processes for enhanced biofuel yields.

3.1.1 Ethanol fermentation by *S. cerevisiae* and *Thermoanaerobacterium* strain AK17

This study investigated the use of *Laminaria digitata* hydrolysate for ethanol production through fermentation by *Saccharomyces cerevisiae* and *Thermoanaerobacterium* strain AK17. The hydrolysate was obtained by enzymatic hydrolysis of dried seaweed, using the enzyme mixture NS81016 (Novonesis). The hydrolysis was stopped after 24h, and due to incomplete hydrolysis of the biomass, a substantial quantity of glucans (laminarin) remained in the hydrolysate [62]. In addition, it also contained fermentable sugars such as glucose and mannitol, which served as the carbon source for microbial growth and fermentation.

Saccharomyces cerevisiae was utilized as a benchmark microorganism for ethanol production due to its established ability to ferment glucose efficiently. The results indicated that *S. cerevisiae* could effectively ferment the monomeric glucose present in the hydrolysate, achieving an ethanol yield of 72% of the maximum theoretical yield based on the glucose consumed (Table 4). However, the yeast showed limitations in its ability to ferment mannitol and glucan oligosaccharides, which restricted the overall conversion efficiency to 38% of the total carbohydrate content in the hydrolysate. This finding highlights the necessity of using more versatile organisms capable of metabolizing a broader range of substrates for the efficient conversion of complex biomass-derived hydrolysates [59,60].

Table 4: Substrate consumptions and ethanol yields of S. *cerevisiae* and *Thermoanaerobacterium* AK17 in cultivations using different amounts of L. digitata hydrolysate as raw material. Data represent the average of three replicate experiments. Ethanol yields were considered to highlight either the added amount of substrate (total) or the substrate utilization profile (partial). This table corresponds to Table 2 in Paper 1.

Strains and Conditions		Free glucose consumption (%)	Sugar consumption in g/L (% of total sugars)	Ethanol yield total (% of theoretical)	Ethanol yield partial (% of theoretical)
AK17	50% hydrolysate	100	7.58 ± 0,5 (56)	0.16 ± 0,01 (32)	0.29 ± 0,01 (57)
	100% hydrolysate	82 ± 2	12.2 ± 0,9 (49)	0.15 ± 0,01 (30)	0.31 ± 0,01 (61)
S. cerevisiae	100% hydrolysate	100	9.3 ± 0,3 (38)	0.14 ± 0,01 (27)	0.37 ± 0,01 (72)

In contrast, *Thermoanaerobacterium* strain AK17 demonstrated a significantly broader substrate utilization capacity, particularly with its novel ability to utilize oligo-laminarin, a type of glucan oligosaccharide (Figure 6). This capability is noteworthy, as it represents the first instance of a *Thermoanaerobacterium* strain being shown to utilize β-1,3-glucans, such as oligolaminarin, for ethanol production. Strain AK17 produced extracellular glucanases that could specifically degrade laminari-oligosaccharides to laminaribiose, which were then transported into the cell for further metabolism. This enzymatic activity enabled AK17 to partially hydrolyze and ferment laminarin-derived oligosaccharides, in addition to glucose and mannitol, leading to a more comprehensive utilization of the carbohydrate content in the hydrolysate [115].

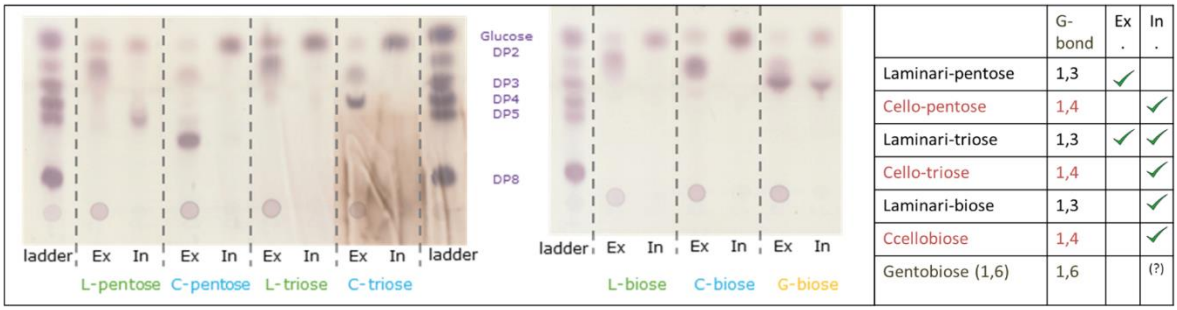

Figure 6: Glucosidase activity of intracellular (In) and extracellular (Ex) fraction of AK17 on different oligo-laminarin and oligo-cellulose. From left to right, different substrates were tested: laminaripentose (L-pentose), cellopentose (C-pentose), laminaritriose (L-triose), cellotriose (C-triose), laminaribiose (L-biose), cellobiose (C-biose) and gentobiose (G-biose). Control spots (substrate without intra- and the extra cellular fraction) are not shown. This figure corresponds to Figure 2 in Paper 1.

Under optimal conditions, *Thermoanaerobacterium* strain AK17 achieved an ethanol yield close to 60% of the maximum theoretical yield, if we only take into account the quantity of substrate consumed. Although acetic and lactic acids were also produced, which reduced the overall ethanol yield, the strain ability to ferment a broader range of substrates, including oligo-laminarin, highlights its potential as a robust candidate for bioethanol production from complex marine biomass. Additionally, the ability of AK17 to grow and produce ethanol at

elevated temperatures (60°C) provides advantages such as reduced contamination risk and lower viscosity of the fermentation broth, facilitating process scalability [66–68].

3.1.2 *L. digitata* hydrolysate utilization by Clostridial strains for production of A/IBE

The second part of this study was conducted by A.M. Lopez-Contreras and focused on the utilization of the same *Laminaria digitata* hydrolysate for the production of acetone, isopropanol, butanol, and ethanol (A/IBE) by fermentation using various *Clostridium* strains. These strains are known for their capability to ferment a wide range of substrates, including complex carbohydrates and sugar alcohols.

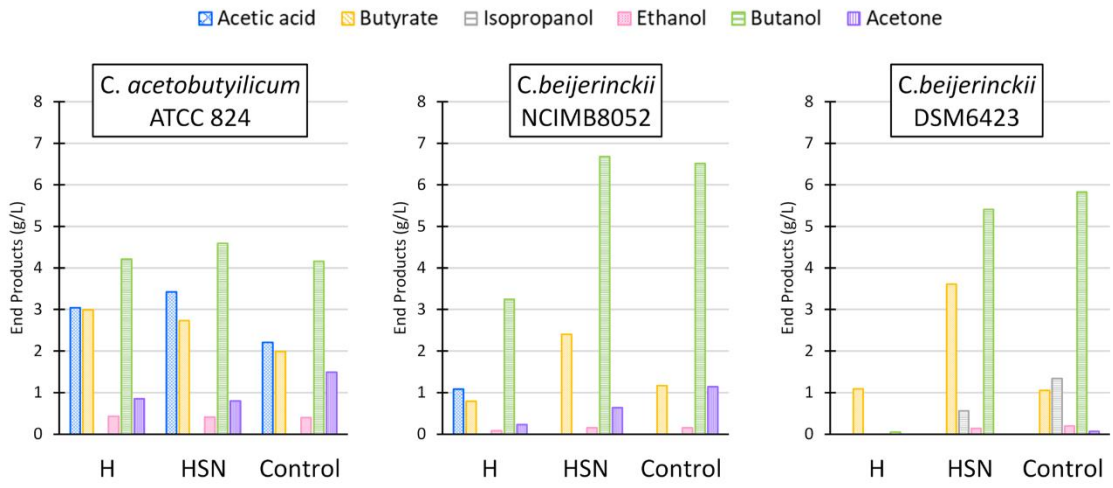

Figure 7: Fermentation products of *L. digitata* hydrolysate by *C. acetobutylicum* ATCC 824 and *C. beijerinckii* strains NCIMB 8052 and DSM 6423 at different conditions: raw hydrolysate (H), hydrolysate with addition of salts and nutrients (HSN), compared to fermentation on pure sugars (control). Substrate concentration was 24 – 26 g/L. Data from 6 days of cultivation. Data represent average of two replicate experiments. This figure corresponds to Figure 3 in Paper 1.

The study evaluated the fermentation performance of *Clostridium acetobutylicum* ATCC 824 and two strains of *Clostridium beijerinckii* (NCIMB 8052 and DSM 6423) (Figure 7). The results showed that all strains could effectively ferment the glucose, mannitol, and glucan components of the hydrolysate, albeit with varying efficiencies and product profiles. *C. acetobutylicum* demonstrated complete consumption of glucose and high utilization of mannitol, achieving ABE yields of approximately 0.22 g/g across all tested conditions. In contrast, *C. beijerinckii* strains exhibited variable fermentation performances depending on the medium composition and nutrient availability, with the NCIMB 8052 strain reaching a partial ABE yield of 0.34 g/g under nutrient-supplemented conditions [63,116].

Interestingly, all Clostridium strains were able to utilize the small amount of lactic acid present in the hydrolysate, which suggests potential applications for using ensiled seaweed as a feedstock, where sugars are partially converted into lactic acid during storage. Moreover, the production of butanol was significantly higher in cultures grown on hydrolysate compared to those grown on pure sugar substrates, indicating the beneficial impact of using mixed substrates for solventogenic fermentation [117].

This study provides a comprehensive evaluation of the potential of *Laminaria digitata* hydrolysate as a substrate for microbial fermentation, demonstrating the feasibility of producing valuable biofuels, including ethanol, acetone, isopropanol, and butanol from brown seaweed. The findings also highlight the importance of selecting appropriate microbial strains and optimizing fermentation conditions to maximize product yields and improve process sustainability [118,119].

3.2 Increase ethanol production by *Thermoanaerobacterium* strain AK17 by metabolic engineering (paper 2)

This study focused on the metabolic engineering of *Thermoanaerobacterium* strain AK17 to enhance ethanol production by eliminating pathways responsible for by-products such as lactate and acetate. By knocking out specific genes, the metabolic flux was redirected towards ethanol production, significantly increasing yield. In addition to the removal of lactate and acetate pathways, the butyrate pathway was also knocked out to prevent compensatory metabolic shifts that could reduce ethanol yield. The final engineered strain, AK17_M6, achieved high ethanol yields from both simple sugars and seaweed hydrolysate.

