



**Lucas Vagueiro de
França**

**Macroalgas como matéria-prima para a cultura de
bactérias marinhas**

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bacteria**



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Master's thesis to be submitted to the University of Aveiro to meet the requirements for the Degree of Master in Industrial and Environmental Biotechnology, performed under the scientific guidance of Prof. Dr. Ana Maria Rebelo Barreto Xavier, Assistant Professor at Department of Chemistry, University of Aveiro, and Dr. Bruno Sommer Ferreira, co-founder and CEO of Biotrend, SA.

To Almerinda.

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Palavras-chave

Alginato; Bactérias extremófilas; Conceito de biorefinaria; Corrosão de biorreatores; Enzimas termófilas; Macroalgas castanhas; Manitol; Laminarina; Projeto BlueGenics; *Rhodothermus marinus*.

Resumo

Alginato, laminarina e manitol podem ascender a 60% do peso seco das macroalgas castanhas. A presença de enzimas de degradação do alginato e da laminarina e a maquinaria para o metabolismo do manitol foram confirmadas pela Matís, um parceiro do projeto europeu BlueGenics. Assim, numa perspetiva de biorrefinaria, *Rhodothermus marinus* pode potencialmente sacarificar e fermentar os hidratos de carbono das macroalgas castanhas para produzir biocompostos com valor comercial, tais como enzimas termoestáveis e carotenóides glicosídicos. *R. marinus* é uma bactéria marinha moderadamente termófila (65°C) e ligeiramente halófila (1,0% NaCl). Por isso, um dos objetivos deste projeto foi o de diminuir a concentração de NaCl no meio de fermentação, uma vez que o cloreto leva a uma menor vida útil dos equipamentos devido à corrosão do aço inoxidável dos biorreatores. O principal objetivo deste trabalho foi o estudo do padrão de crescimento da bactéria *R. marinus* quando cultivada com os principais hidratos de carbono presentes em macroalgas castanhas.

Este trabalho foi realizado com cinco estirpes de *R. marinus*, das quais duas foram aclimatizadas com sucesso ao cultivo no Medium 166, criopreservadas em glicerol e recultivadas em meio líquido, sendo matéria de estudo para os ensaios com diferentes fontes de carbono e sódio em *shake flask*.

Os ensaios de crescimento com diferentes fontes de carbono sugeriram que (i) a estirpe 5 apresentou maior consumo de glucose e crescimento, apesar de nenhuma das estirpes ter consumido toda a glucose disponível no meio; (ii) embora nenhuma das estirpes tenha consumido manitol, a estirpe 5 pareceu ser mais robusta à sua presença; e (iii) as diferenças de crescimento entre controlos e ensaios com alginato e alginato pré-tratado não foram suficientemente significativas para inferir se ocorreu algum consumo de alginato.

Foi testada a substituição parcial e total de NaCl por Na₂SO₄. O processo não foi bem-sucedido, uma vez que o Na₂SO₄ pareceu representar um fator de stress para ambas as estirpes de *R. marinus*. Quando cultivada no Medium 166 contendo apenas metade da concentração padrão de NaCl, a estirpe 5 apresentou um padrão de crescimento similar ao controlo.

Nas condições operacionais impostas nos cultivos em *shake flask* contendo as duas macroalgas castanhas (originais e pré-tratadas) como matéria-prima para crescimento, o manitol não foi consumido. Não foi possível monitorizar a sacarificação e fermentação do alginato e da laminarina. Contudo, os resultados mostraram que as macroalgas castanhas são potenciais matérias-primas num conceito de biorrefinaria, uma vez que foi observado algum crescimento da *R. marinus*.

O resultado mais promissor para o projeto BluGenics foi obtido dos cultivos da estirpe 5 em Medium 166 com 0,500% NaCl e 10,0 g.L⁻¹ de glucose, uma vez que o crescimento com baixo conteúdo em cloreto determina a possibilidade do aumento de escala do processo em biorreator. Por isso, o ensaio foi validado em biorreator controlado de 3 L. O processo apresentou uma $\mu_{\text{máx}}$ de 0,208 h⁻¹, uma concentração máxima de biomassa de 8,75 g.L⁻¹, uma taxa volumétrica de produção de biomassa de 0,295 g.L⁻¹.h⁻¹ e uma taxa volumétrica de consumo de glucose de 0,293 g.L⁻¹.h⁻¹. Algumas estratégias de alimentação foram testadas mas devem ser realizados ensaios adicionais de modo a otimizar o bioprocessamento.

Keywords

Alginate; Bioreactor corrosion; Biorefinery concept; BlueGenics project; Brown macroalgae/seaweeds; Extremophilic bacteria; Laminarin; Mannitol; *Rhodothermus marinus*; Thermophilic enzymes.

Abstract

Alginate, laminarin and mannitol amount up to 60% of dry weight in brown macroalgae. The presence of alginate and laminarin-degrading enzymes and mannitol metabolic machinery have been confirmed by Matis, a partner in European BlueGenics project. Thus, in a biorefinery perspective, *R. marinus* can potentially perform the saccharification and fermentation of brown macroalgae carbohydrates to yield commercial valuable biocompounds, as thermostable enzymes and glycosidic carotenoids. *Rhodothermus marinus* is a moderate thermophilic (65°C) and slight halophilic (1.0% NaCl) marine bacterium. Therefore, one of the objectives of this project was to decrease the NaCl concentration in the fermentation medium, since chloride leads to a lower equipment lifetime due to stainless steel corrosion of bioreactors. The main objective of this work was the study of the bacterium *R. marinus* pattern of growth when cultivated in the main brown macroalgal carbohydrates.

This work was performed with five *R. marinus* strains, two of which were successfully acclimatized to cultivation in Medium 166, cryopreserved in glycerol and recultivated in liquid media, being subject of study in the assays with different carbon and sodium sources in shake flask.

The growth studies with different carbon sources suggested that (i) strain 5 presented higher glucose consumption and growth, even though none of the strains consumed all the glucose available in the media; (ii) although none of strains consumed mannitol, strain 5 seemed to be more robust to its presence; and (iii) the growth differences between the controls and the assays with alginate and pretreated alginate were not significant enough to infer if any alginate consumption occurred.

It was tested a partial and total substitution of NaCl by Na₂SO₄. The process was not successful, since Na₂SO₄ seem to represent a stress factor to both *R. marinus* strains. Interestingly, the strain 5, when cultivated in Medium 166 containing only a half of NaCl standard concentration, presented a similar growth pattern to control.

In the operational conditions imposed in shake flask cultivations containing two tested brown macroalgae (original and pretreated) as feedstock for growth, mannitol was not consumed. It was not possible to monitor the alginate and laminarin saccharification and fermentation. Although, the results showed that brown macroalgae are a potential feedstock under the biorefinery concept, since some *R. marinus* growth was observed.

The more promising result to BlueGenics project was obtained from shake flask cultivations of strain 5 in Medium 166 with 0.500% NaCl and 10.0 g.L⁻¹ glucose, since the growth with low chloride content determinates the feasibility of the scale-up of the process to bioreactor. Because of that, the assay was validated in 3L controlled bioreactor. The process presented a μ_{max} of 0.208 h⁻¹, a maximum biomass concentration of 8.75 gX.L⁻¹, a volumetric biomass production rate of 0.295 g.L⁻¹.h⁻¹ and a volumetric glucose uptake rate of 0.293 g.L⁻¹.h⁻¹. Some feeding strategies were tested but further assays have to be performed in order to optimize the bioprocess.

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Abbreviations

ASCO	Milled <i>Ascophyllum nodosum</i>
CBP	Consolidated BioProcessing
CESAM	“Centro de Estudos do Ambiente e do Mar” – Aveiro University
CM	Cytoplasmic Membrane
C-source	Carbon source
EEZ	Exclusive Economic Zone
EU	European Union
FDA	USA Food and Drug Administration
G	Guluronic acid unit
GRAS	Generally Recognized as Safe
HPLC	High-Performance Liquid Chromatography
HVLV	High-Value and Low-Volume
IS	Ionic Strength
LAM	Milled <i>Laminaria digitata</i>
M	Mannuronic acid unit
MG	α -mannosylglycerate
MGA	α -mannosylglyceramide
Na-source	Sodium source
N-source	Nitrogen source
OD ₆₀₀	Optical Density at wavelength of 600 nm
OM	Outer Membrane
OVAT	One Variable at a Time
PL	Peptidoglycan Layer
P-source	Phosphorus source
R&D	Research and Development
ROW	Reverse-Osmosis Water
r _s	Volumetric substrate uptake rate
r _x	Volumetric growth rate
SSF	Simultaneous Saccharification and Fermentation
VFAs	Volatile Fatty Acids
μ_{\max}	Maximum specific growth rate

1. Biotrend – the traineeship company

Biotrend, - Inovação e Engenharia em Biotecnologia, S.A. (**Figure 1**) is a company headquartered since 2010 in Biocant Park (Cantanhede, Coimbra), the first Portuguese park totally dedicated to Research and Development (R&D), Innovation and Technology Transfer in biotechnology. The company was founded in 2000 as a spin-off of Instituto Superior Técnico (Lisbon) with the main objective of developing bioprocesses for industrial biotechnology.

Biotrend focuses on developing bio-based processes for delivering sustainable value. The company specialties are process development, integration and scale-up, including all main steps from strain screening to fermentation optimization in pilot scale.

The mission of Biotrend is based on putting own know-how and resources to the service of the clients, providing contract services and developing in-house research programs applicable in new technologies to potentially be licensed out to industrial clients. Biotrend also participates in European Union (EU) collaborative projects concerning the full bio-based economy value chain such as BioREFINE-2G, Brigit, Bugworkers, O4S, Transbio, Seafront and BlueGenics, the last two dealing the expanding marine economy.

Biotrend facilities include one office space, one bench-scale bioprocess development lab, including one room for media preparation and reagent manipulations and one room with laminar flow chamber for aseptic manipulations and analytical equipment; and one pilot facility dedicated to fermentation and downstream scale-up.

The present work was originated from the EU-funded collaborative project BlueGenics (**Figure 1**) and was also supported by further initiatives in the topic of marine biotechnology that are currently being expanded at Biotrend. The main objective of the BlueGenics project is to exploit marine genomics, from gene to bioactive products in order to develop a blue bio-based industry. In this collaboration, Biotrend is responsible for assessing the feasibility of translating processes based on marine microorganisms isolated during the project from lab to pilot scale. The project involves 15 additional partners.



Figure 1 – Logos of Biotrend and BlueGenics.

2. Introduction

Presently, the world economy is still based on fossil raw-materials although the fossil fuel reserves keep depleting and climate changes increasing. The R&D is focused on alternative and sustainable energy and chemicals. The biorefinery concept has emerged as a potential solution to transform a fossil-based economy into a more sustainable bio-based economy (Cherubini, 2010).

Due to the high carbohydrate content present in macroalgae, several works prospect some species as a potential feedstock for biorefinery (Jung et al., 2013). Currently, the commercial value of macroalgae is related to food and colloids industries (Wei et al., 2013). However, the harvesting of macroalgae specifically for bioethanol production have been envisaged. Although the macroalgae exploitation in Europe is currently restricted to natural stocks, some EU projects aim to explore the mass cultivation. Furthermore, in some regions, the macroalgae are considered as a waste, since huge drift macroalgae masses are washed up on the coast (Smetacek and Zingone, 2013).

The biochemical profile of macroalgal biomass differs of terrestrial biomass, which leads to the need of novel microorganisms to effectively saccharify and metabolize macroalgal carbohydrates. Alginate (polysaccharide consisting of linked mannuronic acid (M) and guluronic acid (G) units), laminarin (polysaccharide consisting of glucose units) and mannitol amount up to 60% of dry weight in common brown macroalgae (Jensen, 1993).

Rhodothermus marinus is a thermophilic and slight halophilic marine bacterium (Alfredsson et al., 1988, Silva et al., 1999). The genome of *R. marinus* has been sequenced and genes encoding alginate-degrading enzymes have been identified, cloned and expressed by the company Matís Ltd., one of the BlueGenics project partners. The presence of laminarin-degrading enzymes and mannitol metabolic machinery have also been confirmed (Hreggviðsson, 2015). Therefore, *R. marinus* can potentially be cultivated using brown macroalgae carbohydrates as Carbon source (C-source) to yield HVLV (High-Value and Low-Volume) products of interest such as thermostable enzymes and glycosidic carotenoids, in a biorefinery perspective.

In this way, this project was planned, aiming to investigate the cultivation of *R. marinus* resorting to macroalgal biomass as feedstock. From an industrial viewpoint, chlorinated media leads to a lower equipment lifetime due to corrosion, particularly if high-salt media is thermally sterilized prior to fermentation. So, one of the concerns of this project was to decrease the sodium chloride concentration in the fermentation media in order to be feasible on a larger scale. The BlueGenics is a long-term project, so the main objective assigned to this master's thesis was restricted to the study of the *R. marinus* pattern of growth when cultivated in the main brown macroalgal carbohydrates, in a laboratorial scale. This topic was initially somewhat marginal for the BlueGenics project, but emerged as a focal

point of future collaborative work between Biotrend, Matís, SilicoLife, the Technical University of Denmark, the University of Lund and the Norwegian University of Science and Technology.

A simplified outline of the stipulated work is presented on **Figure 2**.

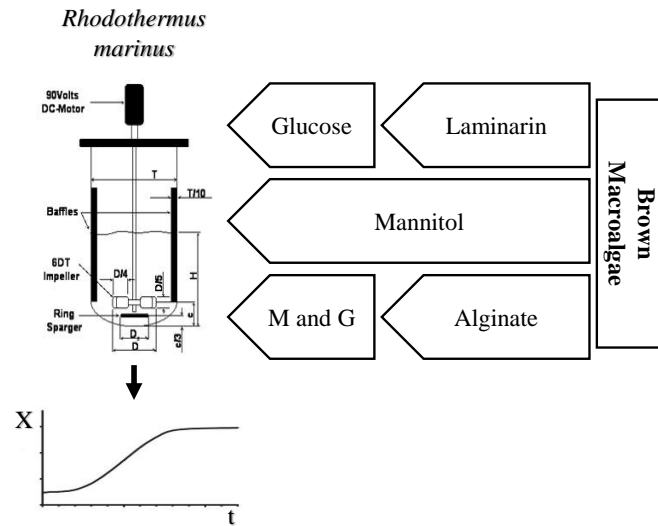


Figure 2 – Cultivation of *R. marinus* in brown macroalgal carbohydrates.

The work developed at Biotrend consisted of stocking the *R. marinus* strains supplied by the EU-project and verifying the partners premises, in order to maximize the biomass yield. The studies carried out were as follows:

- First stage - growth with glucose, mannitol and alginate as C-sources;
- Second stage - growth with varying concentrations of sodium chloride and a different non-chlorinated salt;
- Third stage - growth in defined media supplemented with different macroalgal biomass;
- Fourth stage - validation in controlled bioreactor of the promising results obtained in previous assays (performed in shake flask).

3. State of the Art

3.1. Extremophiles – Marine Bacteria

Extremophiles are described as organisms that can live and reproduce in harsh environments (Schiraldi and De Rosa, 2002). In the past 50 years, extremophiles have been isolated from worldwide locations such as Antarctic, deep sea and geothermal sites at various depths. The capability of extremophiles to survive in extreme conditions is generally related to specific structural and metabolic features that can be interesting for several biotechnological applications. More specifically, extremophilic bacteria are promising sources of highly bioactive primary and secondary metabolites that represent potential valuable compounds.

In marine environments, that provide a large range of adverse conditions such as low/elevated temperatures, high salinities, high pressures and sudden changes on the pattern of light, bacteria are widespread (Dash et al., 2013). The frequent variation of environmental conditions in seawater led to the rich genetic and physiologic diversity preserved by marine bacteria which obviously represents an interesting subject for research. Furthermore, marine habitats represents more than 90% of total biosphere volume (Lauro et al., 2009), including not only marine water but also sediments, sea hydrothermal structures and characteristic microbial flora of other marine organisms (Dash et al., 2013).

The vast features found in marine bacteria have triggered several studies of comparison with terrestrial ones. The majority of marine bacteria exhibit tolerance to higher pressures than the terrestrial bacteria (Zobell and Morita, 1957). Certain marine bacteria have the ability of forming motile spores which distinguishes them from the terrestrial counterparts (Buerger et al., 2012). Particular marine bacteria can grow and reproduce at temperatures as high as 95° C (*Aquifex pyrophilus*) (Huber et al., 1992). Some thermophilic marine bacteria are able to nitrify and fix nitrogen (Ruby and Jannasch, 1982). Marine bacteria usually require Na⁺ and K⁺ for growth and maintenance of osmotic balance between the cytoplasm and the surrounding environment (Macleod and Onofrey, 1957). It has been observed that a diversity of genetic material in marine microorganisms is correlated to alternative mechanisms of obtaining energy, metabolizing different type of compounds and surviving in those conditions. The resulting metabolic plasticity potentiates better sensing of given environmental stimuli, its corresponding integration and physiological response to adversity (Dash et al., 2013).

Most current knowledge about marine bacteria is related to their association with interesting antibiotic production (Okami, 1986), heavy metal and hydrocarbon bioremediation (Margesin and Schinner, 2001, Rainbow, 1995), antibiofilm formation (antifouling) activity (Jiang et al., 2011) and antibiotic resistance capability (Eom et al.,

2013). However, the marine bacteria potentialities remain almost unexplored, due to little knowledge about their genomics (Hartmann et al., 2014) and bioprocessing conditions.

Portugal holds one of the most extensive Exclusive Economic Zones (EEZ) in the world (1.8 million Km² (Seaaroundus.org, 2014)), which represents a strong economic, social and cultural impact. There are already some research groups such as CESAM (“Centro de Estudos do Ambiente e do Mar” – Aveiro University) dedicated to projects in marine biotechnology. However, given the richness and biodiversity of Portuguese coastal regions, it is imperative to improve the R&D in this area. Especially in Azores, the biotechnological potential of thermophilic bacteria collected in hydrothermal vents has been studied, namely the expression of thermostable amylases, cellulases and proteases (Barreto et al., 2014).

3.1.1. Marine Bacteria – mechanisms of adaptation

Marine bacteria show very quick responses to changing environmental conditions, making them versatile and potential biocatalysts or sources of biocatalysts for industrial bio-based processes. The chemotaxic mechanisms of marine bacteria are quite developed. It was described that marine bacteria are more adaptable to pH variations than terrestrial and other marine microorganisms (Takeuchi et al., 1997) but the mechanisms are unknown. Bacteria whose habitats are superficial marine waters are adapted to ultraviolet radiation pattern changes once they developed different mechanisms such as nucleotide excision, photoenzymatic and recombinational repair (Joux et al., 1999). The marine bacteria are constantly exposed to changes of temperature. Often, they overcome this problem by symbiosis with or even through pathogenic infection of other organisms. For example, when a sudden change of temperature occurs, *Vibrio shiloi* adheres to a coral host by a β -D-galactopyranoside receptor and infect epidermal cells (Banin et al., 2001).

The special feature belonging to some marine bacteria of facultative halophilicity is an interesting mechanism of adaptation which is detailed in the next section.

3.1.1.1. Osmoadaptation

Water is a key reagent for most biochemical reactions on the cell. Consequently, the available intracellular water determines the survival and viability of the microorganisms, being inversely proportional to the intracellular osmolarity (the concentration of salts and non-ionic solutes) (Galinski, 1995). The environmental osmolarity influences the intracellular osmolarity due to the osmotic pressure applied to the cell. Sudden changes in the environment osmolarity generate water fluxes that can cause cell cytotoxicity (hypotonic media) or dehydration/plasmolysis (hypertonic media). Interestingly, cells usually require the maintenance of an intracellular osmolarity slightly superior than that observed in the

medium to generate the required cell turgor (Csonka, 1989). Given the shifting salinities occurring in seawater, marine bacteria developed strategies to quickly adapt their metabolism to extremely high environmental osmolarities. Two osmoadaptation strategies seem to be the most common mechanisms used by marine bacteria for balancing osmotic stress: “salt-in” and compatible solutes accumulation (Empadinhas and Da Costa, 2008).

3.1.1.1.1. Salt-in

The salt-in mechanism is typical of some members of the archeobacterial family *Halobacteriaceae* (Lanyi, 1974) which conserve the osmotic equilibrium maintaining similar intracellular salt concentration to extracellular media. Consequentially, an increase in the medium osmolarity leads to the exposure of the cytoplasm to high salinities and low water activity which generally requires adaptations in the amino acid composition of cellular proteins (e.g. amino acid enrichment in aspartyl and glutamyl or substitutions for weakly hydrophobic residues (Lanyi, 1974)). At high salinities, the cell needs to attract a hydration net due to the low water activity in the medium. Also modifications in the tertiary and quaternary structure of the proteins such as additional loops with anionic amino acids potentiate their stabilization and the maintenance of their native structure (Zaccai et al., 1986). When a sudden reduction of salinity occurs and the cations in the cytoplasm are removed, the protein conformation is affected due to the repulsive forces resulting from the negative net of modified proteins (Sleator and Hill, 2002) - **Figure 3**. This mechanism is generally associated to microorganisms that are strictly confined to high salinity environments.

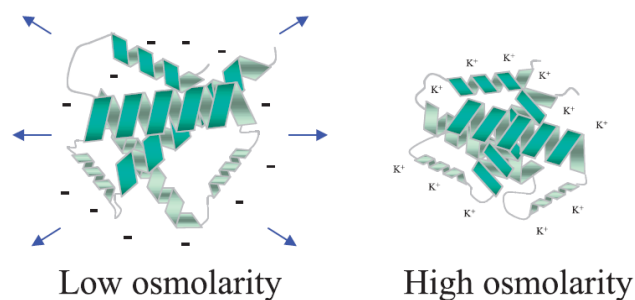


Figure 3 – Effects of low and high osmolarity on the enzymes structure of halophiles presenting “salt-in” mechanism (Dash et al., 2013).

3.1.1.1.2. Compatible solutes

Compatible solutes are low molecular-weight organic compounds (Sleator and Hill, 2002). These compounds are referred as compatible solutes due to their great cell compatibility even at high concentrations (Sleator and Hill, 2002). Such solutes are highly

soluble compounds, neutral at pH 7 and usually do not interact with cellular proteins (Sleator and Hill, 2002). Thus, the microorganisms easily accumulate these osmoprotective compounds to high intracellular concentrations by synthesis, uptake or both (Sleator and Hill, 2002). The accumulation of compatible solutes permits a higher flexibility to salinity fluctuations than the “salt-in” mechanism. Besides that, it was described that the required genetic changes are minimized (Yancey et al., 1982). The mechanism involves two sequential responses when the cells are exposed to elevated osmolarity: 1) an increase of intracellular concentration of paired K^+ and glutamate and almost immediately 2) an accumulation of increasing amounts of compatible solutes in the cytoplasm (Epstein, 1986). So, at high medium osmolarity, the accumulation of compatible solutes leads to an isotonic condition and maintain the protein hydration because of their preferential exclusion from the protein surface (steric incompatibility) - **Figure 4**. At low medium osmolarity, the cells rapidly release some inner compatible solutes (those having bidirectional transporters such as glycine betaine (Sleator and Hill, 2002)) to the extracellular medium in order to achieve osmotic equilibrium (Sauer and Galinski, 1998). It has been observed that the compatible solutes also stabilize protein structure against freezing, high temperatures and drying (Lippert and Galinski, 1992), increase the cell volume (Sleator and Hill, 2002) and can be catabolized by other microorganisms (Empadinhas and Da Costa, 2008).

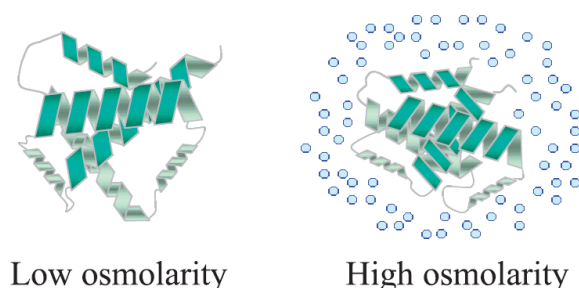


Figure 4 – Effects of low and high osmolarity on the enzymes structure of halophiles presenting compatible solutes mechanism (Dash et al., 2013).

The main compatible solutes produced by bacteria were summarized by Empadinhas and Da Costa (2008): aminoacids (such as alanine, glutamate, glutamine, glycine betaine and proline), glycerate, phosphodiester derivatives, polyalcohols (sorbitol, mannitol and glucosylglycerol) and some sugars (sucrose and trehalose).

3.1.1.2. Low salinity-stress

Halophilic bacteria can require more than 20% NaCl for growth (Kushner, 1985). For these microorganisms, the dilution of NaCl in the medium becomes a stress factor. On the other hand, chlorinated media enhances the corrosion rate of stainless steel equipment (Anon, 1963). In this sense, the cultivation of extremely halophiles in industrial bioreactors

is troublesome. Possible solutions for the problem are to adapt the industrial strains of halophiles to lower NaCl concentrations or to substitute NaCl for non-chloride salts in order to reduce the equipment depreciation.

Wong and Liu (2008) investigated the response of *Vibrio vulnificus* to low NaCl stress. *V. vulnificus* is an halophilic, Gram-negative, marine bacterium (Wong and Liu, 2008). The experiments were based on the comparison between the adaptive response of a pre-adapted group to low NaCl content and a control group. The results showed that the adapted group was more resistant to a lethal low salinity than the control group. Furthermore, the authors verified that the adaptation process applied in the exponential phase was more effective than that in the stationary phase. For the verified adaptation times of exposure to adaptation (30, 60 and 90 minutes), the more effective (higher bacterial resistance) was that of 30 minutes. The effect of the addition of osmoprotectants to the medium was also evaluated. The supplementation with glycine betaine and sucrose effectively enhanced the survival of the cells under lethal low salinities (Wong and Liu, 2008). The supplementation with peptone was less effective due to the presence of low amounts of proline, the most significant osmoprotectant between the other free amino acids (Wong and Liu, 2008).

Barclay (2002) patented a process for reducing corrosion of bioreactor during fermentation providing sodium in the form of non-chloride salts, namely Na₂SO₄. The author warns that some strains can exhibit significant aggregation even though maintaining the growth rate.

3.1.2. *Rhodothermus marinus* – physiology and genetics

Rhodothermus marinus is a marine bacterium belonging to phylum *Bacterioidetes* (Alfredsson et al., 1988), class *Sphingobacteria* and family *Crenotrichacea* (Garrity et al., 2004). Alfredsson et al. (1988) firstly isolated a *Rhodothermus* sp. from sub-marine (2-4 meters depth) alkaline hot springs at Isafjardardjup, Iceland. Since then, *R. marinus* was isolated in three more geothermal habitats in Iceland (Petursdottir et al., 2000), in Stufe di Nerone, Italy (Moreira et al., 1996), on the island of Monserrat, Argentina (Silva et al., 2000) and at two sites in São Miguel island (Azores), Portugal (Praia da Ribeira Quente (Nunes et al., 1992) and Ferrara (Silva et al., 2000)).

R. marinus is a microorganism with 476 nm diameter, 2-2.5 µm length (Alfredsson et al., 1988) and a polar flagellum (Nunes et al., 1992) not related to mobility. Alfredsson et al. (1988) reported the absence of spore and lipid granule formation and the presence of an external capsule when grown in carbohydrate-rich medium. Most strains forms reddish colonies (Alfredsson et al., 1988). The major pigments found in strain DSM 4253 consists of glycosidic carotenoids and their acyl derivatives (Lutnaes et al., 2004).

The main parameters of growth of *R. marinus* and its suitable substrates are summarized in **Table 1** (Parts A and B, respectively).

Table 1 – Main parameters of growth (A) and suitable substrates (B) for *R. marinus* (Alfredsson et al., 1988, Nunes et al., 1992, Silva et al., 1999).