3.2.1 Knocking out of the lactate and acetate pathway in *Thermoanaerobacterium* strain AK17

The initial modification involved deleting the *ldh* gene, which encodes lactate dehydrogenase, resulting in strain AK17_M1 (*Δldh*) (Figure 8). This genetic alteration effectively abolished lactate production, as confirmed by fermentation assays where lactate levels were undetectable. Consequently, ethanol yield increased by 20% compared to the wild-type strain AK17, reaching 0.35 g ethanol per g glucose (Figure 9). This enhancement is attributed to the successful rerouting of carbon flux from lactate towards ethanol, consistent with findings in other thermophilic bacteria where similar deletions led to a 10-23% increase in ethanol production [69,120]. The ethanol-to-acetate ratio remained similar to the wild type, indicating that the elimination of lactate production did not affect acetate formation.

Following the success of the *ldh* knockout, the study proceeded with the deletion of the *ack* and *pta* genes, encoding acetate kinase and phosphotransacetylase, respectively (Figure 8). The resulting strain, AK17_M2 (*Δack/pta*), showed no detectable acetate production, confirming the effective disruption of the acetate pathway. However, this modification led to a substantial metabolic shift, with a fivefold increase in lactate production and a 60% decrease in ethanol yield, dropping to 0.14 g ethanol per g glucose (Figure 9). This result suggests a compensatory response to the loss of acetate production, where the redox balance was disrupted, favoring lactate production as an alternative electron sink. Such shifts in metabolic pathways reflect the organism's adaptation to maintain redox homeostasis and energy generation, as seen in other studies where deletion of acetate pathways induced increased lactate production [69,120].

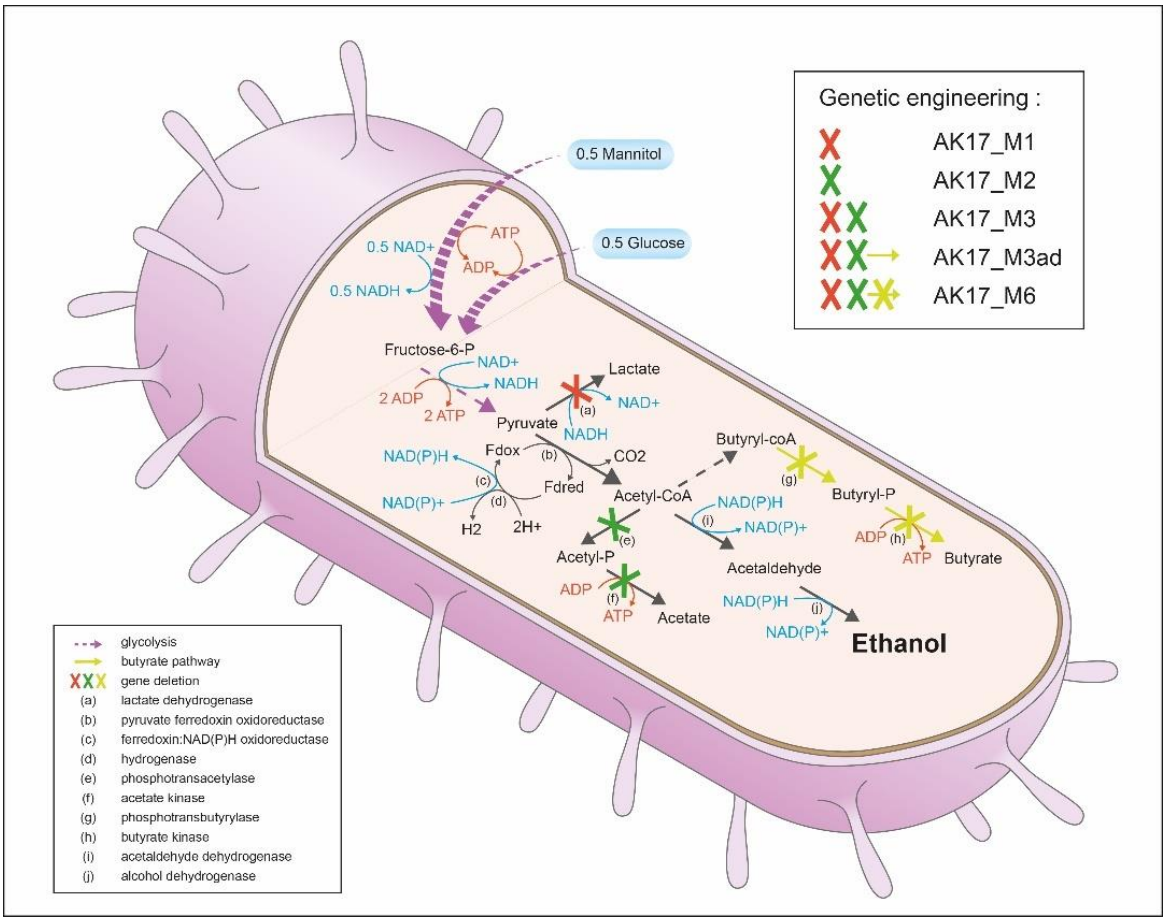

Figure 8: Overview of the metabolic pathways in *Thermoanaerobacterium* AK17. The pathways for production of ethanol, acetate, lactate and butyrate in *Thermoanaerobacterium* AK17 are shown. Positions in the pathways where genes have been deleted are indicated by crosses (red cross: *ldh* knockout; green cross: *ack/pta* knockout; yellow cross: *bk/ptb* knockout) and the designations of the corresponding engineered strains are shown in the box in the upper right corner. The enzymes responsible for different steps in the pathways are indicated by letters (a–j), and the corresponding enzymes are shown in the explanatory box in the lower left corner of the figure. This figure corresponds to Figure 1 in Paper 2.

To eliminate both by-products and maximize ethanol production, a double knockout strain, AK17_M3 (*Δldh, Δack/pta*), was constructed (Figure 8). This strain successfully produced ethanol as the sole detected fermentation product, with no measurable levels of lactate or acetate. Ethanol yield increased by 58% compared to the wild type, achieving 0.45 g ethanol per g glucose, which corresponds to approximately 88% of the maximum theoretical yield (Figure 9). This significant improvement demonstrates the efficacy of the double knockout in channeling metabolic flux exclusively towards ethanol production. However, the strain exhibited instability, with a reversion to acetate production observed after multiple subcultures. This suggests the activation of compensatory metabolic pathways that bypass the knocked-out genes, reflecting the complex regulatory mechanisms governing metabolic fluxes and redox balance in *Thermoanaerobacterium* [121].

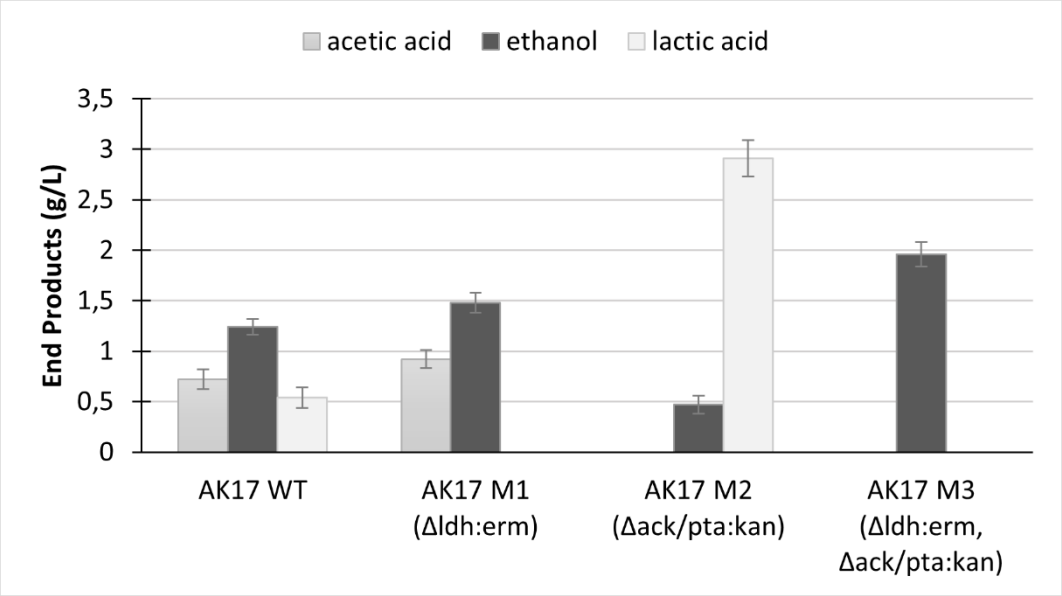

Figure 9: End product formation of AK17 (wild type = WT), and the new engineered strains, in media containing 4.5 g/L glucose. Samples were taken at 0 h (inoculation) and after 24 h (end of fermentation). Acetic acid (dark gray), ethanol (black) and lactic acid (light gray). The absence of a bar indicates the product was below the detection limit. Data represent the average of three replicate experiments. Standard deviations are shown as error bars. This figure corresponds to Figure 2 in Paper 2.

3.2.2 Acetate revertant and butyrate pathway knockout in strain AK17_M3 (*Δldh:erm Δack/pta:kan*)

The reversion to acetic acid production was accompanied by a restoration of cell density to levels similar to the wild-type strain, suggesting that the cells had adapted to regain the ability to produce acetate. Genomic analysis of the reverted strain AK17_AM3ad identified potential compensatory mutations in the genes encoding phosphotransbutyrylase (PTB) and butyrate kinase (BK). These enzymes belong to the same families as the deleted enzymes, acetate kinase (AcK) and phosphotransacetylase (PTA), and were hypothesized to have dual specificity, compensating for the lost acetate production (Figure 8).

Enzymatic assays confirmed that AK17_AM3ad showed activity on substrates for both acyltransferases and phosphotransferases, supporting the hypothesis that PTB and BK could restore acetate production in the absence of PTA and AcK (Figure 10) [122].