Part A		Part B	
Temperature		Suitable substrates	
Range	54-77 °C	2-Oxoglutarate	-
Optimum	65 °C	Asparagine	+
pH		Aspartate	+
Range]5.0;9.0]	Cellobiose	+
Optimum	7.0	Citrate	-
NaCl		Galactose	+
Range	0.5-6.0 %	Glucose	+
Optimum	1%	Glutamine	-
KCl		Glycerol	-
1%	no growth	Lactose	+
Kinetics		Malate	-
μ_{\max} (1% NaCl; 65°C)	0.61 h ⁻¹	Proline	-
Generation time	80 min	Sorbitol	-
Other		Starch	+
Anaerobic growth (with nitrate)	-	Succinate	-
Production of nitrite	-	Xylose	+

R. marinus strains usually grows at temperatures ranging 54 to 77 °C (65 °C as optimum) and salinities ranging 0.5 to 6.0 % NaCl (1.0 to 2.0 % NaCl as optimum, depending on the medium) (Alfredsson et al., 1988, Silva et al., 1999), being therefore a moderate thermophile and a slight halophile. The optimum pH for growth is 7.0 (Alfredsson et al., 1988) and it was shown that there is no growth at pH 5.0 or below (Gomes et al., 2000) and pH above 9.0 (Nunes et al., 1992). *R. marinus* was classified as heterotroph and strictly obligate aerobe (Bjornsdottir et al., 2006). It was also observed that *R. marinus* does not grow with no NaCl in the medium (Alfredsson et al., 1988). The optimal salinity for *R. marinus* hardly depends on the growth temperature (Silva et al., 1999). In literature, the maximum specific growth rate (μ_{\max}) was 0.61 h⁻¹, calculated for cultivations in 1.0 % NaCl and 65 °C (Silva et al., 1999).

It has been reported that most strains of *R. marinus* can catabolize hexoses such as glucose and galactose and disaccharides such as lactose, maltose, and sucrose (Alfredsson et al., 1988). The existence of the encoding gene for the enzyme mannitol 2-dehydrogenase indicates that *R. marinus* potentially catabolizes mannitol (Nolan et al., 2009). Gomes and Steiner (1998) reported that *R. marinus* can also catabolize more complex carbohydrates such as starch, xylan, mannan and galactomannan and consume diverse nitrogen sources such as yeast extract, cotton seed protein, meat and fish peptone. *R. marinus* does not reduce

nitrate (Alfredsson et al., 1988), is resistant to aminoglycoside antibiotics (Alfredsson et al., 1988) and not resistant to ampicillin, lincomycin and tetracycline (Bjornsdottir et al., 2006).

The **Figure 5** shows an electron micrograph of *R. marinus* strain DSM 4252 cells. A more detailed view (*R. marinus* strain R-18) shows that the cell consists of a Cytoplasmic Membrane (CM), a Peptidoglycan Layer (PG) and an Outer Membrane (OM) in agreement with Gram-negative classic representation. The forming furrow of dividing cells is marked with "F".

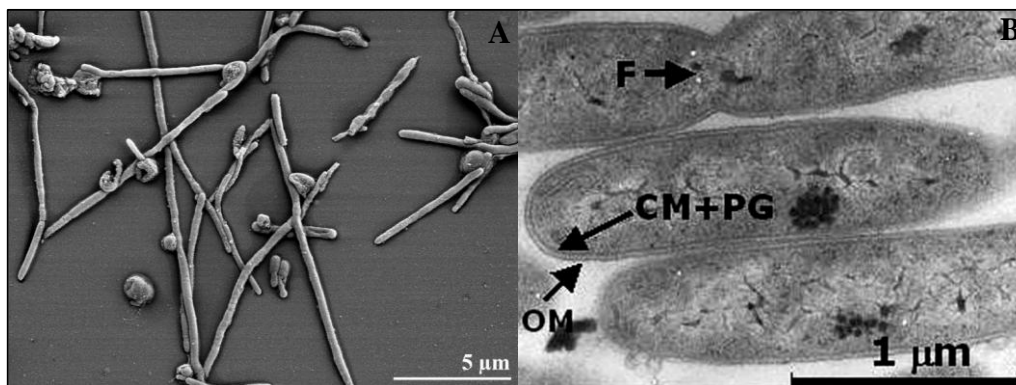


Figure 5 – Micrographs of *R. marinus* DSM 4252 (A) and R-18 (B) obtained by scanning electron microscope (SEM) (Bjornsdottir et al., 2006, Nolan et al., 2009).

The complete genome of *R. marinus* DSM 4252 was firstly sequenced and annotated by Nolan et al. (2009). It was observed that it is about 3.39 Mb long and includes a 125 kb plasmid. The metabolic pathways and biosynthesis mechanisms were explored with the characterization of certain precursors involved in citric acid cycle (Bjornsdottir et al., 2006).

The actual knowledge about *R. marinus* pool of enzymes is directly related to the biotechnological potential of thermostable enzymes, namely polysaccharide hydrolases. Most of them have been isolated and expressed in an alternative host (generally *E. coli*) in order to potentiate massive production. Main *R. marinus* polysaccharide hydrolases and respective optimal temperatures are presented on **Table 2**.

Table 2 – Main *R. marinus* polysaccharide hydrolases – optimal temperatures.

Enzyme	Host	T _{opt} (°C)	References
Cellulase	-	100	Hreggvidsson et al. (1996)
Glucanase	<i>E.coli</i>	85	Spilliaert et al. (1994)
Laminarinase	<i>E.coli</i>	88	Krah et al. (1998)
Xylanase	-	90	Dahlberg et al. (1993)
Mannanase	<i>E.coli</i>	85	Politz et al. (2000)
Chitinase	<i>E.coli</i>	70	Hobel et al. (2005)
Pullulanase	-	80	Gomes et al. (2003)
Amylase	-	85	Gomes et al. (2003)
Trehalase	<i>E.coli</i>	65	Jorge et al. (2007)

A cellulase (endo- β -1,4-glucanase) was detected by cultivation of *R. marinus* DSM 4253 (Hreggvidsson et al., 1996). It was described as an extremely thermostable hydrolase due to its half-life time of 3.5h at 100°C. The enzymatic products were glucose, cellobiose, cellotriose and a mixture of larger oligosaccharides. Other glucanases were overproduced in *E. coli* (β -Glucanase and Laminarinase) and also seem to be thermostable and functional (Krah et al., 1998, Spilliaert et al., 1994).

Dahlberg et al. (1993) described that *R. marinus* is able to grow in xylans because of the secretion of thermostable xylanolytic enzymes. The production of *R. marinus* xylanases for their use in the enzymatic treatment of lignocellulosic pulp was patented (Dahlberg et al., 1995). *R. marinus* is also able to grow in locust bean gum because of a high β -mannanase activity (Gomes and Steiner, 1998).

A chitinase gene was isolated from *R. marinus* genome (Hobel et al., 2005) and expressed in *E. coli*. It was shown that the isolated chitinase is an endoacting enzyme that releases chitobiose as main product and can hydrolyze deacetylated chitosan. Gomes et al. (2003) screened strains of *R. marinus* for the production of pullulanases and amylases and showed the evidence of activity of both enzymes for strain ITI990. Jorge et al. (2007) observed that the overexpression of a trehalase gene present in *R. marinus* genome generates a functional enzyme whose maximum activity temperature coincides with optimal *R. marinus* growth temperature.

More recently, four different alginase (alginate lyase) encoding genes isolated from *R. marinus* have been characterized and expressed by the Icelandic company Prokazyme. This company maintain a R&D cooperation with Mafís ltd., a partner in the BlueGenics project. Prokazyme has developed some works with *R. marinus* and already markets a laminarinase (ThermoActive™ Laminarinase) from selected strains. The available information for alginases is summarized on **Table 3**.

Table 3 – *R. marinus* alginases – main characteristics (Prokazyne, 2014).

Enzyme	T _{opt} (°C)	Stability at 70° C	pH _{opt}	Action pattern
AlyRm1	87	5 h	7.2	endo-/exo-
AlyRm2	81	>16 h	6.5	endo-
AlyRm3	75	8 h	5.5	endo-
AlyRm4	81	>16 h	6.5	exo-

It was observed that the four enzymes are much more thermostable than other known alginases. Furthermore, they present a very high range of optimal conditions for industrial application (temperature, stability time, pH and action pattern), whereby the company is applying for a patent that is currently pending.

Also restriction endonucleases (Hjörleifsdottir et al., 1996), DNA ligases and DNA polymerases were screened (Hjörleifsdottir et al., 1997) with the goal to develop technologies for DNA cloning and amplification, due to the thermostability of these enzymes.

It was observed that *R. marinus* responds to increasing osmolarity by accumulating low amounts of intracellular compatible solutes such as glutamate, glucose and trehalose beyond K⁺ by “salt-in” mechanism (Nunes et al., 1995, Silva et al., 1999). Nunes et al. (1995) also detected the accumulation of two major compatible solutes, α -mannosylglycerate (MG) and α -mannosylglyceramide (MGA). A primary accumulation of MG was described as result of increasing salinity of the medium. At salinities nearby the maximum for the *R. marinus* growth (6% NaCl), MGA becomes the dominant compatible solute present on the cell.

3.2. The Biorefinery Concept

Several definitions of biorefinery can be found in the literature. Among them, the most clear and accepted seems to come from “biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy” (IEA, 2007). Analogously to petroleum refinery concept, which consists of producing fuels, chemicals and materials from the fractionating of crude fossil reserves, a biorefinery comprises the expertise, raw materials and equipment necessary for producing biofuels, biochemicals, biomaterials and bioenergy from biomass conversion (Cherubini, 2010). The biorefinery concept purposes to gradually convert the actual fossil fuel-based economy into a sustainable bio-based economy. This replacement requires not only scientific and technological developments in biotechnology, genetics and process engineering but also a social mentality transformation. In recent years, the society has acknowledged the benefits that arise from a renewable sources-based economy and started to fund R&D projects related to this proposal (Cherubini, 2010).

The main operations of biorefinery were summarized by Kamm and Kamm (2006): the biomass containing the desired precursors for biotransformation (feedstock) is separated, mainly by physical methods; the separated precursors are subjected to chemical and biological treatments; the products are recovered and the by-products should be recirculated or treated before being discarded to for the environment.

One of the most important biorefinery characteristics is the sustainability of the processes. Accordingly, its implementation should not negatively impact both the involved ecosystems (minimizing generated gases and toxic wastes) and the economy more than the analogous processes developed so far (Ragauskas et al., 2006). However, the most used feedstock for biorefinery concepts has been crop biomass, which significantly affects the world economy due to the competition in prices of food (Jung et al., 2013). This concern led to the classification of edible raw materials as first generation feedstocks. Second generation feedstocks (non-edible raw materials such as lignocellulosic biomass, agricultural residues, industrial by-products and municipal wastes) have gradually attracted more interest, not only because of the aforementioned food concern, but also because the higher range of availability worldwide (Ho et al., 2014).

The production of biofuels has been the first focus of the biorefinery development (Cherubini, 2010) given the world energetic dependence on fossil fuels reserves which are running out. Because of that, the industrialization of biofuels (essentially bioethanol and biodiesel production) has been quite developed. However, the majority of bioethanol production are still based on first generation dedicated feedstocks such as sugarcane (Brazil, Colombia and India) and corn (USA and China) (Cheng and Timilsina, 2011). Lignocellulosic biomass is a suitable feedstock for bioethanol production but presents lower bioethanol yields than crop biomass, which is currently preferred due to economic reasons (Jung et al., 2013). The feedstocks for biodiesel production are also mostly based on first generation feedstocks such as palm oil and soybean (Atabani et al., 2012).

Recently, a third generation feedstock concept emerged, consisting of quickly growing aquatic autotrophic organisms, i.e. algae (both micro- and macroalgae) (Jung et al., 2013). The potential of macroalgal biomass as feedstock for biorefinery proposals is discussed in the next chapter. Some authors classify the genetic engineered algal biomass as a fourth generation feedstock for biorefinery proposals (Lü et al., 2011).

The biorefinery nomenclature according to the feedstock generation is sometimes controversial. In recent years, the R&D established four biorefinery systems as models of study: the “Whole-Crop” biorefinery including mill-dry feedstocks such as sugarcane and cereals; the “Lignocellulosic Feedstock” biorefinery including nature-dry feedstocks (cellulose-containing biomass and organic wastes); the “Green” biorefinery, using nature-wet feedstocks such as switchgrass and immature cereals; and the “Two-Platform”

biorefinery, a concept that combines a sugar platform and a syngas platform – detailed information in “Advances in Biochemical Engineering” (Kamm and Kamm, 2006). More recently, other biorefinery models have emerged as cases of study, such as “Marine Biorefinery”, whose feedstock is based on marine biomass (Cherubini et al., 2009), the focus of this Master’s thesis.

The application of multiple feedstocks for achieving a multi-product output ensure more dynamics and robustness to the concept – called as a “phase-III biorefinery”. Unlike petroleum refineries, the biorefineries are expected to be developed in dispersed areas and to exhibit a whole range of setups. Accordingly, the development of Integrated Biorefining Complexes (clusters of bio-based platforms) could allow surrounding industries to incorporate the own by-products in the input flows of the biorefinery to enhance the utilization of biomass components (Cherubini, 2010) and reduce spending on feedstock transportation.

3.3. Macroalgae – general considerations

Algae are a very large group of organisms including microalgae (single or filamentous unicellular forms) and macroalgae (multicellular form). Macroalgae (also colloquially called seaweeds for benthic habitats) are characterized as eukaryotic, photosynthetic and multicellular organisms possessing some lower plants characteristics and consisting of a leaf-like thallus without vascular tissues (Lobban and Harrison, 1994). The thallus color derives from the presence of different natural pigments and chlorophylls and determines the phylogenic classification (group or *phylum*) of macroalgae including some members of green (*Chlorophyta*), red (*Rhodophyta*) and brown (*Phaeophyta*) algae (Sze, 1998). The described dominant pigments are chlorophyll a and b in green macroalgae, phycocyanin and phycoerythrin in red macroalgae and fucoxanthin in brown macroalgae (Lin and Qin, 2014). Some authors describe macroalgae based on morphology (functional-form): coarsely-branched, crustose, filamentous, jointed-calcareous, sheet and thick-leathery macroalgae (Hurd et al., 2014).

Macroalgae are described as primary producers because their capability of using light, CO₂ and/or HCO₃⁻ for the production of organic compounds and oxygen (Gao and McKinley, 1994). Due to the higher diffusion rate of HCO₃⁻ in seawater, most macroalgae uptake HCO₃⁻ rather than CO₂ (Lobban and Harrison, 1994).

Until now, there are about 10 000 known species of macroalgae. Most of them are marine and live attached in suitable surfaces. Some species can live at high depth of sea or in floating forms due to changes of gas content on the cells (Lin and Qin, 2014). The life cycles of macroalgae are diverse, with different combinations of sexual and asexual

reproductive strategies (Roesijadi et al., 2010). The most cost-effective method for farming macroalgae is the vegetative cultivation (McHugh, 2003). Although, some brown macroalgae require a reproductive cycle between generations (McHugh, 2003).