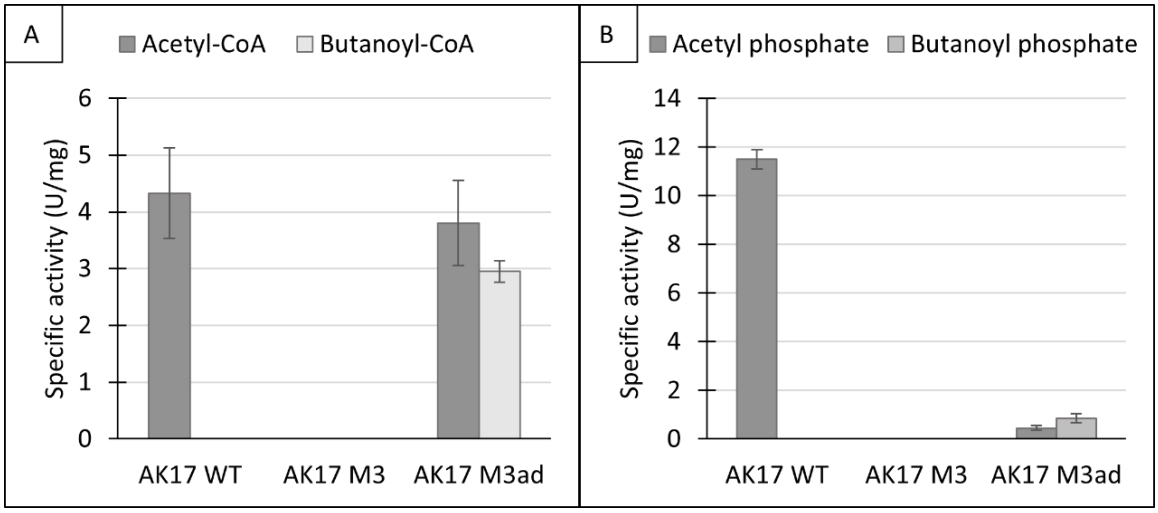

Figure 10: Activity assay of PTA and PTB (A) and AcK and BK (B) from strain AK17 (wild-type = WT) and the two other mutants AK17_M3 and AK17_M3ad. A Acetyl-CoA (dark gray) and Butanoyl-CoA (light gray). B Aceyl phosphate (dark gray) and Butanoyl phosphate (light gray). The absence of a bar indicates the product was below the detection limit. Data represent the average of three replicate experiments. Standard deviations are shown as error bars. This figure corresponds to Figure 3 in Paper 2.

This adaptive reversion likely provided a selective growth advantage by restoring the energy yield associated with acetate production, as an ATP molecule is generated for each acetyl-CoA converted to acetic acid. The restoration of acetic acid production allowed the cells to regain additional ATP, which could enhance growth under the selective pressure of fermentation conditions, thereby explaining the observed phenotype [123]. Such spontaneous mutations and phenotypic reversions are common in genetically modified strains, especially when subjected to repeated cultivation.

To prevent further reversion to by-product formation, a third genetic modification was made by knocking out the butyrate pathway genes *ptb* and *bk*, using the *pyrF* recycling marker system (Figure 8) [124]. This strategy enabled the reuse of antibiotic selection markers, facilitating further genetic modifications. The resulting strain, AK17_M6 (*Δldh Δack/ptaΔbk/ptb*), was stable across more than ten subcultures and produced ethanol as the sole volatile end product with a high yield of 0.47 g ethanol per g glucose, close to the theoretical yield of 91% (Figure 11). The elimination of the butyrate pathway effectively prevented the compensatory acetate production observed in the previous strain, demonstrating the importance of comprehensively addressing potential metabolic bypasses in engineered strains.

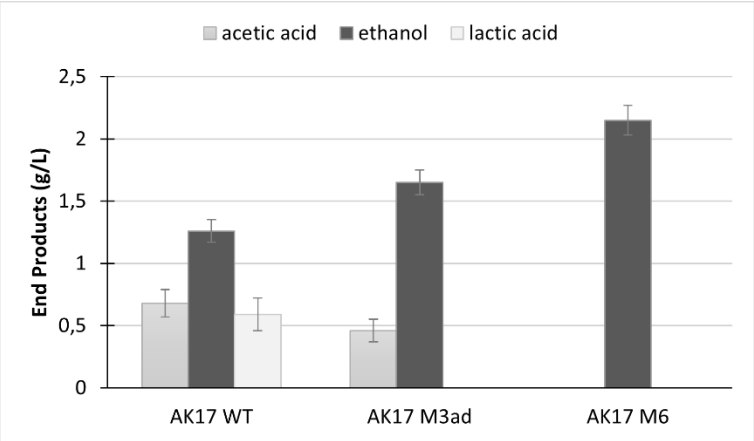

Figure 11: End product formation of AK17_WT and strains AK17_M3ad and AK17_M6, on media containing 4.5 g/L glucose. Samples were taken at 0 h (inoculation) and after 24 h (end of fermentation). Acetic acid (dark gray), ethanol (black) and lactic acid (light gray). The absence of a bar indicates the product was below the detection limit. Data represent the average of three replicate experiments. Standard deviations are shown as error bars. This figure corresponds to Figure 4 in Paper 2.

3.2.3 Fermentation on mixed sugars and seaweed hydrolysate

This part assessed the fermentation capabilities of the wild-type *Thermoanaerobacterium* strain AK17 and the engineered strain AK17_M6 on various substrates, including glucose, mannitol, mixed sugars, and *Laminaria digitata* hydrolysates. The goal was to evaluate the potential for bioethanol production from seaweed biomass.

The wild-type strain AK17 demonstrated a differential response to single carbon sources. When grown on 4.5 g/L glucose, AK17 fully consumed the substrate, achieving an ethanol yield lower than when grown on 4.5 g/L mannitol, from which it produced 35% more ethanol. However, only 65% of the supplemented mannitol was consumed. The engineered strain AK17_M6, designed for optimized ethanol production, achieved an ethanol yield of 90% of the theoretical maximum on both glucose and mannitol, separately. Nevertheless, similar to the wild-type strain, AK17_M6 did not fully utilize all the mannitol. This incomplete utilization of mannitol by both strains suggests a metabolic bottleneck, possibly due to the lower redox potential of mannitol (−0.32 V) compared to glucose (−0.17 V), which requires an additional oxidation step that produces extra NADH, and potentially disturbs the overall redox balance in the cells [125].

In mixed sugar fermentations, AK17_M6 achieved an ethanol yield of 92% of the theoretical maximum, significantly outperforming the wild-type strain AK17, which achieved only 62%. This superior performance of AK17_M6 is indicative of its enhanced metabolic efficiency and the successful redirection of carbon flux towards ethanol production, minimizing the accumulation of by-products and maximizing ethanol yield.

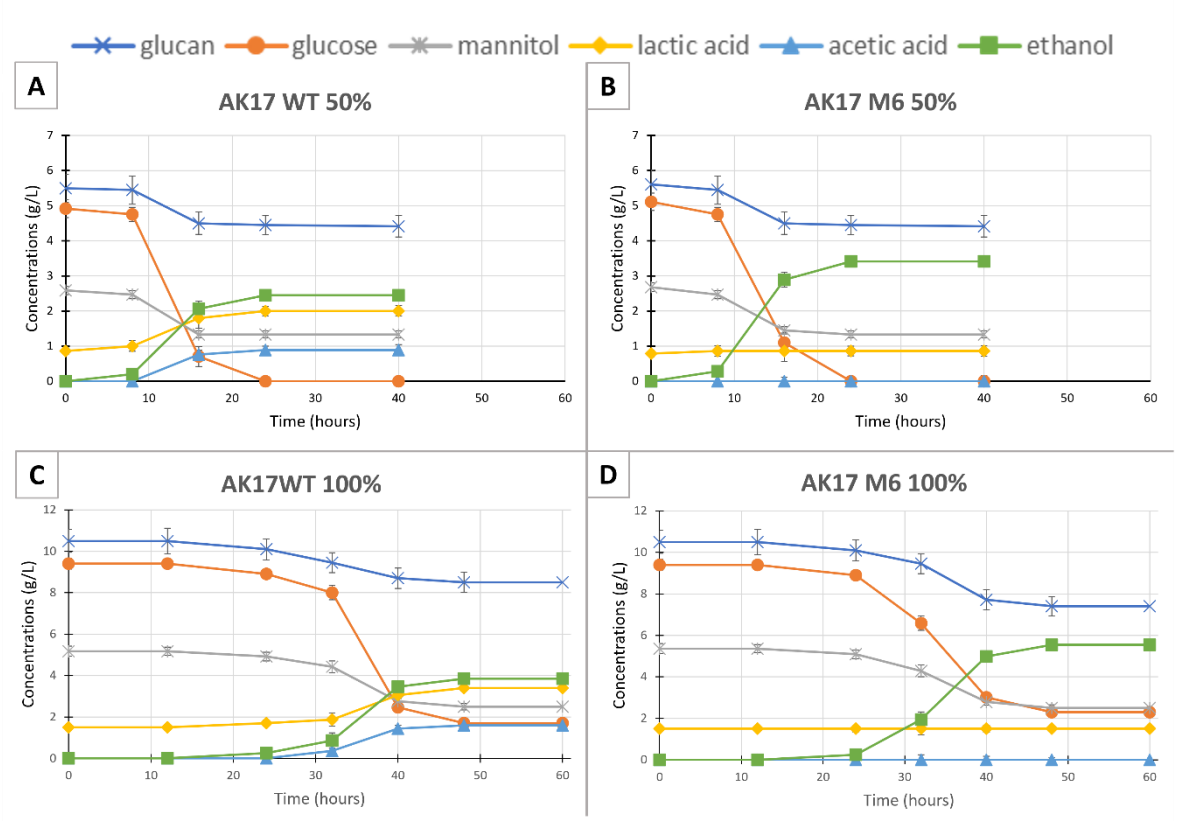

Figure 12: Fermentation kinetics of AK17 (WT) and AK17_M6, in 50% or 100% *L. digitata* hydrolysate. Fermentations were carried out for 40 h with 50% hydrolysate, for both AK17 (A) and AK17_M6 (B) and samples were taken at different times between 0 h (inoculation) and 40 h (end of fermentation). Fermentations were carried out for 60 h with 100% hydrolysate, for both AK17 (C) and AK17_M6 (D) and samples were taken at different times between 0 h (inoculation) and 60 h (end of fermentation). Glucan (blue cross), glucose (orange circle), mannitol (grey cross), lactic acid (yellow diamond), acetic acid (blue triangle) and ethanol (green square). Data represent the average of three replicate experiments. Standard deviations are shown as error bars. This figure corresponds to Figure 5 in Paper 2.

Further evaluations were conducted using *Laminaria digitata* hydrolysates, composed of a mixture of glucose, mannitol, and partially digested glucans (Figure 12). AK17_M6 showed robust fermentation capabilities, consuming 82% of glucose, 29% of glucan, and 43% of mannitol in undiluted hydrolysate (100% concentration) within 48 hours. In diluted hydrolysate (50% concentration), the strain consumed 100% of the glucose, 20% of the glucans, and 50% of the mannitol within 24 hours. In comparison, the wild-type strain AK17 produced ethanol along with lactic acid and acetic acid, with an ethanol yield of approximately 60% under both hydrolysate conditions. AK17_M6, however, produced only ethanol, achieving yields comparable to those observed with glucose and mannitol substrates (around 90% of the theoretical maximum) and an ethanol productivity of 0.32 g/L/h. The performance of AK17_M6 in converting seaweed hydrolysates is competitive with other reported strains. For example, *E. coli* KO11, engineered for mannitol utilization, achieved an ethanol yield of 0.4 g/g sugars with a productivity of 0.22 g/L/h [60], which is lower than that of AK17_M6. Yeast strains such as *S. cerevisiae* have reported bioethanol yields of 71-84% using various seaweed biomasses, but these often require extensive genetic modifications and still do not reach the ethanol yield achieved by AK17_M6 on *L. digitata* hydrolysate [126–128]. Its performance also surpasses that of other engineered

microorganisms, such as *Defluviitalea phaphyphila* Alg1, which produced ethanol from brown macroalgae with a lower productivity of 0.13 g/L/h [65].

The robust fermentation profile of AK17_M6 makes it a promising candidate for the sustainable production of biofuels from seaweed biomass. Further optimization could focus on enhancing substrate range and resistance to inhibitors typically found in industrial hydrolysates, potentially increasing its utility in diverse biofuel production scenarios. The data suggest that AK17_M6 not only has the potential for high ethanol yield but also for maintaining metabolic stability under various fermentation conditions, making it a valuable asset in the development of efficient bioethanol production technologies.

3.3 Development of an enzymatic reaction cascade for the production of KDG from seaweed carbohydrates (paper 3)

This study developed a cell-free enzymatic cascade to convert brown seaweed carbohydrates, specifically alginate and glucose from laminarin, into 2-keto-3-deoxygluconate (KDG), a precursor for bioplastics (Figure 5, page 17). The process involved optimizing a series of enzymatic steps, including alginate depolymerization, cofactor recycling, and conversion of intermediates to KDG, all integrated into a one-pot reaction system. The final enzymatic cascade achieved KDG production using alginate and glucose, with room for further optimization, but it is a proof of concept that this newly designed CFS is working.

3.3.1 Pathway creation and cascading design

The study aimed to develop a cell-free cascading enzymatic process for converting alginate and glucose into 2-keto-3-deoxygluconate (KDG), a valuable compound for bioplastic production. The process begins with the depolymerization of alginate into smaller oligosaccharides, which are further degraded into 4-deoxy-L-erythro-5-hexoseulose uronate (DEH) by specific alginate lyases (Figure 13). Following this, a reductase converts DEH to KDG using NADH as a cofactor, making the regeneration of NADH crucial for the reaction to proceed. To ensure continuous NADH supply, a dehydrogenase oxidizes glucose to gluconate while simultaneously reducing NAD+ to NADH, thus coupling cofactor regeneration with glucose utilization. Finally, a dehydratase converts gluconate into KDG, integrating glucose oxidation into the pathway.

3.3.2 Alginate to KDG cascading

The conversion of alginate to 2-keto-3-deoxygluconate (KDG) was achieved through a two-step enzymatic process, beginning with the depolymerization of alginate and followed by the reduction of 4-deoxy-L-erythro-5-hexoseulose uronate (DEH) to KDG (Figure 13).

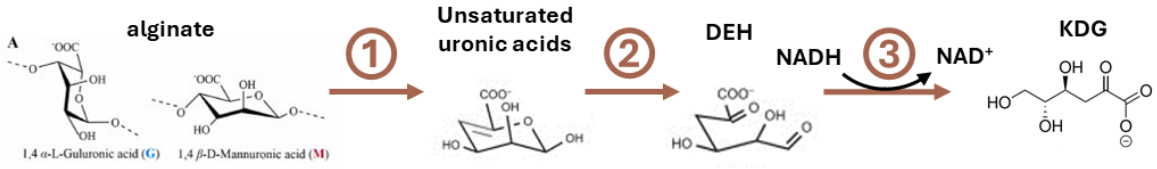

Figure 13: Enzymatic reactions from alginate to KDG. Alginate, made of G and M blocks, is digested by alginate lyases into unsaturated uronic acids (1), which spontaneously convert to DEH (2). DEH is reduced to KDG by the reductase Adh51 and NADH (3).

In the first step, the alginate lyases AlyRm3 and AlyRm4, previously described [111], were employed to break down the alginate into monouronates and then further to DEH. AlyRm3 is an endo-type lyase that cleaves the alginate polysaccharide chain oligosaccharides of different lengths (di, tri-, and tetrasaccharides). AlyRm4 is an exo-type lyase that cleaves the alginate and alginate oligosaccharides from the reducing end, resulting in the production of unsaturated monosaccharides. Alginate digestions were performed with single enzymes or both combined and followed over a period of 22 hours, with a pH varying from pH 5.0 to pH 9.0 (Figure 14). AlyRm4 showed activity between pH 6.5 and pH 9.0, whereas AlyRm3 between pH 5.5 and pH 9.0, with an optimum between pH 6.5 and 7.0. It was therefore decided to continue the next steps at pH 6.8. At temperatures above 50°C, a browning reaction was observed, likely due to the degradation products of DEH, which occurs at high temperature, as previously observed [111]. The temperature was then set at 50°C.

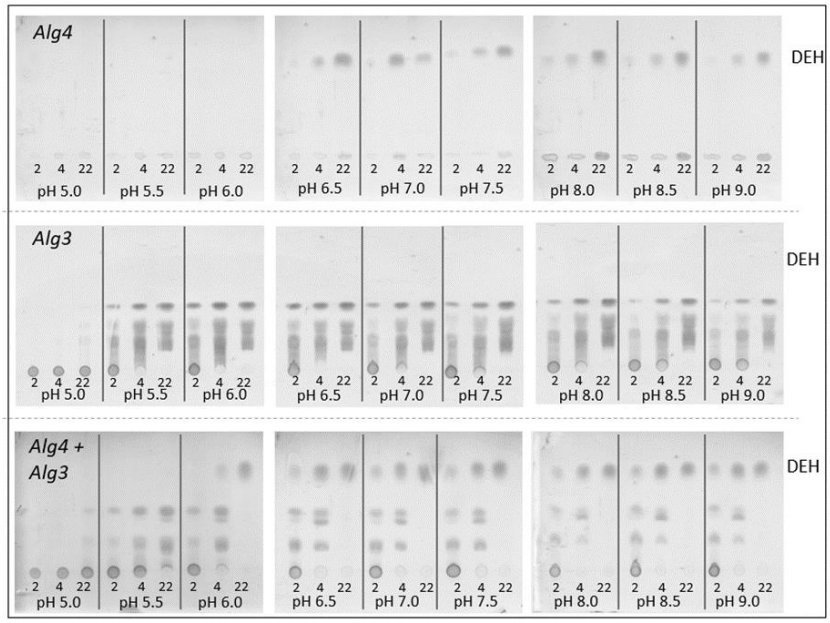

Figure 14: Single and combined enzyme activity of AlyRm3 and AlyRm4 on pH ranging from 5.0 to 9.0 at 45°C. This figure corresponds to Figure 2 in Paper 3.

In the subsequent step (Figure 13), the reductase Adh51 catalyzed the reduction of DEH to KDG using NADH as a cofactor. Kinetic analysis revealed a maximum reaction velocity (Vmax) of 2.5 U/mg protein and a Michaelis constant (Km) of 0.15 mM for DEH et 50°C and pH 6.8, indicating a strong affinity of the reductase for its substrate. When combining these two steps, starting with alginate and a high enough amount of NADH to make sure that all could be converted to KDG, a strong inhibition of the alginate lyases was observed. These

results highlighted the necessity of maintaining NADH at a lower concentration and of regulating its level through recycling.

3.3.3 Recycling of the cofactor and glucose to KDG cascading

The recycling of NADH and the conversion of glucose to KDG were integrated into the cascading enzymatic process to ensure efficient cofactor regeneration and maximize the production of 2-keto-3-deoxygluconate (KDG). The glucose dehydrogenase GDH16 was employed to oxidize glucose into gluconate while simultaneously regenerating NADH from NAD+. This step was optimized at a pH of 6.8 and a temperature of 50°C. Under these conditions, the enzyme demonstrated a specific activity of 68 U/mg and a Michaelis constant (Km) of 1.9 mM for glucose. This rapid conversion ensured a continuous supply of NADH, which would be immediately available for the subsequent reduction of DEH to KDG, effectively coupling the oxidation of glucose with the reduction step in the cascade (Figure 15).