The macroalgae industry represents 6.5 billion euros per year worldwide (FAO, 2012). Currently, the food products based on macroalgae such as aonori, kombu, nori and wakame represents 83-90% of total macroalgae industry value (Wei et al., 2013). Most remaining value refers to the utilization of phycocolloids (discussed in Chapter 3.3.2) for biotechnological and pharmaceutical proposals (Roesijadi et al., 2010). Some application of macroalgal biomass for fertilizers and animal feed has been described. The macroalgal biofuels industrial value is still negligible (Roesijadi et al., 2010). Portugal was one of the world's largest producers of agar in 1980 decade but nowadays this industry is residual. In north region, the traditional mix of brown macroalgae “Sargaço” is still used as fertilizer (Netalgae.eu, 2012). In Azores, the traditional “tremoço do mar”, also a brown macroalgae, is widely consumed as a snack (Patarra et al., 2014). Some recent commercial projects, such as AlgaPlus, may contribute to the sustained and sustainable growth of macroalgae-based businesses in Portugal.

It is important to mention that huge drift macroalgae masses are washed up on the coast frequently. There are many reports that *Ulva* spp. (green macroalgae) are found on shore in many locations of Europe (Smetacek and Zingone, 2013). Traditionally, the biomass is used as fertilizer. The occurrence of this phenomenon, often called “green tides” (**Figure 6**), is increasing due to sea pollution. However, this biomass is not yet used by industries maybe because its availability are unpredictable. So, “green tides” are still considered as a waste and an environmental problem (Smetacek and Zingone, 2013).



Figure 6 – Photo of a “green tide” in Brittany, France (Smetacek and Zingone, 2013).

3.3.1. Biochemical Composition

For biotechnological applications, the analysis of the chemical compounds available in macroalgal biomass is crucial to investigate a promising biotransformation and to design and optimize the technological process. It is described that dry macroalgal biomass are generally comprised of 25–60% of carbohydrates (Jung et al., 2013), the most significant compounds available for bioprocessing. The typical composition of macroalgal biomass is presented on **Table 4**. The biochemical profile of macroalgae is particularly variable seasonally even within the same phylum (Khairy and El-Shafay, 2013).

Table 4 – Typical composition (approximated) of green, red and brown macroalgae.

Components	% w/w		
	Green (<i>Ulva</i> sp.)	Red (<i>Gracilaria corticata</i>)	Brown (<i>Laminaria</i> sp.)
Polysaccharides			
Agar	ND	18	ND
Alginate	ND	ND	23
Carrageenan	ND	18	ND
Cellulose	18	ND	6
Fucoidan	ND	ND	5
Laminarin	ND	ND	14
Ulvan	20	ND	ND
Starch	2	ND	ND
Mannitol	ND	ND	12
Lipids	2	6	2
Proteins	19	15	12
Ash content	24	21	26
References	Bruton et al. (2009)	Omar et al. (2013)	Reith (2005)

ND – not determined.

The carbohydrate content of green macroalgae represents 25–50% w/w of dry biomass (Jensen, 1993). Green macroalgae, as the remaining macroalgae, generally present small amounts of starch (maximum 4% w/w of dry biomass) (Bruton et al., 2009). The distinctive polysaccharide present in green macroalgae is ulvan that typically represents 20% w/w of dry biomass. Cellulose is the other main component, generally representing 18% w/w of dry biomass.

Red macroalgae usually present 30–60% w/w of dry biomass in carbohydrates (Jensen, 1993). The distinctive polysaccharides present in red macroalgae are agar (18% w/w of dry biomass) and carrageenan (18% w/w of dry biomass) (McHugh, 2003), both galactans. Red macroalgae usually present starch granules (called floridean starch) which are structurally similar to higher plant starch granules (lacking amylose). These granules can represent up to 80% of cell volume (Yu et al., 2002).

The carbohydrate content of brown macroalgae is up to 60% w/w of dry biomass (Jensen, 1993). The most important distinctive polysaccharides of brown macroalgae are alginate (23% w/w of dry biomass) and laminarin (14% w/w of dry biomass). A high mannitol content is also reported (12% w/w of dry biomass).

Macroalgal biomass is significantly different from phyto-biomass in terms of the biochemical profile, and it is much less known by the scientific community. The distinctive polysaccharide included in macroalgal cell structures are agar, alginate, carrageenan, fucoidan, laminarin and ulvan. The structural representations of these polysaccharides are presented on **Figure 7**.

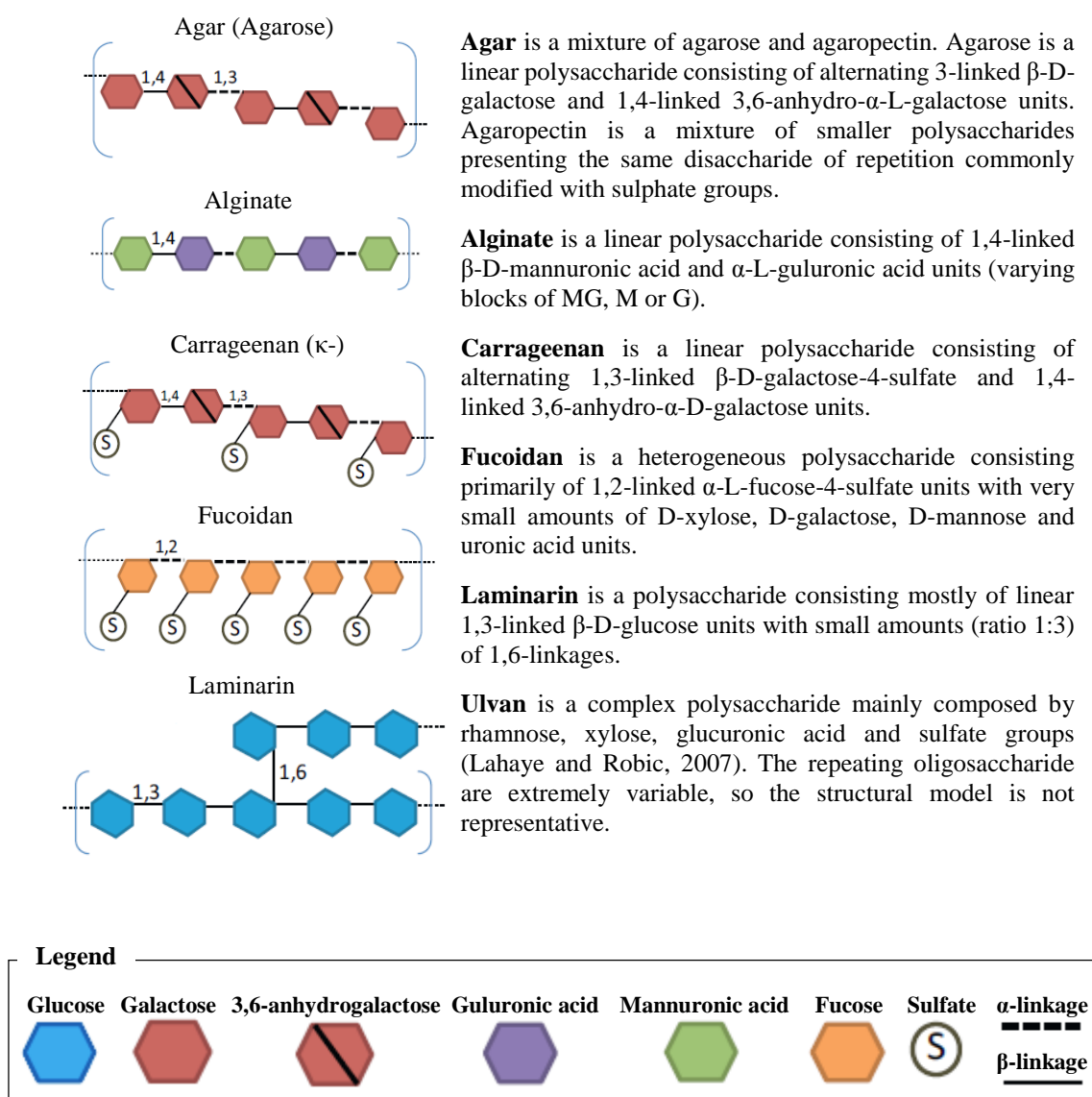


Figure 7 – Structural representation of typical polysaccharides present in macroalgal biomass (adapted of (Wei et al., 2013)).

The protein content of macroalgae varies accordingly to species and seasonal period. Generally, the protein content in brown macroalgae is lower than in green and red macroalgae (**Table 4**). A large part of the macroalgal amino acids content consists of aspartate and glutamate (Fleurence, 1999).

Generally, macroalgae present small amounts of lipids (maximum 6% w/w of dry biomass). The majority of the lipid content is in the form of saturated fatty acids (51.9–67.4%), mostly palmitic acid (38.0–59.8%) and myristic acid (4.5–12.4%) (Rohani-Ghadikolaei et al., 2012).

As shown on **Table 4**, macroalgae show high amounts of ashes. Rohani-Ghadikolaei et al. (2012) evaluated the proximate mineral composition of representative macroalgae and observed high amounts of potassium, magnesium and iron. Macroalgae selectively absorb and accumulate minerals from seawater. For this reason, the macroalgal mineral composition are seasonal and very specific with respect to the location of harvesting (Azmat et al., 2006).

It have been shown that several bioactive compounds are present in macroalgal biomass having anti-inflammatory, antidiabetic and antimutagenic properties (Andrade et al., 2013).

3.3.2. Phycocolloids

The term phycocolloids refer to algal colloids, the most relevant commercial polysaccharides worldwide (Bixler and Porse, 2011). They are described as high molecular weight polysaccharides found in the cell wall of algae that usually form colloidal solutions (Cardoso et al., 2014). These compounds are used as gelling agents, stabilizers and thickeners mostly in biotechnology, cosmetics, food, medical and pharmaceutical industries (Cardoso et al., 2014). Currently, the most commercially significant phycocolloids are agar, alginates and carrageenans (Roesijadi et al., 2010). These polysaccharides are obtained in high amounts from macroalgal biomass usually by alkaline or hot water treatments (Cardoso et al., 2014). The main applications of the three major colloids obtained from macroalgal biomass are summarized on **Table 5**.

Table 5 – Main applications of agar, alginate and carrageenan (adapted from Cardoso et al. (2014)).

Phycocolloids				
Applications	Agar	Alginate	Carrageenan	Function
Biotechnology				
Electrophoresis gel	✓		✓	Gelling
Biocatalysts Immobilization		✓	✓	Matrix
Solid media	✓			Gelling
Cosmetics				
Shampoos		✓		Vitalization interface
Toothpaste			✓	Viscosity
Food				
Chocolate milk			✓	Keep the cocoa in suspension
Gums and sweets	✓		✓	Gelling, texturing
Juices	✓		✓	Viscosity, emulsifier
Low calorie gelatins			✓	Gelling
Milk ice-cream			✓	Prevent ice crystals formation
Sauces and condiments	✓		✓	Thicken
Medicine and Pharmaceutics				
Laxatives		✓		Indigestibility and lubrication
Metal poisoning			✓	Binds metal
Tablets		✓	✓	Encapsulation
Other Industries				
Paints		✓		Viscosity and glazing
Paper making	✓	✓	✓	Viscosity and thickening

The agar gels are formed in water solution by hydrogen bonds between the polysaccharide molecules (Paolucci et al., 2011). Due to this property, the interior of the formed matrix exhibits a great amount of water that moves almost without spatial restrictions. The agar gels are thermally reversible and formed by cooling hot aqueous solution. The agar viscoelastic strength is affected by sulfate substitution level (lower substitution level represents stronger gels) (Phillips and Williams, 2000). Accordingly, agarose is the gelling fraction of agar, since it is low in sulfates. Agar presents gelling, stabilizing and thickening properties and is classified as a GRAS (Generally Recognized as Safe) product by the USA Food and Drug Administration (FDA). Additionally, agar is tasteless (no salts requirement) and presents intestinal-regulating and satiating characteristics (Lahaye, 1991). Because of these reasons, agar is used in the processed food industry including condiments, gums, juices, sauces and sweets. Agar is a good ingredient for low-calorie products since it is not digestible by humans (Lahaye, 1991). Biotechnological applications of agar include preparation of solid culture media and

separation of macromolecules by electrophoresis (specifically purified agarose) (McHugh, 2003).

The term alginate is used to define the alginic acid, its derivatives and salts (Cardoso et al., 2014). The segments of guluronic acid units (G-blocks) present more folded and rigid structural conformation than segments of mannuronic acid units (M-blocks), so the length of G-blocks determines the properties of alginate gels. In aqueous solution, an ionic cross-linkage between divalent cations present in the medium and COO^- groups of guluronic acid units occurs (Paolucci et al., 2011). A network with a typical conformation of “egg-box” is created (Grasdalen et al., 1981) which is stable and thermo-irreversible, so the gels do not melt at high temperatures. Alginates are GRAS and their properties of emulsifying, gelling, stabilizing and thickening led to some application in food industry, especially for improving textural quality of beer and wine. Various applications are described in cosmetics, medicine, pharmaceuticals and painting industries. Particularly in biotechnology, several works have been described about biocatalysts immobilization (Elnashar et al., 2013).

The carrageenan structure is highly variable. At least 15 different carrageenan structures have been described (Cardoso et al., 2014). The most relevant are kappa (κ -), iota (ι -) and lambda (λ -) carrageenans. The κ -carrageenan structure is described on **Figure 7**. The ι -carrageenan is similar to κ -carrageenan, except that the anhydrogalactose is sulfated at carbon 2. The λ -carrageenan consists of alternating 1,3-linked β -D-galactose-2-sulfate and 1,4-linked α -D-galactose-2,6-disulfate units (Paolucci et al., 2011). The κ -, ι - and λ -carrageenans are typically brittle gel-forming, elastic gel-forming and non-gelling, respectively. Strong gel formation in κ - and ι -carrageenan requires a gel-inducing cation, generally K^+ and Ca^{2+} respectively (Cardoso et al., 2014). Carrageenans (also GRAS) are one of the most commercially utilized emulsifier/stabilizer agents in the food industry (Pereira et al., 2009). For example, κ - and λ -carrageenans are ideal stabilizers for the suspension of cocoa in milk chocolate (Van De Velde and De Ruiter, 2002). Other food applications include texturing cheese and preventing ice crystals formation in milk ice-creams. The immobilization of biocatalysts in carrageenan beads have also been used (Elnashar et al., 2013).

3.4. Macroalgae as a potential feedstock for biorefinery

Recently, macroalgae have attracted attention as a potential feedstock for biorefineries (Jung et al., 2013) due to their high content in polysaccharides. As mentioned in the previous chapter, biorefinery feedstocks have been based mainly on crop and some lignocellulosic biomass (both designated as terrestrial biomass).

Several authors claim that macroalgae have high potential to partly replace terrestrial biomass as biorefinery feedstock in order to achieve a more sustainable bio-based economy. Some reasons are presented: macroalgae do not require land and freshwater for cultivation (Lobban and Harrison, 1994), they can be cultivated in salt water and municipal wastewater; the production yield of macroalgal biomass per cultivation area is higher than the terrestrial biomass yield (Bixler and Porse, 2011); the macroalgal photosynthetic efficiency can be three times higher than the terrestrial biomass photosynthetic efficiency (Jung et al., 2013); there is lower raw material competition for food industry than crop cultivations since the macroalgae application is geographically more restricted (Bixler and Porse, 2011); the macroalgal structure lacks lignin and hemicellulose (present in lignocellulosic biomass) which theoretically provides an easier depolymerization and lower amount of inhibitors in the hydrolysates (Bixler and Porse, 2011).

Microalgae can also represent a useful feedstock for biorefinery concepts. However, some problems have been found too (Pan et al., 2011). The open-air mass-cultivation of such fragile organisms generally leads to contamination problems (Wang et al., 2014). The separation of tiny and buoyant (due to the high lipid content) cells from culture medium presents technical and economic issues (power and time consuming stage, often involving an energy-intensive centrifugation step) (Sydney et al., 2011). Some authors refer that filamentous microalgae would be a better candidate for mass-cultivation, since they are more resistant to contaminations and can be harvested by a single filtration step (Wang et al., 2014), but the insights about that are at a very early stage. None of the aforementioned problems affect the macroalgae mass-cultivation. The biochemical profiles of micro- and macroalgae are quite distinct whereby the intended final product(s) will determine their use as biorefinery feedstocks in the coming years.

There are some barriers to macroalgal biomass implementation as feedstock in a biorefinery concept. It is important to note (**Figure 7**) that many monomers present in macroalgal biomass are generally not found in terrestrial biomass (Wei et al., 2013). Despite the macroalgal polysaccharides depolymerization is expectedly much easier, the desired total conversion of “unusual” fermentable monosaccharides can represent a technological challenge due to the low knowledge about the metabolism of those monomers. Since macroalgal physiology and morphology are quite different of terrestrial biomass (Lobban and Wynne, 1981), the lack of know-how and specific technology for genetic and metabolic manipulation still exists. The massive cultivation of macroalgae has increased in the last years (Jung et al., 2013): in 2012, it was noted that the majority of total production came from farms (FAO, 2012). Despite this, the world production of total macroalgae was two orders of magnitude less than the world production of sugarcane (the most produced energy crop in 2012) (Jung et al., 2013). Despite the great potential of untapped natural stocks for a large scale application as feedstock, an increase in macroalgae farming area is obviously

needed to prevent fluctuations in biorefineries feeding (Wei et al., 2013). Correlated to the need of increasing the massive production, possible environmental impacts may arise (Gunaseelan, 1997) that deserve attention such as alteration of natural habitats, nutrient depletion and changes in biodiversity (De Silva, 1992). It should be referred that the high concentrations of NaCl present in macroalgal biomass can be a huge technical problem for applying it to a bio-based process, since salts may inhibit microbial growth and enzymatic activity (Klinke et al., 2004), in addition to the aforementioned corrosion issues on bioprocessing equipment.

The increase of the macroalgal biomass production for biorefinery concepts leads to the need of understanding and improving the farming technology and the experience of the main macroalgae producers (East Asian countries such as China, Philippines and Indonesia) (Roesijadi et al., 2010). The current production of red and brown macroalgae is much higher than green macroalgae, which is negligible. The most commercially important macroalgae species are *Laminaria japonica* (brown) and *Eucheuma* sp. (red), representing more than 50% of total world production in 2010 (Jung et al., 2013). Those species seem to be the most promising feedstocks since there is already more practical know-how, production infrastructure and logistics of supply.

On **Figure 8**, a possible configuration for a biorefinery based on macroalgal biomass is presented.

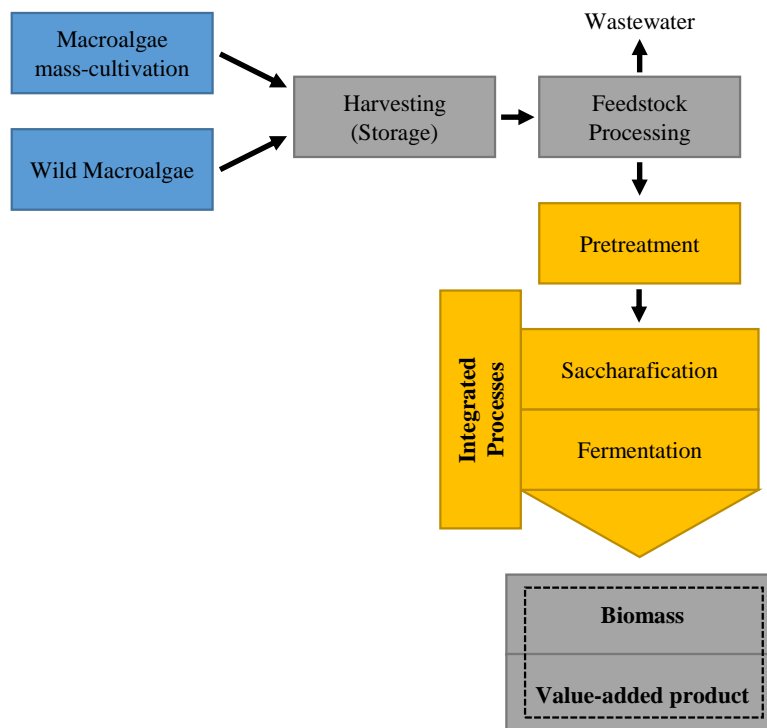


Figure 8 – Main steps of a macroalgal biomass-based biorefinery.

Macroalgae are present, as natural or cultivated stocks, in three locations worldwide: nearshore waters (generally attached to rocks), offshore waters and terrestrial systems (Wei et al., 2013). The nearshore stocks are the most harvested macroalgae worldwide. In Europe, harvesting is almost restricted to natural stocks whereas in Asia, harvesting is based in cultivated stocks that represents 89% of World production. In Portugal, the natural stocks are based on red macroalgae (spread along the continental Azorean coasts) (Netalgae.eu, 2012). The manual harvesting of macroalgae is still common in some countries but the increasing demand for phycocolloids led to the implementation of mechanical harvesting systems. In a biorefinery concept, after dewatering and crushing steps (designated as feedstock processing on **Figure 8**), the macroalgal biomass should be pretreated and saccharified in order to be used as feedstock for fermentation (Wei et al., 2013).

Similarly to classic biorefineries, macroalgal biomass-based biorefinery requires at least two unit operations including pretreatment-saccharification and fermentation. It is often difficult to dissociate the pretreatment and saccharification steps since most of the pretreatment methods usually originate some polysaccharide degradation.

3.4.1. Pretreatment and Saccharification

Biomass pretreatment is essential and one of the most expensive steps in bioprocessing. An effective pretreatment destroys and fragments the cell wall and improves the following step of biomass hydrolysis to fermentable sugars. In fact, the pretreatment of macroalgal biomass is easier than terrestrial biomass due to the lack of lignin and its “soft” organization in its cell wall structure, but there are less insights about it. Some physical and physic-chemical methods have been developed in this sense.

Some physical pretreatments such as mechanical fragmentation (milling), irradiation and ultrasonication (Li et al., 2014) can be applied for obtaining smaller particles (higher surface area-volume ratio) in order to achieve higher saccharification efficiency of macroalgal biomass. Applying gamma irradiation on *Undaria* sp. (brown macroalgae) biomass, Yoon et al. (2012) found that the cell wall was seriously damaged and the concentration of fermentable sugars increased even without a step of saccharification. Zhou and Ma (2006) used ultrasound to degrade the cell wall of *Porphyra yezoensis* (red macroalgae) and found that the degradation rate effectively increases with the increase of ultrasonic power, higher temperatures and lower initial pH.

Several physic-chemical methods were developed for pretreating terrestrial biomass and began to be applied to macroalgal biomass pretreatment. Choi et al. (2012) applied a hydrothermal pretreatment method (high-temperature liquefaction) for improving the saccharification yields of green macroalgae *Ulva pertusa*. Applying 15 MPa and 150 °C for

15 min before the enzymatic hydrolysis step, the authors observed that 61.1 % of total glucose was released. The main advantage is no prerequisite of corrosion-resistant materials for the enzymatic hydrolysis step since the solvent used in the method is pure water. Viola (2009) tested the effect of the steam explosion pretreatment in the green macroalgae *Ulva armoricana* and observed that the method can improve the cellulose hydrolysis. Some research is necessary for transferring the pretreatment technologies used in terrestrial biomass for macroalgal biomass (Jung et al., 2013).

The saccharification of macroalgal biomass is usually the critical step for success of the bioconversion processes. Mainly dilute-acid and enzymatic hydrolysis have been explored in order to develop the macroalgal biomass-based biorefinery.

Meinita et al. (2012b) reported that the saccharification with dilute-acid (0.2 M H₂SO₄) at high temperatures (130 °C for 15 min) can be useful for treatment of raw macroalgal biomass. However, during the acidic hydrolysis two compounds were formed that are known as growth inhibitors: hydroxymethylfurfural (originated by the hexoses degradation) and levulinic acid (originated by the degradation of hydroxymethylfurfural). So it was needed to add a supplementary step of detoxification (Meinita et al., 2012a) for removing these inhibitors, which can limit the effectiveness of the described method. More detoxification research is needed to carry out the process in this sense. The alkaline hydrolysis has been extensively used in lignocellulosic biomass processing. The main effect is the removal of lignin (no present in macroalgae), so these methods are rarely used in macroalgal biomass processing (Li et al., 2014).

Enzymatic hydrolysis is another potential approach for saccharifying the macroalgal polysaccharides. The problem is that macroalgal biomass usually consists of more than one type of “unusual” polysaccharide. Consequently, for achieving high yields, multi-enzymatic complexes or additional pre-treatment steps with specific enzymes such as agarases, alginases (alginate lyases), carrageenases, fucoidanases (fucoidan hydrolases), laminarinases (licheninases) and ulvanases (ulvan lyases) may be required (Jung et al., 2013). Although enzymatic hydrolysis requires less energy, the enzyme production has not yet sufficient demand, which increases the process cost. On **Table 6**, some organisms that produce enzymes capable of degrading macroalgal polysaccharides are presented.

Table 6 – Some of known organisms producers of macroalgal polysaccharides hydrolases (Alderkamp et al., 2007, Colle et al., 2011, Holtkamp et al., 2009, Jung et al., 2013, Reddy et al., 2008).

Enzyme	Microorganism	Obs.
Agarase	<i>Alteromonas</i> sp. C-1	β -agarase, extra-
	<i>Bacillus</i> sp. MK03	β -agarase, extra-
	<i>Pseudomonas atlantica</i>	β -agarases, extra-/memb-
	<i>Thalassomonas</i> sp. JAMB-A33	α -agarase, intra-
	<i>Vibrio</i> sp. JT0107	β -agarase, extra-
Alginase	<i>Alginovibrio aquatilis</i>	extra-, endo-
	<i>Alteromonas</i> sp. H-4	intra-/extra, exo-
	<i>Asteromyces cruciatus</i>	endo-
	<i>Dendryphiella arenaria</i>	
	<i>Pseudoalteromonas elyakovii</i> IAM 14594	extra-, exo-
	<i>Rhodothermus marinus</i>	
	<i>Sphingomonas</i> sp. A1	
Carrageenase	<i>Alteromonas fortis</i>	ι -carrageenase
	<i>Pseudoalteromonas carrageenovora</i>	λ -carrageenase
	<i>Zobellia galactanivorans</i>	κ -carrageenase, extra-
Fucoidanase	<i>Pseudomonas atlantica</i>	
	<i>Pseudoalteromonas citrea</i> KMM3296	
Laminarinase	<i>Chaetomium indicum</i>	
	<i>Pseudoaltermonas issachenkonii</i> KMM 3549	
	<i>Rhodothermus marinus</i>	
Ulvanase	<i>Persicivirga ulvanivorans</i>	endo-

The first organism studied in detail for being involved in agar hydrolysis was *Pseudomonas atlantica*, a marine bacterium (Morrice et al., 1983). β -agarases I and II (extra-cellular and membrane associated, respectively) were isolated, purified and characterized. β -agarases I hydrolyze agarose to neoagarobiose and β -agarases II additionally hydrolyze neoagarobiose to galactose residues. Mainly β -agarase I has been overproduced in *E.coli* and commercialized by companies such as Thermo Fisher Scientific, as research-grade product, for digestion of agarose gel from electrophoresis assays in order to efficiently recover DNA or RNA fragments. Another marine bacterium (*Alteromonas* sp. strain C-1) was identified as producer of high levels of an extracellular agarase (Leon et al., 1992). The enzyme production is accompanied by a decrease in bacterial growth and seems to be repressed by the presence of glucose.

Sawabe et al. (1997) isolated the marine bacterium *Alteromonas* sp. strain H-4 from brown macroalgae and reported the production of intra- and extra-cellular alginases and the utilization of alginate as carbon source. The extra-cellular alginase shows degradation activity for both G-blocks and M-blocks. The enzymatic degradation of alginate by marine fungi such as *Asteromyces cruciatus* and *Dendryphiella arenaria* has also been observed

(Schaumann and Weide, 1990). There are some works about overexpression of alginases in *E. coli*, namely from the bacterium *Pseudoalteromonas elyakovii* and *Sphingomonas* sp. (Ma et al., 2008, Yoon et al., 2000).

Potin et al. (1991) isolated the marine bacteria *Zobellia galactanivorans* from marine macroalgae and observed a κ -carrageenase activity. Also ι - and λ - carrageenases were isolated from marine bacteria, namely *Alteromonas fortis* and *Pseudoalteromonas carrageenovora*, correspondingly (Guibet et al., 2007, Michel et al., 2001).

Interestingly, Daniel et al. (1999) and Bilan et al. (2005) observed that the protein extracts from digestive systems of the *Pecten maximus* and *Littorina kurila* (marine mollusks) show fucoidan hydrolyzing activities. It was observed that the higher activities are achieved by fucoidanases isolated from marine bacteria such as *Pseudoalteromonas citrea* (Kusaykin et al., 2007).