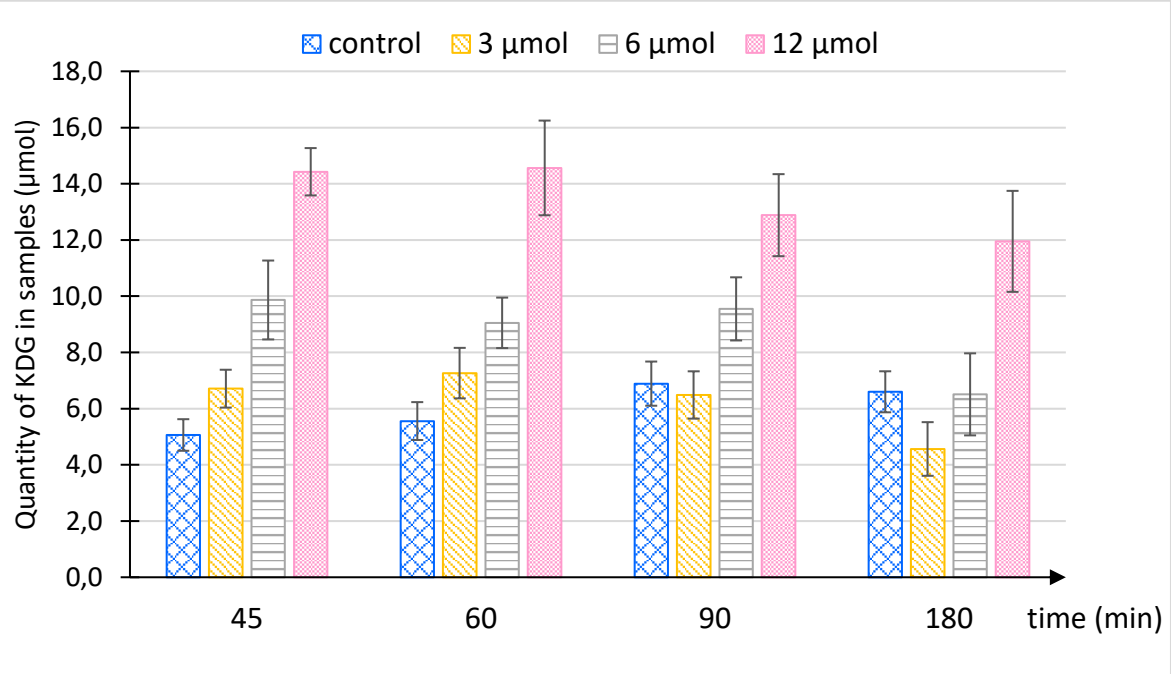

Figure 15: Effect of the glucose on the KDG production from DEH using Adh51 and GDH16 at 50°C. Each reaction contained 65µmol DEH, 3µmol NADH, Adh51 and GDH16 in 100 mM phosphate buffer pH 6.8. Increasing amounts of glucose were added: 3 µmol (orange), 6 µmol (grey) and 12 µmol (pink). No glucose was added for the control (blue). Samples were taken at different times, prepared and analyzed by LC-MSMS to quantify the KDG (See material and methods). Each bar represents the average of three replicates, and the error bars are also displayed. This figure corresponds to Figure 7 in Paper 3.

Following glucose oxidation, the generated gluconate was converted to KDG via a dehydration reaction catalyzed by the dihydroxyacid dehydratase DhadSs, previously described [112]. However, DhadSs was identified as the bottleneck of the process due to its relatively low activity and slow turnover rate (Figure 166). The enzyme operates at a much lower efficiency compared to the other enzymes in the cascade, significantly slowing down the overall conversion process of gluconate to KDG, which is a similar problem in other CFS [96,99,112]. This limitation highlighted the need for further optimization of the DhadSs

or the exploration of alternative enzymes that could perform the dehydration reaction more efficiently, thereby enhancing the overall yield of KDG production.

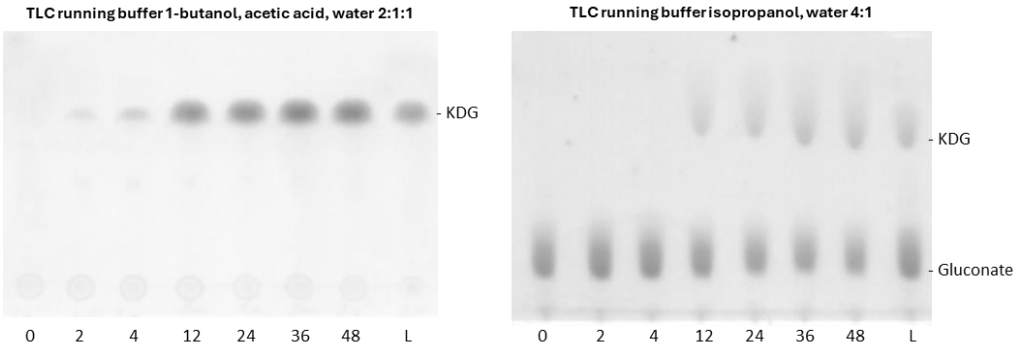

Figure 16: KDG production from gluconate using DhadSs overtime. Reaction mixture contained gluconate (30 µmol), DhadSs and was incubated at 50°C in 100 mM phosphate buffer pH 6.8. Samples were taken at different times (0/2/4/6/24/36/48 hours) and run on TLC, using either 2:1:1 1-butanol, acetic acid, water (left) or 4:1 isopropanol, water (right) as running buffer. The ladder (L) shows the bands for KDG and gluconate. This figure corresponds to Figure 10 in Paper 3.

3.3.4 KDG Production from Alginate and Glucose in One Cascading Reaction

The final step was to produce KDG at a small scale using all the enzymes in a single mixture with alginate and glucose and NAD+ as the starting cofactor (Figure 17).

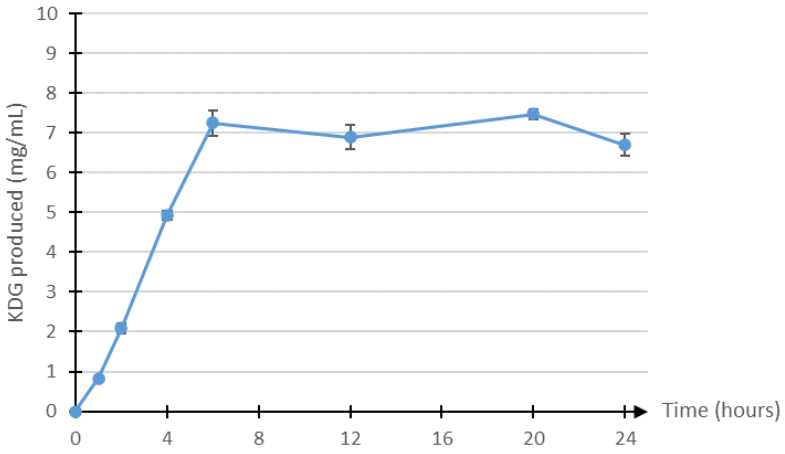

Figure 17: KDG production from alginate and glucose in a single cascading reaction. The reaction contained alginate (10mg/mL), glucose (10mg/mL), NAD+ (0,01 mg/mL) and the enzymes AlyRm3, AlyRm4, Adh51, GDH16 and DhadSs in 100 mM phosphate buffer pH 6.8 and incubated at 50°C for 48h. Samples were taken at different times, prepared, and analyzed by LC-MS/MS to quantify the KDG. Each point represents the average of three replicates, errors bars are also displayed. This figure corresponds to Figure 11 in Paper 3.

KDG was produced during the first 7 hours, and then production stopped. Theoretically, 10 mg/mL KDG could be obtained from the alginate side and another 10 mg/mL from the glucose side. However, as described earlier, the glucose side is less efficient compared to the alginate side, and it is suggested that most of the KDG produced originates from the alginate. If we only consider the alginate, the KDG yield would be around 73%. Recently, the purity

of the alginate used in this study was assayed, and it actually contained around 75% polysaccharide, while the remaining 25% accounted for salts and moisture. This meant that the actual yield of KDG from alginate could be closer to 100%. If we also take glucose into account, the KDG yield lowers to 35% of the theoretical yield (or 50% if we consider the alginate purity). Nonetheless, in the first 7 hours, a productivity of 1.0 g/(Lh) KDG was achieved with a catalyst loading of 0.01 g of catalyst per g of KDG. This productivity is still behind current industrial standards using cell factories, like 13.4 g/(L·h) of alanine produced from fumarate by *E. coli* and *P. dacunhae* [129]. Comparison with the productivity of other CFS also shows that it is in the lower range, for instance, compared to the synthesis of m2,3-butanediol reaching 11.3g/(Lh) [130] or synthesis of α-ketoglutarate reaching 2.8 g/(Lh) [108]. However, it is better than the production of many other platform chemicals using cell-free systems, such as alanine produced from glucose at 0.17 g/(L·h), L-lactate produced from glucose at 0.55 g/(L·h), and cysteine produced from glucose at 0.11 g/(L·h) [96,99,100].

Ultimately, the first in vitro enzymatic reaction cascade from alginate and D-glucose to KDG was established, comprising five enzymes and NAD+ as the only cofactor. After identification and characterization of suitable biocatalysts, a thorough optimization of the different enzyme conditions (temperature, pH, cofactor concentration) increased the overall cascade effectiveness resulting in a more cost-efficient process. Although we could demonstrate a successful production of KDG from glucose, this step is still considered as the main bottleneck of the reaction cascade. In order to further increase the efficiency of the cascade and enable the scale-up of the production of KDG, there is a need to find better enzymes or improved the current one.

3.4 Cloning and production of biocatalysts for brown seaweed carbohydrates valorization (paper 4)

During the course of this work, novel enzymes acting on laminarin were also cloned and produced from genetic material originating from the marine environment as well as from genes in the MATIS culture collection (Table 3). This work resulted in the isolation and characterization of novel interesting laminarin-degrading enzymes from a polysaccharide utilization locus in the marine bacterium *Muricauda lutaonensis*.

3.4.1 Laminarin polysaccharide utilization locus in the marine bacterium *Muricauda lutaonensis*

The effective enzymatic breakdown of seaweed biomass requires enzymes tailored to marine polysaccharides. These enzymes are often encoded by genes clustered in operons called polysaccharide utilization loci (PULs). A PUL is a generally regulated, colocalized gene cluster that encodes enzyme and protein ensembles required for the saccharification of complex carbohydrates, such as laminarin. There is a significant lack of information about PUL responsible for laminarin degradation in most marine species, including species belonging to the genus *Muricauda*. Following the sequencing and annotation of the genome of *Muricauda lutaonensis* (strain ISCAR-4703) at Matís, a specific PUL was discovered with conserved synteny in the genus *Muricauda*, including a cluster of genes encoding potential enzymes relevant to laminarin utilization (Figure 18). Two enzymes from the GH17

family were encoded in the PUL: MlGH17A and MlGH17B. The enzyme MlGH17B was recently shown to be a β-1,3-glucanosyltransglycosylase (**Paper 5**), motivating further analysis of its homolog MlGH17A in the PUL to clarify their respective roles.