Laminarinases were already isolated from marine mollusks (*Aplysia Juliana*), fungi (*Chaetomium indicum*) and bacteria (*Pseudoalteromonas issachenkonii*). Recently, two ulvanases were isolated from a culture of the marine bacterium *Persicivirga ulvanivorans* (Nyvall Collén et al., 2011).

Physicochemical and enzymatic hydrolysis can be combined to maximize the saccharification. Ge et al. (2011) reported that an acidic treatment step applied to macroalgae biomass before the enzymatic hydrolysis enhanced the final content of monosaccharides (27.8% w/w glucose/dry biomass) for fermentation by increasing the contact surface between the biocatalysts and the polysaccharides.

3.4.2. Fermentation and Integrated Processes

Some works have been done in order to develop the production of certain building blocks from macroalgal biomass fermentation (Jung et al., 2013). It is possible to obtain Volatile Fatty Acids (VFAs) from the anaerobic digestion of macroalgae hydrolysates. Gupta et al. (2011) described the production of lactic acid and methane from the anaerobic digestion of heat-treated brown macroalgae. The tested brown macroalgae strains contain a high content of laminarin, which is fermentable by the lactic acid bacteria used in the experiment. The macroalgal structural polysaccharides can be applied as carbohydrate-based building blocks by itself (without a fermentation step). Ducatti et al. (2009) reported a procedure for chemically converting red macroalgae polysaccharides into a C-glycosyl aldehyde. Agar was described as a low cost material suitable for being converted to building blocks whose total synthesis usually is more expensive.

There are already several works about biofuels production from macroalgal biomass. Gunaseelan (1997) reported that the digestion of macroalgal biomass can exhibit higher

biogas (CH₄ + CO₂) production rates (0.31–0.48 m³ CH₄ kg⁻¹) than lignocellulosic biomass (0.32–0.42 m³ CH₄ kg⁻¹) because of the aforementioned lower content of inhibitors related to the non-existence of lignin. Several works related to bioethanol production from fermentation of macroalgal biomass were described too. Horn et al. (2000) showed that the yeast *Pichia angophorae* can simultaneously ferment mannitol and laminarin from brown macroalgae to produce bioethanol with a maximum yield of 43% w/w.

Contrary to microalgae which present 10-20% lipids/dry biomass (Becker, 1994), the low lipid content of macroalgae (**Table 4**) is not interesting for direct production of fatty-acid fuel, namely biodiesel. Other possible bioproducts extractable from macroalgae are amino acids (Lammens et al., 2012) but also microalgae are potentially more interesting for that purpose (40-60% w/w protein/dry biomass).

Some authors refer that the application of a Simultaneous Saccharification and Fermentation (SSF) system (combination of isolated enzymes and fermenting microorganisms in the same bioreactor) to macroalgae biotransformation can be technically and economically convenient, allowing lower contamination and higher energy efficiency. Jang et al. (2012a) used SSF for bioethanol production coupling an industrial enzyme (Termamyl) and the yeast *P. angophorae* (strain KCTC 17574) in the same bioreactor with brown macroalgal biomass as feedstock, achieving 33.3% of maximum theoretical yield.

However, it is difficult to find wild microorganisms that can ferment the most “unusual” releasing sugars. Co-fermentation could be a solution for that, but applying various microorganisms in the same SSF system, can lead to enzyme inhibition and a consequent lower yield of saccharification (Jung et al., 2013). Besides, these systems are usually quite challenging to control due to variations of the microbial population. It should be noted that the fermentation conditions such as high viscosity could inhibit exogenous enzyme activity. To solve this problem, the concept of SSF can be reformulated to become more functional: Consolidated BioProcessing (CBP). CBP consists of applying diverse genetic and metabolic engineering tools with the objective of obtaining the production of the saccharolytic enzymes by the same microorganism that simultaneously hydrolyzes and metabolizes ideally almost all the types of released monosaccharides (Van Zyl et al., 2007) – case study discussed in Chapter 3.4.3.

A third methodology is based on applying genetic engineering to modify the main chemical composition of macroalgae (that would be considered a fourth generation feedstock), but some authors have reported difficulties in their genetic manipulation (John et al., 2011, Qin et al., 2004). Due to the less understanding about macroalgal genomics, the genetic engineering applied in macroalgae has been modeled on established methods for high plants (Lin and Qin, 2014), which leads to slower yield improvements, since the technology is unspecific. Furthermore, the macroalgae culture implies constant water flows

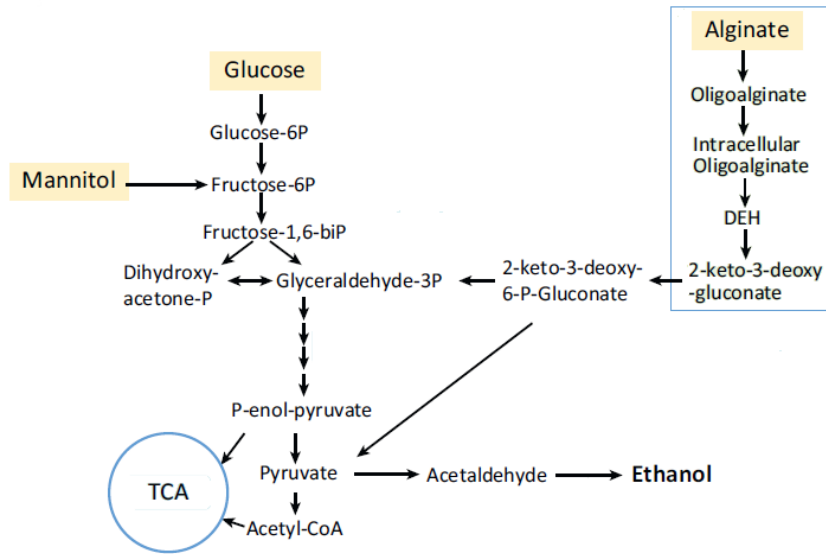
that can lead to a fast gene dispersion which represents an environmental and ethical issue if applied in the open sea.

3.4.3. Case study: brown macroalgae as feedstock for bioethanol production

As mentioned before, macroalgae exhibit some features to be considered an ideal feedstock for biorefinery proposals. Some works have been developed, especially for bioethanol production (Wei et al., 2013). For now, given the compatibility between *R. marinus* pool of enzymes and brown macroalgae carbohydrates, this work will focus on the application of mannitol and alginate, in addition to glucose as C-source for growth.

The potential of brown macroalgae as biorefinery feedstock has not currently been accessed because of the absence of industrial microorganisms to metabolize alginate. As case study, it was chosen a work developed by Wargacki et al. (2012), where the bacterium *Escherichia coli* was genetically modified to degrade, uptake and metabolize alginate and to produce bioethanol (CBP platform). In addition, *E. coli* naturally feature metabolizes mannitol and glucose (laminarin monomer) and represents a well-characterized Gram-negative bacterium allowing the comparison with *R. marinus*.

Wargacki et al. (2012) described that the alginate lyase activity can occur extracellularly, in the periplasmatic space and intracellularly. Only oligoalginates with 2-5 monomers are translocated into the cell. The alginate monomers spontaneously rearrange to 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) that is reduced to 2-keto-3-deoxygluconate (KDG), a metabolite that enters to the Entner-Doudoroff pathway (EDP). Glucose and mannitol catabolism pathways are present in wild strain. A summarized version of the pathway is presented on **Figure 9**.



TCA - Tricarboxylic Acid Cycle

Figure 9 – Metabolic pathway of the CBP (reported by Wargacki et al. (2012)) for bioethanol production by the fermentation of brown macroalgae carbohydrates (adapted of Wei et al. (2013)).

A preliminary test with a 5% sugar mixture of alginate, mannitol and glucose (ratio of 5:8:1) was performed to select the optimum fermentation conditions. Using the brown macroalgae *Saccharina japonica* as a model substrate, this CBP platform enabled an enhanced bioethanol production with a maximum yield of 41.0% w/w ethanol/total sugars (80% of maximum theoretical yield). The application of *R. marinus* in a similar system has the advantage of not being necessary to perform such heavy genetic modifications since the wild strain potentially presents the enzymatic machinery to saccharifying alginate and laminarin and to catabolize the releasing monomers and mannitol.

4. Objectives

Main objectives:

- a) To study the growth of *R. marinus* strains (supplied by Matís) in different defined media and conditions in order to select the strains with highest observed biomass yield;
- b) To study the growth of selected strains using the main carbohydrates of macroalgae as C-source;
- c) To reduce the chlorinated salts concentration in the growth media in order to enable the feasibility of the process in standard industrial equipment;
- d) To study the growth of selected strains in defined media supplemented with macroalgal biomass;
- e) For the first time, establish a protocol for the production of *R. marinus* in controlled bioreactor.
- f) Produce enough biomass to be sent to NeaNAT at University of Naples (also a partner of BlueGenics) for the analysis of the presence of novel bioactive compounds.

5. Materials and Methods

5.1. Microorganisms and maintenance

R. marinus strains were kindly supplied by Dr. Guðmundur Óli Hreggviðsson, from Matís, an Icelandic research company, participating in the BlueGenics project. Five different strains (numbered from 1 to 5) in Marine Broth agar plates.

The strains 1, 4 and 5 were grown in Medium 166 (composition provided by Dr. Viggó Martenisson, from Matís) and Marine Broth agar plates at 65°C and maintained at 4°C. As starter cultures, the same strains were grown in liquid Medium 166 at 60°C and stored in cryotubes with 30.0% glycerol (final v/v concentration) at -80°C.

5.2. Growth Media

The growth media formulation is presented on **Table 7**.

Table 7 – Medium 166 and Marine Broth (Difco™) formulations.

Part A - Medium 166		Part B - Marine Broth (Difco™)	
Components	Concentration (g.L ⁻¹)	Components	Concentration (g.L ⁻¹)
NaCl	10.0 ^a	NaCl	19.5
Yeast extract	1.00	MgCl ₂	5.90
Peptone	1.00	Peptone	5.00
Tryptone	1.00	MgSO ₄	3.24
Starch	0.500	CaCl ₂	1.80
K ₂ HPO ₄ ·3H ₂ O	0.393	Yeast extract	1.00
Sodium pyruvate	0.300	KCl	0.550
Proline	0.300	NaHCO ₃	0.160
MgCl ₂ ·6H ₂ O	0.200	Ferric citrate	0.100
Na ₂ CO ₃	0.180	KBr	8.00 × 10 ⁻²
Nitriloacetic acid	0.138	SrCl ₂	3.40 × 10 ⁻²
CaCl ₂ ·2H ₂ O	2.40 × 10 ⁻²	H ₃ BO ₃	2.20 × 10 ⁻²
Ferric (III) citrate	1.23 × 10 ⁻³	Na ₂ HPO ₄	8.00 × 10 ⁻³
FeSO ₄ ·7H ₂ O	6.99 × 10 ⁻⁴	Na ₂ O ₃ Si	4.00 × 10 ⁻³
MnCl ₂ ·4H ₂ O	2.50 × 10 ⁻⁴	NaF	2.40 × 10 ⁻³
ZnSO ₄ ·7H ₂ O	2.11 × 10 ⁻⁴	NH ₄ NO ₃	1.60 × 10 ⁻³
CoCl ₂ ·6H ₂ O	1.50 × 10 ⁻⁴	pH adjusted to 7.50-7.60 with 1.00N NaOH	
CuCl ₂ ·2H ₂ O	2.50 × 10 ⁻⁵		
Na ₂ MoO ₄ ·2H ₂ O	2.50 × 10 ⁻⁵		
H ₃ BO ₃	1.00 × 10 ⁻⁵		
NiCl ₂ ·6H ₂ O	1.00 × 10 ⁻⁵		

pH adjusted to 8.0 with 1N NaOH

^a Variable component (1.00% w/v NaCl by default)

Medium 166 is composed by several stock solutions and is totally prepared at Biotrend (intern data). The Medium 166 agar plates preparation includes the addition of 28.0 g.L⁻¹ agar. The Marine Broth is a commercial growth medium manufactured by Difco™, whose preparation is to suspend 37.4 g of powder in 1 L of ROW (Reverse-Osmosis Water). Marine Broth agar plates preparation includes the addition of 15.0 g.L⁻¹ agar.

5.3. Incubation, sampling and sterility

The inocula and fermentation shake flasks were incubated in an Innova 43R shaker with 50 mm orbit (New Brunswick Scientific).

All the assays with pure cultures were carried out under sterile conditions in a laminar flow chamber Telstar Bio II Advance 4. The media and stock solutions were sterilized by autoclaving for 15-30 min, depending the autoclave charge or liquid volume.

5.4. Analytical Methods

5.4.1. Biomass determination

5.4.1.1. Optical Density (600 nm)

All growth studies were monitored measuring OD₆₀₀ (Optical Density at wavelength of 600 nm) in a Shimadzu UV-1700 spectrophotometer. All data were registered and stored by PC acquisition. The calculation of the real OD₆₀₀ is presented below. Dilutions were performed by adding ROW.

$$OD_{600} = (OD_{600_{\text{reading}}} \times \text{dilution factor}) - OD_{600_{\text{media}}}$$

5.4.1.2. Dry Cell Weight (DCW)

Protocol:

- a) Aliquots of 1.000 mL of fermentation broth were pipetted into previously dried and weighed microtubes (technical duplicates);
- b) The aliquots were centrifuged during 1 min at 13000 rpm;
- c) The supernatant was removed and the pellet was washed with 1 mL of a Sea Salts (Sigma-Aldrich) solution and resuspended;
- d) The centrifugation step was repeated once;
- e) After one more wash, the microtubes were dried at 70°C for at least 48h, weighed and the DCW (gX.L⁻¹) was determined.

5.4.2. High-Performance Liquid Chromatography (HPLC)

Organic acids and sugars were quantified using a Shimadzu LC-20AD IVD and a Shimadzu LC-2010 CHT, respectively, both equipped with a 96-well plate autosamplers. HPLC columns and operation conditions are logged on **Table 8**.

Table 8 – HPLC operation conditions for quantification of organic acids and sugars.