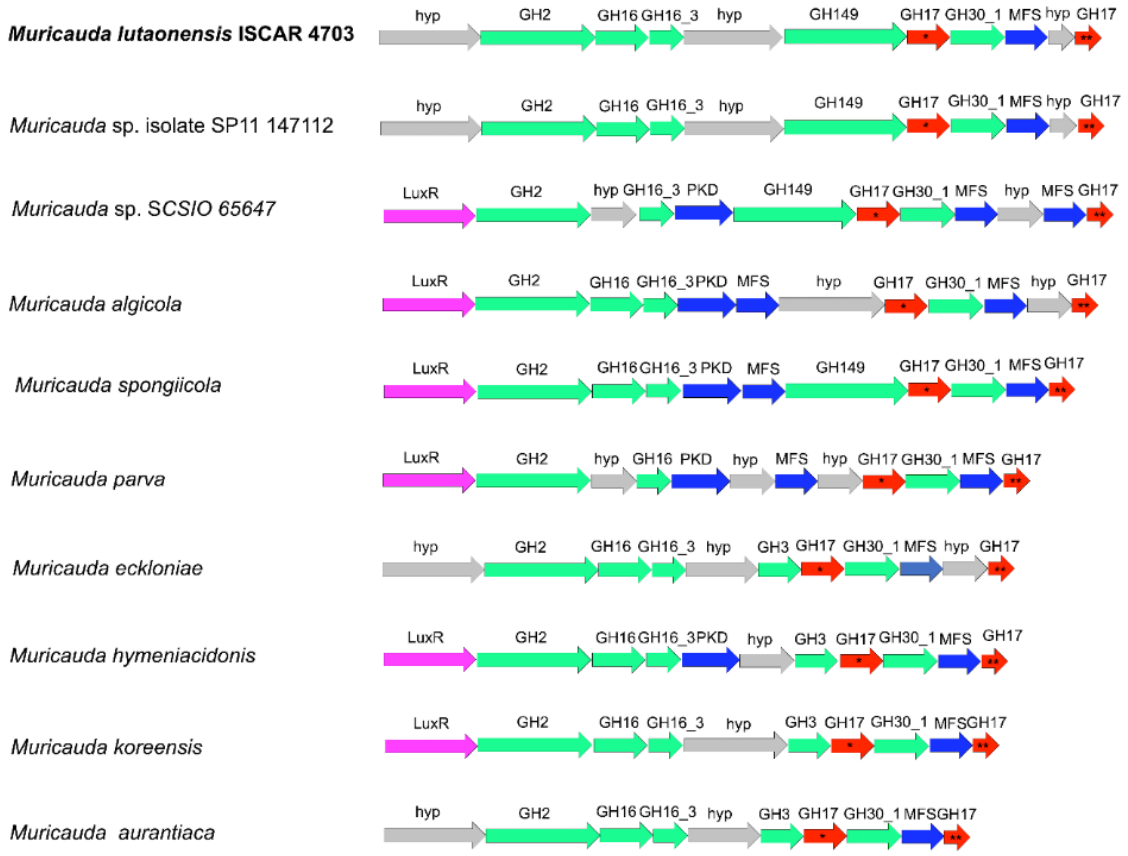

Figure 18 : Gene clusters available in *Muricauda* species/strains. MlGH17A and MlGH17B and their homologues are shown by (*) and (**) respectively. This figure corresponds to Figure 1 in Paper 4.

3.4.2 Initial cloning and characterization of the GH17 laminarinase MlGH17A

In this project, I contributed to the initial phase of functional characterization of MlGH17A. The gene was successfully cloned into a suitable expression vector using *E. coli* as a heterologous host. Different constructs were tested, for instance, with the addition of the maltose-binding protein (MBP) as a fusion protein to increase the solubility, or with the addition of a histidine tag for easier purification. To maximize the amount of soluble enzyme produced, the expression was carried out under different conditions, varying the temperature, rhamnose concentration, and cell density during induction. The best conditions for producing soluble MlGH17A were obtained using the MBP construct, with an incubation at 30°C after induction instead of 37°C (Figure 19A). The fusion protein was then incubated with Ulp1 (Ubiquitin-like-specific protease 1) to remove the MBP, and the enzyme was purified by metal ion affinity chromatography using a HisTrap column. The activity of the enzyme was verified on laminaripentose (Figure 19B). Preliminary activity assays confirmed its ability to hydrolyze laminaripentose in laminaritriose and laminaribiose, supporting its annotation as a laminarinase. This enzyme is therefore a potential new candidate for laminarin

degradation (to be combined with a glucosidase, such as the ones from *R. marinus* [114]) in the first step (laminarin to glucose) of the *de novo* pathway described in **Paper 3**.

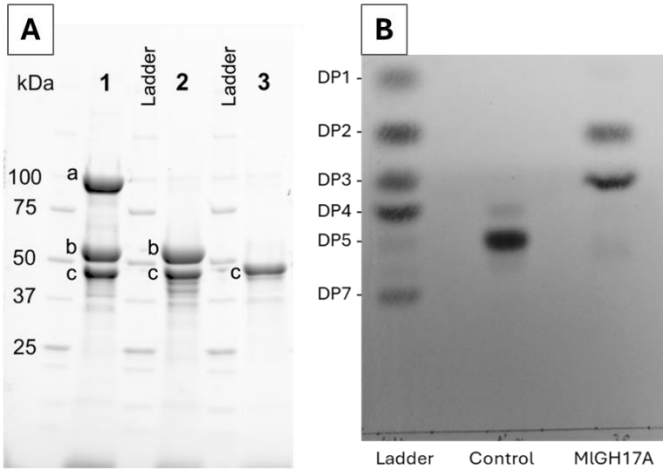

Figure 19 : Expression and initial characterization of MlGH17A. A: SDS-PAGE analysis of MlGH17A. Lane 1 shows the MBP-fused MlGH17A after purification using an MBP-Trap column; lane 2 illustrates the enzyme along with the MBP after being cleaved with Ulp1; lane 3 demonstrates the purified enzyme after affinity chromatography using a His-Trap column. Precision Plus Protein Dual Color Standards (Bio-Rad) molecular-mass marker was used to indicate the molecular weight. Band a represents MBP3-Smt3-MlGH17A (103 kDa), and bands b and c represent MBP-Smt3 (55 kDa) and MlGH17A (48 kDa) respectively. B: TLC analysis of MlGH17A. Laminaripentose was incubated either with extract from non-induced cells (control) or with purified MlGH17A for 1h at 37°C. MlGH17A seems to cleave laminaripentose in DP3 and DP2.

3.4.3 Further enzymatic characterization and structural analysis

Following the initial cloning and characterization, the enzyme was subjected to a detailed biochemical and structural analysis by other collaborators. This included kinetic profiling, substrate specificity studies using diverse β-glucans, and structural analysis through homology modeling of the enzyme. Thermal stability and pH profiling indicated moderate robustness, with optimal activity at neutral pH and temperatures ranging from 40 to 50°C. The enzyme was shown to have high specificity for β-1,3-glucan linkages and demonstrated a unique active-site architecture consistent with GH17 family members. These findings, while beyond the scope of my own contributions, provide valuable insights into the mechanistic basis of laminarin hydrolysis and support the strategic use of this enzyme in synthetic pathways targeting seaweed-derived carbohydrates.

3.5 Other work attempted without publications

This part focuses on work which were performed during this project but did not reach the expected results. Therefore, they could not be included in peer-reviewed publications; however, they are still part of this PhD thesis.

3.5.1 Attempt to increase macroalgal utilization in AK17

The objective was to identify genes that could be functional in *Thermoanaerobacterium* AK17 and would allow it to degrade the monouronates (DEH) from alginate and the

laminarin and subsequently clone them into a replicative plasmid (previously engineered by Matís). The oxydo-reductase adh51 from *Rhodothermus marinus* was selected to convert the DEH into KDG, which could then be fluxed into the glycolysis through the Entner-Doudoroff pathway present in AK17.

The gene encoding Adh51 was cloned into a replicative plasmid and expressed in AK17, and its activity was successfully tested *in vitro*. Nonetheless, AK17 was unable to grow on monouronates, obtained beforehand by incubating alginate with both AlyRm3 and AlyRm4 (Table 3). This highlighted the need for a specific transporter. Two transporters were selected, one from *Sphingomonas sp.A1* [131] and the other from *Defluviitalea phaphyphila* [65]. They were both composed of several sub-units, and the resulting plasmids to be cloned in AK17 were above 13kb. After many trials, no clones could be obtained. The size of the plasmid could decrease the transformation efficiency, and the expression of membrane proteins could also lead to metabolic disruptions (if they accumulate in the cytoplasm). Membrane proteins also contain a signal peptide, allowing them to be directed to the membrane when expressed. This signal peptide can also lead to metabolic disruption. Alternatively, the transporter genes could have been inserted stepwise into the chromosome to avoid the transformation of excessively long insertion sequences and thereby obtain stable transformants. However, it was considered to be too extensive and would not fit into the timeline, so it would need to be executed outside the scope of this project.

For the laminarin degradation, several laminarinases were selected from the Matis database, from different species. Laminarinases are secreted enzymes, and therefore, they also contain signal peptides within their sequence to allow their secretion. These signal peptides are usually host-specific and can cause disruptions when trying to clone them in other hosts. Three laminarinases, identified and characterized by researchers at Matís, were selected and their encoding genes cloned into AK17 with and without their signal peptide sequences: *amo78, amo176* and *amo939* (Table 3). Cloning and expression were successfully achieved with amo78, without a signal peptide encoding sequence. The recombinant enzyme was active, but the activity was only present in the cytoplasm, and not extracellularly. As the enzyme needed to be secreted to access laminarin, several combinations of signal peptides and carbohydrate-binding domains (CBDs) upstream of the laminarinases were designed. As no signal peptides or CBD had been described in AK17, they were chosen from the ones that had been identified within its closest relatives, like *Thermoanaerobacterium Thermosaccharolyticum* and *Clostridium acetobutylicum* [132]. Four CBDs were tested with their native signal peptide and with the signal peptide from the laminarinase. Unfortunately, no clones could be obtained in AK17, indicating that the constructs were likely lethal to the bacteria.

3.5.2 Attempt to implement 1,2-propanediol production in AK17

The production of 1,2-propanediol (1,2PD) was also investigated in AK17 and in the best-known producer *Thermoanaerobacterium* HG8. The 1,2PD pathway has been identified and requires only three enzymes: a methylglyoxal-synthase (MGS), a methylglyoxal-reductase (MGR), and a glycerol dehydrogenase (GDH). The gene encoding for the MGS was present in AK17 genome, but its activity had not been verified. The two other genes, encoding for the MGR and the GDH, were not present in AK17 and therefore needed to be cloned recombinantly. The genome of HG8 was sequenced and the specific genes were identified. The *mgs* and *gdh* genes were easily found in HG8 genome, but it was more complex for the

mgr gene, as three potential genes were annotated (and called *mgr1*, *mgr2* and *mgr3*). Two strategies were adopted (Figure 20): on one hand, several constructs were cloned into replicative plasmids and transformed into AK17 WT, AK17 eth- (ethanol mutant), and HG8 (as a positive control). The ethanol mutant strain was also used because it may offer a better redox balance after expressing the 1,2PD pathway compared to AK17 WT. On the other hand, we investigated the engineering of HG8 for increasing 1,2PD production, by knocking out other metabolic pathways, using integrating plasmids and homologous recombination. Numerous constructs were cloned in AK17 with different combinations of the genes of interest (*mgs+gdh+mgr*, *gdh+mgr+mgs*, *gdh+mgr*) under a constitutive promoter in a replicative plasmid but no positive clones could be obtained, which suggested that the constructs were somehow lethal for the bacteria, or that the transformation efficiency decreased drastically. In parallel, HG8 was also engineered by knocking out the lactate (*ldh*) and the ethanol (*adhE*) pathways, respectively by inserting the kanamycin resistance gene and the erythromycin resistance gene. Both resistance genes were inserted into the HG8 genomic DNA, but the *ldh* and *adhE* genes were not knocked out, suggesting that only a single crossover occurred. For a complete knock-out, a double cross-over would be needed, but it could not be reached.

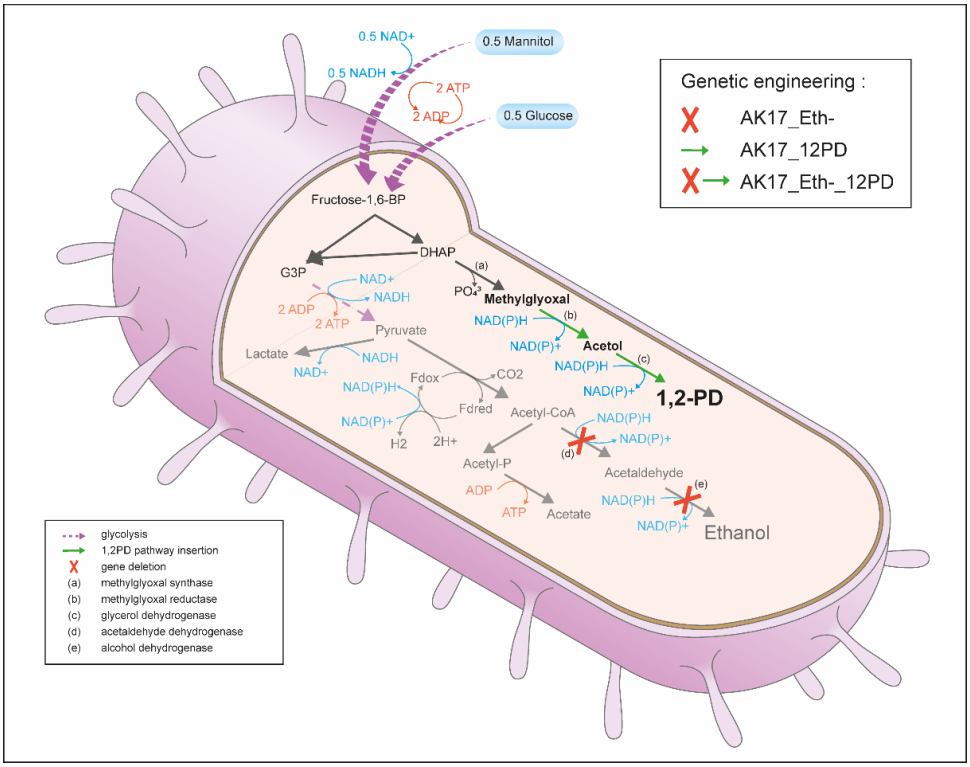

Figure 20 : Overview of the metabolic pathways in *Thermoanaerobacterium* AK17. The pathways for production of ethanol, acetate and lactate are greyed out to highlight the pathway for 1,2PD production. Positions in the pathways where genes have been deleted, are indicated by crosses (ethanol knockout) and the designations of the corresponding engineered strains are shown in the box in the upper right corner. The enzymes responsible for the 1,2PD and ethanol pathways are indicated by letters (a–e), and the corresponding enzymes are shown in the explanatory box in the lower left corner of the figure.

3.5.3 Attempt to construct a genome editing CRISPR-Cas system for AK17

The discovery of CRISPR-Cas systems has revolutionized the field of genome engineering. Identified initially as adaptive immune systems in bacteria and archaea, CRISPR-Cas systems protect microbial cells by recognizing and cleaving foreign genetic material. Their adaptation for genome editing has enabled precise, targeted modifications in a wide variety of organisms, ranging from model species like Escherichia coli to more challenging, thermophilic, and non-model organisms [133]. While class II systems like Cas9 have been most widely used, other endogenous class I CRISPR systems, such as type I and type III complexes, are increasingly being explored for genome editing, particularly in organisms where exogenous Cas9 expression is challenging [134]. Thermostable Cas proteins, such as CaldoCas9 developed from *Geobacillus stearothermophilus*, have recently expanded the possibilities for editing genomes of thermophiles [83]. Building upon these advancements, we explored two different strategies to establish a genome editing tool for *Thermoanaerobacterium* AK17.

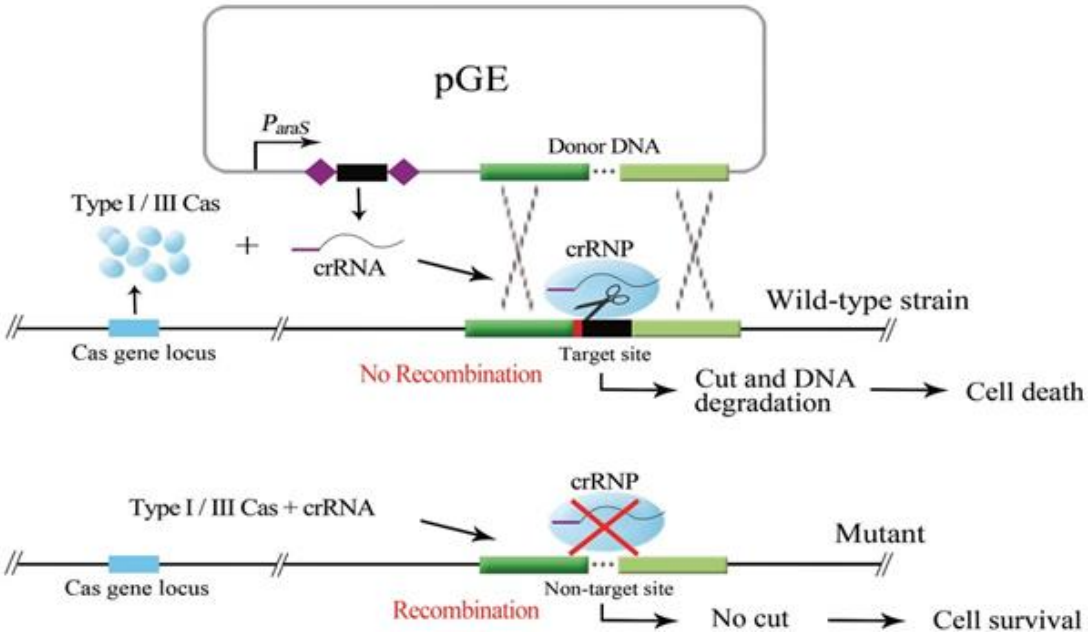

Figure 21 : Example of CRISPR Type I/III mechanism. Two alternative fates for the cells transformed with a pGE plasmid. pGE carrying an artificial mini-CRISPR locus with a single spacer and a donor DNA fragment. The target site is composed of a protospacer and its adjacent sequence, which is to be recognized by a Type I or Type III-B DNA interference system. The donor DNA fragment contains a DNA segment homologous to that flanking the chromosomal target site, but it is altered either by deletion, insertion, or point mutation, such that it is not to be targeted by the endogenous CRISPR systems. If recombination did not occur during transformation, the CRISPR DNA interference selectively targets the wild-type gene for degradation, leading to cell death; if recombination yielded the mutant gene on the chromosome, the mutant cell is devoid of the CRISPR immunity, forming colonies on plates. The figure is adapted from the figure found in Li et al.[134].

Approach 1: Endogenous CRISPR System (Type I-B or Type III-D)

The first strategy aimed to exploit the endogenous CRISPR system present in AK17. Bioinformatic analysis of the AK17 genome revealed the presence of a CRISPR array with repeat sequences matching those identified in closely related strains *Thermoanaerobacterium* DSM 571 and *Thermoanaerobacterium xylanolyticum* LX-11,

suggesting conservation of the system across these strains. Spacer sequences were extracted from the genomes of the three strains and compared against public and proprietary databases to identify potential protospacers. Approximately 20 protospacers were identified, showing high sequence identity to the extracted spacers. Analysis of these targets allowed the inference of a protospacer-adjacent motif (PAM) preference of 5'-TNNTNNN, characteristic of a type I-B system. However, based on the genetic organization of the system, we could not definitively distinguish whether the CRISPR system was of type I-B or type III-D, a similar approach to that described by Li et al. [134] (Figure 21).