HPLC parameters	Organic acids	Sugars
Column	Rezex RHM-Monosaccharide H ⁺ (8%)	Rezex RPM-Monosaccharide Pb ⁺² (8%)
Column size	300 × 7.8 mm	300 × 7.8 mm
Eluent	5.00 mM H ₂ SO ₄	Milli-Q water
Eluent flow rate	600 μL.min ⁻¹	600 μL.min ⁻¹
Volume of injection	20.0 μL	20.0 μL
Oven temperature	35°C	85°C
Detector	UV (Ultraviolet)	RI (Refraction Index)
Samples dilution	4×	5×
Diluent	50.0 mM H ₂ SO ₄	ROW

5.4.3. Ammonia and Phosphate determination

The ammonia concentration of samples was determined by a colorimetric procedure based on the reaction of ammonia, sodium hypochlorite and phenol catalyzed by sodium nitroprusside that originates indophenol, which was measured with a spectrophotometer at 640 nm. The phosphate concentration was also determined by a colorimetric method, based on the reaction of phosphate and ammonium molybdate, whose product was reduced by ascorbic acid, originating β-keggin ion that was measured with a spectrophotometer at 850 nm.

5.4.4. pH

The pH of the media and the final fermentation samples were measured in a Mettler Toledo Seven Compact pH/ion S220.

5.5. Acclimatization and Cryopreservation of *R. marinus* strains

For stocking the *R. marinus* cultures, the strains were replated to Medium 166 and Marine Broth agar plates (performed in duplicate) in sterile conditions and incubated at 65°C during 48 h.

For stocking the strains in cryotubes, it was necessary to acclimatize the strains through successive transfers from the seed culture until both less cell aggregation and higher OD₆₀₀ were observed. The inocula of strains 1, 4 and 5 were prepared taking a single colony from Medium 166 and Marine Broth agar plates to liquid Medium 166 and Marine Broth, respectively, supplemented with 10.0 g.L⁻¹ glucose. The shake flasks closed with cotton stoppers were incubated at 60°C and 150 rpm in duplicate. At this stage, a working volume of 25 mL of media in 100 mL shake flasks was used.

After 2-3 seed transfers, the cryotubes from cultures incubated in Medium 166 were prepared as mentioned bellow.

Protocol:

- a) The OD₆₀₀ of the cultures were periodically monitored. It was stipulated that once an OD₆₀₀ of 5.000 was reached, the cultures were ready for cryopreservation;
- b) A culture aliquot of 1180 µL was transferred to each cryotube previously sterilized with 621 µL of 87.0% glycerol;
- c) Potential contaminations were checked, plating the remaining culture in agar plates that were incubated at 65°C for 48 hours;
- d) The cryotubes were stored at -80°C.

5.6. Evaporation Study

The water evaporation of different shake flasks with various working volumes was monitored when incubated at 60°C and 150 rpm. For that, the shake flasks were dried and weighed before adding known weights of ROW. The assays were performed in duplicate. After incubation, the shake flasks were weighted periodically over 72 h. The calculation of the evaporation percentage is presented below.

$$\% \text{ Evaporation (w/w) at t} = \frac{w(\text{shake flask} + \text{initial RO water}) - w(t)}{w(\text{initial RO water})} \times 100$$

In Equation, w (t) represents the weight of the shake flask with ROW at time t. The slope of the % Evaporation in function of t (h) represents the evaporation rate (%.h⁻¹) of the assay.

An additional evaporation study was performed in bioreactor. The volume of 2 L ROW was added to a 3 L bioreactor (New Brunswick BioFlo 110 Fermentor) at 60°C stirred with two Rushton turbines at 300 rpm, and aerated with an air flow of 0.5 vvm. A condenser with circulating tap water was installed at the gas exhaust prior to the outlet sterile filter. After 72h, the volume of ROW remaining in the bioreactor was measured.

5.7. Growth Studies in shake flask

5.7.1. Growth Conditions

For studying the effect of different C-sources and Na-sources on the growth pattern of *R. marinus*, an “One Variable at a Time” (OVAT) approach was adopted. All the assays were performed in duplicate for strains 4 and 5. The standard conditions used as control were:

- Assays in 100 mL shake flasks with 50 mL working volume;
- Medium 166 with 1.00% NaCl (w/v) by default;
- Supplementation with 10.0 g.L⁻¹ glucose by default;
- Incubation at 60°C and 150 rpm.

5.7.2. Inocula

The inocula for the growth studies in shake flask were prepared by adding culture from stocked cryotubes to Medium 166 supplemented with 10.0 g.L⁻¹ glucose (targeted initial OD₆₀₀ of 0.100). The calculation of the required cryotube seed volume for inocula preparation is presented below.

$$V_{\text{cryotube}} \text{ (mL)} = \frac{\text{OD}_{600_{\text{target}}}}{\text{OD}_{600_{\text{cryotube}}}} \times \frac{1.801 \text{ mL}}{1.180 \text{ mL}} \times V_{\text{culture}} \text{ (mL)}$$

, where 1.801 mL and 1.180 mL are the total and the seed culture transferred into the cryotube, respectively.

The inocula were incubated at 60°C and 150 rpm during 24 h in duplicate, with varying working volumes. The calculation of the required inocula seed volume for fermentation cultures preparation is presented bellow (targeted initial OD₆₀₀ of 0.100).

$$V_{\text{inoculum}} \text{ (mL)} = \frac{\text{OD}_{600_{\text{target}}}}{\text{OD}_{600_{\text{inoculum}}} - \text{OD}_{600_{\text{Medium 166}}}} \times V_{\text{culture}} \text{ (mL)}$$

5.7.3. C-sources studies

5.7.3.1. Glucose as C-source

The standard C-source available in all the following assays was glucose. Because of that, a characterization of the pattern of growth with 10.0 and 2.00 g.L⁻¹ glucose was carried out as control models. Unfortunately, no assays with laminarin were carried out because the compound was not delivered on time.

5.7.3.2. Mannitol as C-source

For monitoring the assays with mannitol, a HPLC calibration with D-Mannitol (Prolabo) was performed in Rezex RPM-Monosaccharide Pb+2 (8%) LC Column, with a retention time of 27.526 min. The strains were grown in 10.0, 9.0 and 8.0 g.L⁻¹ mannitol, supplemented with 10.0, 1.00 and 2.00 g.L⁻¹ glucose, respectively (**Figure 10**).

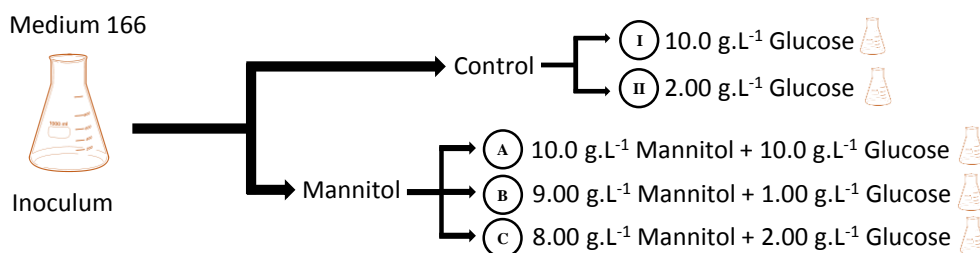


Figure 10 – *R. marinus* cultivation in Medium 166 supplemented with mannitol.

5.7.3.3. Alginate as C-source

For monitoring the assays with alginate, HPLC calibrations with Sodium Alginate (Prolabo) and dilute-acid hydrolyzed Sodium Alginate were performed in Rezex RHM-Monosaccharide H⁺ (8%) LC Column. The strains were grown in 8.00 g.L⁻¹ sodium alginate and pretreated sodium alginate, both supplemented with 2.00 g.L⁻¹ glucose (**Figure 11**).

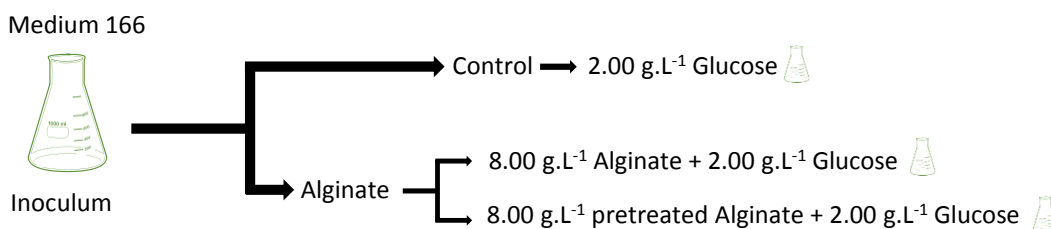


Figure 11 – *R. marinus* cultivation in Medium 166 supplemented with alginate.

The alginate pretreatment consisted in autoclaving 20.0 g.L⁻¹ sodium alginate in 0.100 N H₂SO₄ solution for 15 min (adapted from Chandia et al. (2001)). After the pretreatment,

the pH was neutralized with 0.500 N NaOH and the solution was pasteurized at 70° C for 1h before being added to shake flasks (40% v/v). Different concentrations of hydrolyzed alginate were analyzed by HPLC. Aliquots of alginate solution were analyzed too.

In a preliminary study, different amounts of alginate were dissolved in 0.500 N H₂SO₄ and exposed to domestic microwave (Fagor, 2450 MHz) for 4 minutes as described by Chhatbar et al. (2009).

5.7.4. Na-source studies

The Na-source (sodium source) studies were based on the approach adopted by Barclay (2002) that consists of providing sodium in the form of non-chloride salts, namely Na₂SO₄. The calculations were carried out taking into account the sodium molarity and the ionic strength of 1.00% NaCl. The NaCl ionic strength (IS) contribution was calculated as described in following equation.

$$IS = \frac{1}{2} \sum_{i=1}^n C_i \times z_i^2$$

In equation, C_i is the molar concentration and z_i is the charge number of ions i. The sum was calculated considering the NaCl ions in the media.

Additional assays with substitution of just a half of the NaCl contained in the media (0.500%) were carried out. The schematic representation of performed assays is presented on **Figure 12**.

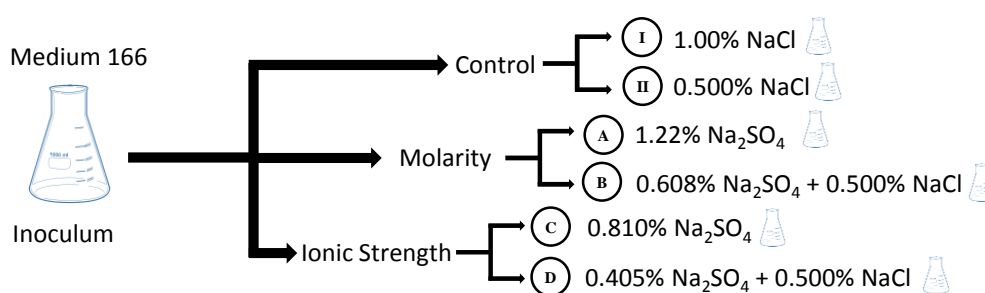


Figure 12 – *R. marinus* cultivation in Medium 166 with different concentrations of NaCl and Na₂SO₄.

5.7.5. Macroalgae as feedstock

Two species of brown macroalgae were kindly supplied by Matís. The biomass was sent in the form of a dry powder (**Figure 13**).

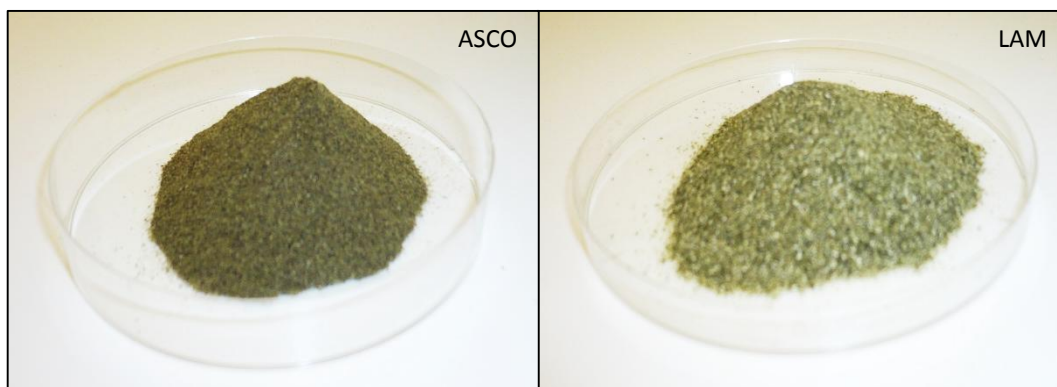


Figure 13 – Milled macroalgae powder – ASCO and LAM.

ASCO biomass is from *Ascophyllum nodosum* milled to < 0.212 mm. The available biochemical profile (w/w) is: dietary fiber, 44%; ash, 27%; NaCl, 9.2%; protein, 8.1%; and fat, 1.8%. LAM biomass is from *Laminaria digitata* milled to < 0.315 mm. The available biochemical profile (w/w) is: dietary fiber, 36%; ash, 31%; NaCl, 15%; protein, 5.4%; and fat, 0.6%. It is described a large amount of trace elements such as phosphorus and iron in both ASCO and LAM (internal data from Matís, ltd).

In this study, the *R. marinus* strains were grown in 8.00 g.L^{-1} ASCO, LAM, pretreated ASCO, pretreated LAM, all supplemented with 2.00 g.L^{-1} glucose (**Figure 14**).

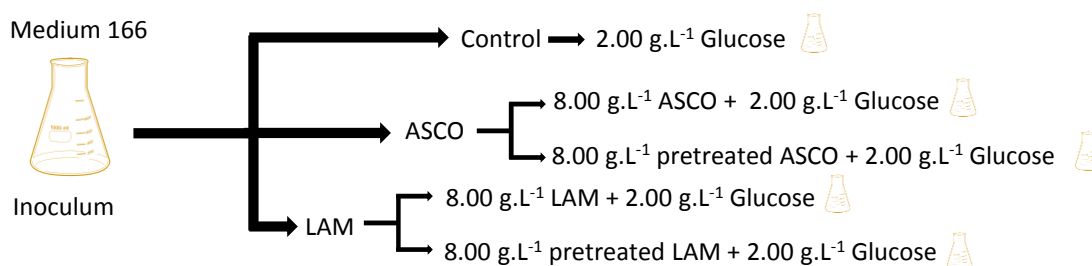


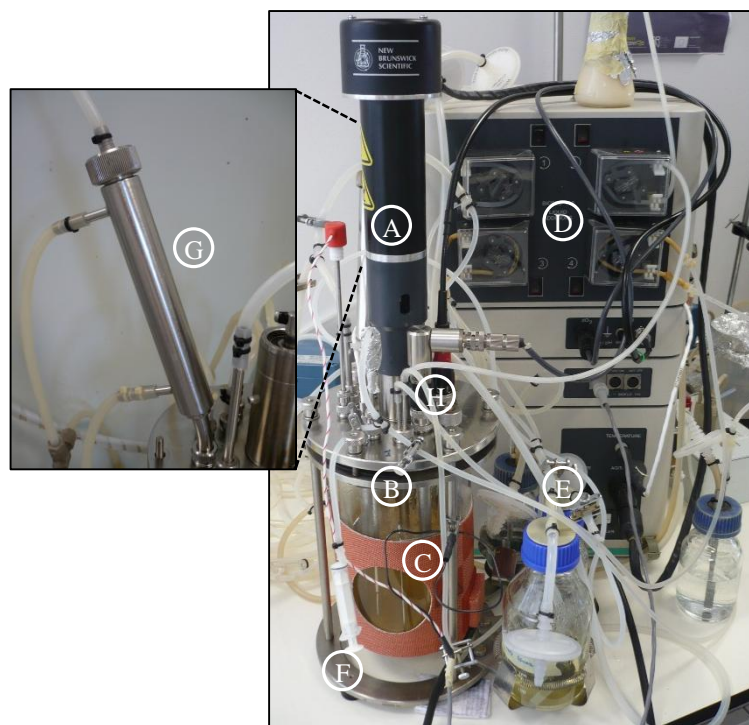
Figure 14 – *R. marinus* cultivation in Medium 166 supplemented with ASCO and LAM (original and pretreated biomass).