Based on this analysis, vectors were designed to validate the system for genome editing. We targeted the β-galactosidase (*β-gal*) gene for knockout. The vector included a CRISPR spacer specific for the *β-gal* gene adjacent to a suitable PAM sequence, along with homologous arms upstream and downstream of the β-gal coding sequence to facilitate homologous recombination-based repair after DNA cleavage. In principle, only cells that successfully repaired the break by recombination, resulting in deletion of the target gene, would survive, while unmodified cells would suffer lethal genome cleavage. Despite numerous transformation attempts, varied conditions, and repeated culturing, no pure knockout clones could be obtained. PCR analysis of recovered colonies consistently revealed a mixed genotype, showing both the wild-type and knockout alleles simultaneously. The reason for the persistence of mixed populations remains unclear, but possibilities include incomplete cleavage, recombination inefficiency, or coexistence of edited and non-edited plasmid forms within the cells.

Approach 2: Exogenous CaldoCas9 System

The second approach sought to introduce an exogenous, thermostable Cas9 system into AK17. For this, we used CaldoCas9, a Cas9 variant derived from *Geobacillus stearothermophilus* LC300, developed at Matís and previously demonstrated to function in thermophilic species such as *Thermus thermophilus* and *Rhodothermus marinus* [83]. This system was chosen for its high thermostability and proven efficiency at temperatures comparable to AK17 optimal growth range. A vector was constructed containing the *cas9* gene, a sgRNA expression cassette targeting *β-gal*, and homologous arms flanking the target gene. However, due to the inclusion of all necessary elements, the final plasmid size approached 10 kb. Despite multiple transformation attempts and optimization of transformation protocols, no successful clones were obtained, possibly due to the large size of the plasmid.

Although neither approach led to the successful establishment of a robust genome editing platform in *Thermoanaerobacterium* AK17, the results from the endogenous CRISPR system appear promising. The identification of conserved CRISPR arrays, putative PAM sequences, and preliminary mixed-genotype observations suggest that, with further optimization, genome editing via the endogenous CRISPR system could become feasible in AK17. These efforts represent an essential first step toward developing genetic tools for *Thermoanaerobacterium* strains, an area where genetic manipulation techniques are still in their early stages of development.

4 Objectives reached and limitations

The research presented in this thesis aimed to explore and optimize the use of brown seaweed carbohydrates for the production of ethanol and 2-keto-3-deoxygluconate (KDG) through both microbial fermentation and cell-free enzymatic processes, respectively. The following objectives were set and successfully achieved throughout the studies:

Objective 1 (O1): Assess the fermentation capabilities of *Thermoanaerobacterium* strain AK17 for ethanol production from brown seaweed carbohydrates.

This objective was achieved by evaluating the fermentation performance of *Thermoanaerobacterium* strain AK17 on various substrates derived from brown seaweed, such as glucose, mannitol, and seaweed hydrolysates. The strain demonstrated effective ethanol production, particularly from mannitol, and showed adaptability to different fermentation conditions, confirming its suitability for further optimization and genetic engineering efforts.

Objective 2 (O2): Genetically engineer *Thermoanaerobacterium* strain AK17 to enhance ethanol production by deleting specific metabolic pathways.

This objective was achieved by systematically deleting the metabolic pathways responsible for the formation of lactate, acetate, and butyrate in *Thermoanaerobacterium* strain AK17. These genetic modifications redirected the metabolic flux towards ethanol production, significantly increasing ethanol yield and minimizing the production of unwanted by-products. The final engineered strain, AK17_M6, maintained stable ethanol production at high yields over multiple subcultures, demonstrating the effectiveness of the genetic engineering strategy. Further work would include increasing the ethanol tolerance and the substrate load to reach industrial standards.

Objective 3 (O3): Develop and optimize a cell-free enzymatic cascade for converting alginate into KDG.

This objective was met by designing and optimizing a cell-free enzymatic cascade that efficiently converts alginate, a polysaccharide from brown seaweed, into 2-keto-3-deoxygluconate (KDG). The process involved selecting specific enzymes for each step of the conversion, optimizing reaction conditions such as pH and temperature, and ensuring cofactor availability. The resulting cascade achieved the conversion of alginate to KDG, but the high amount of NADH required caused an inhibition and highlighted the need for a recycling system.

Objective 4 (O4): Develop and optimize a cell-free enzymatic cascade for converting glucose into KDG, ensuring continuous cofactor regeneration.

To achieve this objective, a separate enzymatic cascade was developed to convert glucose into KDG while ensuring continuous regeneration of NADH, a necessary cofactor for the reduction step in the alginate cascade. By integrating enzymes that effectively recycle NADH during the oxidation of glucose to gluconate, and the addition of another enzyme converting gluconate to KDG, this new enzymatic cascade could regenerate NADH and produce KDG at the same time, even though in small amounts. Further work would involve enzymatic engineering or finding faster enzymes to increase the overall KDG yield from that pathway. In addition, for the conversion of laminarin to glucose, the new laminarinase MlGH17A was cloned and characterized. It represents another candidate that could be used in the CFS to produce KDG from laminarin.

Objective 5 (O5): Integrate and evaluate the combined enzymatic cascades for the simultaneous production of KDG from alginate and glucose.

This objective was accomplished by integrating the alginate and glucose enzymatic cascades into a single, one-pot reaction system. The combined cascades enabled the production of 2-keto-3-deoxygluconate (KDG), utilizing a synthetic pathway that facilitated cofactor regeneration. The integration of all enzymatic steps in one pot demonstrated the feasibility of this approach, despite the conversion of gluconate to KDG being limited. Further investigations would be directed at optimizing the glucose to KDG steps for potential scaling up of this integrated system for industrial applications.

5 Conclusion and future prospects

The research conducted across three studies focused on the metabolic engineering of microbial systems and the development of cell-free enzymatic cascades for the efficient conversion of brown seaweed biomass into valuable compounds, specifically ethanol and 2-keto-3-deoxygluconate (KDG).

The first study assessed the fermentation abilities of *Thermoanaerobacterium* strain AK17 and other microbial strains using seaweed hydrolysates. The goal was to evaluate if these strains are suitable for producing ethanol and other biofuels such as acetone, butanol, and isopropanol (ABE/IBE). Strain AK17 showed promising ethanol production when grown on seaweed hydrolysates. It was found that AK17 could utilize oligo-laminarin because of both extracellular glucanases, which break down oligolaminarin into disaccharides, and intracellular glucanases, which break down laminaribiose into glucose units. The study also examined the abilities of various clostridial strains for ABE/IBE fermentation, highlighting their potential to produce different biofuels. This initial evaluation provided a basis for further genetic modifications of AK17, considering its capacity and adaptability to generate ethanol from seaweed-derived carbohydrates.

Building on the findings from the first study, the second study focused on the metabolic engineering of *Thermoanaerobacterium* strain AK17 to enhance ethanol production. Genetic modifications were made to knock out pathways responsible for producing lactate, acetate, and butyrate, which are by-products that lower ethanol yield. The initial knockout of the *ldh* gene eliminated lactate production and increased ethanol yield, but further deletion of the *ack* and *pta* genes caused an increase in lactate production due to a disrupted redox balance. Creating a double knockout strain, AK17_M3, eliminated both lactate and acetate production, redirecting carbon flux entirely towards ethanol. However, this strain showed instability over multiple subcultures, reverting to acetate production due to compensatory mutations. To fix this, additional modifications were made by deleting genes responsible for the butyrate pathway, resulting in strain AK17_M6, which maintained stable, high-yield ethanol production. This study demonstrated the effectiveness of targeted metabolic engineering in optimizing microbial strains for biofuel production, while also highlighting the complexity of microbial metabolism and the need for comprehensive genetic strategies to achieve stable production.

The third study shifted focus from microbial fermentation to developing a cell-free enzymatic cascade for the conversion of seaweed carbohydrates into 2-keto-3-deoxygluconate (KDG), a precursor for bioplastics. The enzymatic cascade integrated multiple steps, including the depolymerization of alginate by alginate lyases, the reduction of the resulting DEH to KDG by a reductase, and the oxidation of glucose to regenerate NADH using glucose dehydrogenase. A dihydroxyacid dehydratase catalyzed the final conversion of gluconate to KDG. The study successfully demonstrated the feasibility of a one-pot reaction system, achieving a KDG yield of 35% from alginate and glucose under

optimal conditions. However, the slow activity of the dihydroxyacid dehydratase was identified as a bottleneck, indicating the need for further enzyme optimization or the selection of an alternative enzyme to improve the overall efficiency of the process. This research highlights the potential of cell-free systems for the sustainable production of valuable biochemicals from renewable resources, offering a promising alternative to traditional microbial fermentation.

The primary goal of this research was to develop efficient microbial and cell-free systems for converting brown seaweeds into valuable biochemical products, particularly ethanol and KDG. Through metabolic engineering of *Thermoanaerobacterium* strains and the design of a cascading enzymatic process, significant advancements were made in optimizing ethanol production and establishing a viable method for KDG synthesis. The engineered strains demonstrated enhanced ethanol yields with minimized by-product formation, while the enzymatic cascade successfully converted seaweed carbohydrates into KDG, despite challenges with enzyme efficiency. These studies contribute to the development of sustainable biotechnological processes for industrial applications, utilizing renewable resources to produce biofuels and bioplastics. Ultimately, this thesis lays the groundwork for future research aimed at further optimizing enzyme activities, enhancing strain stability, and expanding the range of substrates that can be efficiently converted in cell-free systems. By advancing the understanding of microbial metabolism and enzymatic processes, this research supports the broader goal of developing scalable and sustainable technologies for the bio-based economy.

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Papers

Paper 1: Evaluation of *Laminaria digitata* hydrolysate for the production of bioethanol and butanol by fermentation

Paper I

Paper 2: Metabolic engineering of T*hermoanaerobacterium* AK17 for increased ethanol production in seaweed hydrolysate

Paper II

Paper 3: Development of an enzymatic reaction cascade for the production of KDG from seaweed carbohydrates

Paper III

Paper 4: Characterization of a GH17 laminarinase, MlGH17A, from a laminarin polysaccharide utilization locus in the marine bacterium *Muricauda lutaonensis*

Paper IV