The macroalgae pretreatment consisted in autoclaving 20.0 g.L^{-1} macroalgal biomass in $0.100 \text{ N H}_2\text{SO}_4$ solution for 15 min (adapted from Jang et al. (2012b)). After the pretreatment, the pH was neutralized with $0.500\text{-}5.00 \text{ N NaOH}$ and the solution was pasteurized at 70° C by 1 h before being added to shake flasks (40% v/v). Aliquots of the stock solutions were analyzed by HPLC.

5.8. Fermentation in Bioreactor

At this stage, it was used a 3L bioreactor (New Brunswick BioFlo 110 Fermentor) agitated with two Rushton-style impellers and aerated through a ring sparger. The bioreactor was coupled with an auto-sampler system and an acquisition computer with BioCommand®

Software (Eppendorf, Inc.). A condenser with circulating tap water was installed at the gas exhaust prior to the outlet sterile filter. The vessel containing 1.375L of Medium 166 with 0.500% NaCl (w/v) was autoclaved at 121 °C for 30 min. After sterilization, 75 mL of a sterile 200.0 g.L⁻¹ glucose solution was pumped into the bioreactor. The bioreactor was inoculated with 50 mL of seed culture, and the fermentation was maintained at 60 °C with an initial agitation rate of 200 rpm and an aeration rate of 1.50 L.min⁻¹. The seed culture consisted of a 24h shake flask culture of *R. marinus* strain 5 (standard Medium 166) incubated at 60°C and 150 rpm. The fermentation broth contained 1.5L of medium with 10.0 g.L⁻¹ glucose and an effective initial OD₆₀₀ of 0.105. The minimal dissolved oxygen (DO₂) was set to 25% of medium saturation and controlled by adjusting the agitation speed in a cascade mode, accordingly to previous studies performed at Biotrend. The pH was maintained at 7.00 +/- 0.10 by an automated addition of 4.00N NaOH and 2.00N H₂SO₄. Foam formation was controlled by an automated addition of 30.0% (v/v) Simeticone. The scheme of the bioreactor is presented on **Figure 15**.



A- Agitation system; B- Bioreactor vessel; C- Thermal jacket; D- Feeding pumps; E- Feed solutions (including base, acid and antifoam); F- Sampling syringe; G- Condenser with circulating tap water - gas exhaust; H- Sensor probes (pH and DO).

Figure 15 – Bioreactor scheme.

The sampling was carried out periodically by syringe or by automatic sampler. The samples were analyzed in duplicate (OD₆₀₀, DCW, HPLC, ammonia and phosphate).

At the end of the fermentation, the broth was collected and centrifuged. The wet biomass was separated and frozen in order to be sent to the Prof. Alfonso Mangoni at University of Naples.

5.8.1. Feeding strategies

When the glucose concentration was maintained without visible growth, a fed-batch with a pulse feeding sterile $(\text{NH}_4)_2\text{SO}_4$ solution was carried out to a final concentration of 1 g.L^{-1} (called Feed N). Then, a second fed-batch with a pulse feeding filtered KH_2PO_4 solution was carried out to a final concentration of 1.00 g.L^{-1} (called Feed P). With no visible change in growth parameters, a third fed-batch with a pulse feeding concentrated glucose solution into the bioreactor was carried out to increase the glucose concentration by 10.0 g.L^{-1} (called Feed C). Lastly, a fourth feed with filtered yeast extract was injected into bioreactor to a final concentration of 1.00 g.L^{-1} (called Feed YE).

6. Results and Discussion

6.1. Acclimatization and Cryopreservation of *R. marinus* strains

In order to increase the internal stock of *R. marinus* strains for the following activities, the cultures in solid media sent by Matís were replated in both Medium 166 and Marine Broth agar plates. Only strains 1, 4 and 5 grew in both media. Strains 2 and 3 did not grow and so they were not tested anymore.

Preliminary studies with *R. marinus* strains revealed that, when incubated directly from agar plates to liquid media, the cultures usually develop as cell aggregates. An “Acclimatization” was adopted, in which the strains were cultivated through 2-3 successive transfers from the seed culture until less cell aggregation and higher OD were observed.

At this stage, the reference Medium 166 was used as the defined media for growth studies since cultures acclimatized in Marine Broth presented low OD (< 3.000), compared to cultures acclimatized in Medium 166 (>5.000). The acclimatized cultures in Medium 166 were cryopreserved.

After stocking the selected batches of strains 1, 4 and 5 in cryotubes, the effectiveness of the cryopreserved cultures as pre-inoculum was tested. Inocula prepared from stocked cryotubes of strains 4 and 5 presented similar growth than observed immediately before the cryopreservation. On the other hand, the starter cultures of strain 1 presented little growth when transferred to fresh media. Due to this, the following growth studies proceeded only with strains 4 and 5 in parallel. The process of acclimatization and preparation of cryotubes with *R. marinus* strains is presented on **Figure 16**.

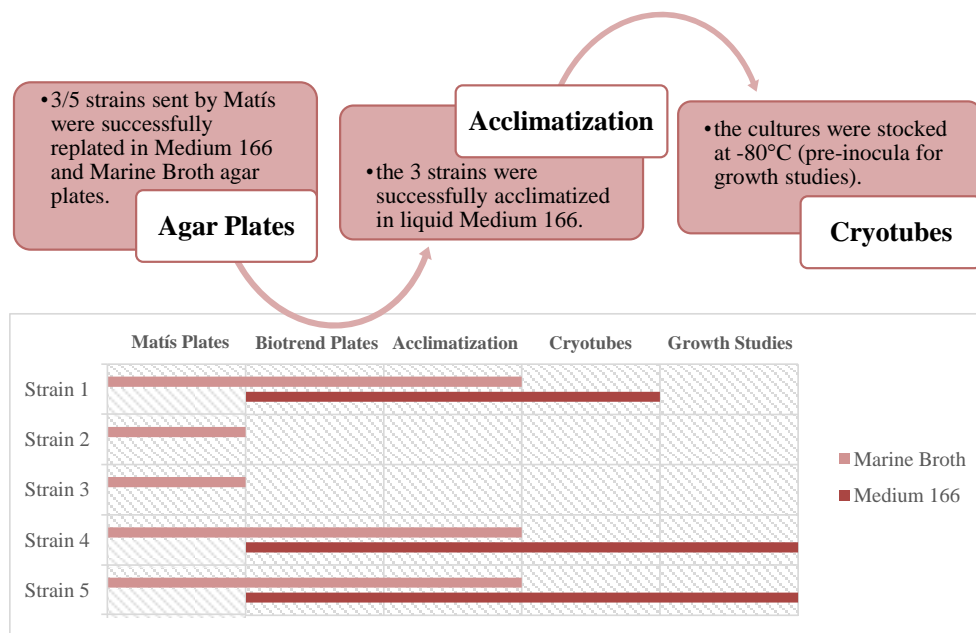


Figure 16 – Process of acclimatization and cryopreservation of *R. marinus* strains.

The cryopreservation of pre-inocula was required to make sure that the starter cultures had always the same conditions, which is important in comparative studies. Further, the preparation of the inocula became faster and more practical.

6.2. Evaporation Study

The growth studies in shake flask with *R. marinus* strains were carried out at 60°C. At high temperatures, the media water loss by evaporation is not negligible in shake flask cultivations. The high evaporation rates in saline cultures imply increasing osmotic stress during the fermentation. Besides that, the monitoring of the assays is directly affected by the concentration of the media components and the biomass inside the shake flask. A preliminary study during the acclimatization step showed that after 72h of incubation, more than 50% of the media (both Medium 166 and Marine Broth) had evaporated from 100 mL shake flasks with 25 mL working volume. In order to confirm the evaporation effect during the incubation of the cultures, an evaporation study with ROW in different shake flasks and working volumes was carried out. An additional study in a stirred (300 rpm) and aerated (0.5 vvm) 3L bioreactor filled with 2L ROW and coupled with a condenser with circulating tap water was performed. The results are presented on **Figure 17**.

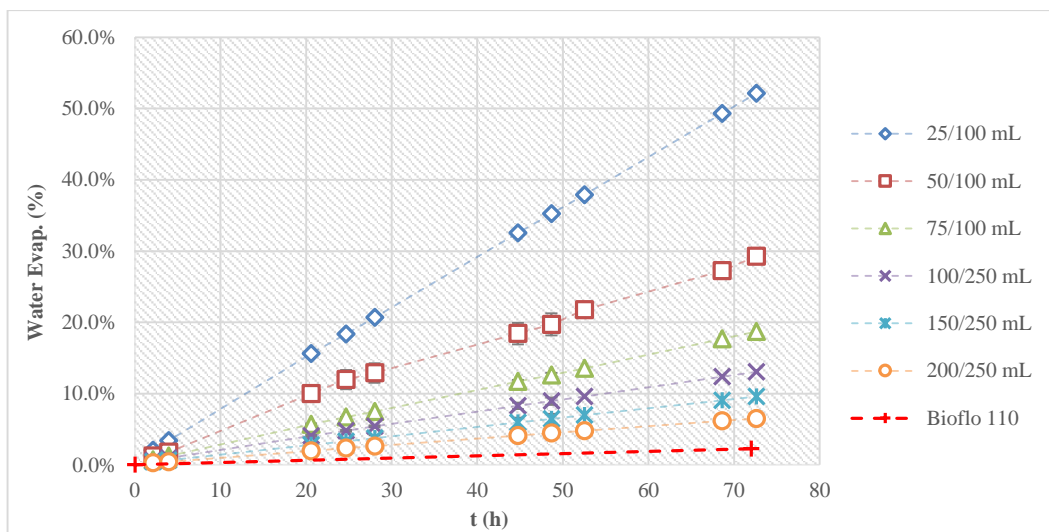


Figure 17 – Evaporation study performed at 60°C with 100 and 250 mL shake flasks and a bioreactor (2L working volume).

A linear relationship between the time of incubation and the water loss by evaporation was observed in all assays. As expected, with higher working volumes, lower evaporation rates were obtained, since the cross section area of the liquid surface is smaller and the specific area (area of the liquid surface divided by total volume) is lower. The 100 mL shake flasks (total volume) were chosen over 250 mL shake flasks due to technical issues (more simultaneous assays per incubator and less spending of reagents for culture media preparation). For 100 mL shake flasks, the evaporation rates of 0.709, 0.388, 0.254%.h⁻¹

were obtained for 25, 50 and 75 mL working volumes, respectively. Given the high evaporation rate observed with 25 mL working volume and the low head-space with 75 mL working volume, the more balanced option seemed to be the 50 mL working volume.

Some alternatives such as keeping water in the incubator base or adding sterile water during the fermentation were considered but any of the processes could not be used routinely and reproducibly. Taking the correspondent implications into account, such as the low mass and oxygen transfer in the system, the growth studies in shake flask were performed as preliminary comparative studies for a future fermentation in controlled bioreactor to validate the results. An evaporation rate of 0.0313 %·h⁻¹ was obtained by coupling a condenser in Bioflo 110 bioreactor, which represents only 2.02% water loss after 65h of incubation, which is quite acceptable.

6.3. Growth Studies with *R. marinus* strains

6.3.1. General Considerations

The optimum incubation temperature for *R. marinus* cultivation recommended in the literature is 65°C (Alfredsson et al., 1988). The incubation temperature of the growth studies performed at Biotrend was limited to 60°C due to equipment restrictions (incubator maximum working temperature).

According to the evaporation study results, it was assumed that the shake flask cultivations should not be prolonged beyond 65 hours, representing a maximum of 25.2% of water loss. Consequently, the growth studies in shake flask were based in low amounts of C-sources in order to avoid substrate inhibition and to observe all the growth phases in such short period of time.

It should be clarified that all the biological data obtained from the samples of shake flask assays was directly affected not only by the *R. marinus* production and consumption of compounds but also by the concentration of those compounds, intrinsic to the high evaporation rate. The correspondent ratios could not be strictly quantified, since the evaporation rate depends on unmeasurable variables such as the compaction degree of the cotton stop over the time and the water loss during the sampling.

The value bioproduct in this work was the biomass, since the studied compounds by BlueGenics partners are intracellular. The main objective is to obtain the highest biomass concentration in the shortest fermentation time (higher productivity). It was observed that the relationship between OD₆₀₀ and DCW varies during the fermentation (data not shown), probably because of morphological changes in *R. marinus* cells, so the calibration with the DCW of the final sample would not be scientifically correct. Another option could be to perform DCW assays for all the samples, but the collected volume in shake flask cultivations was not enough. Because of that, the main parameter of comparison between fermentations was OD₆₀₀. The dilutions for OD₆₀₀ measurements were carried out with ROW, since a

previous study revealed that no significant difference is observed between dilutions with ROW and saline solution for immediate spectrophotometric readings.

Because of all the aforementioned considerations, the growth studies should be seen as comparative tests to guide towards the establishment of a narrower space of experimental conditions to be tested in bioreactor. The presented standard deviations refer to biological duplicates measurements.

6.3.2. C-source studies

6.3.2.1. Glucose as C-source

The determination of the growth pattern in Medium 166 supplemented with 10.0 g.L^{-1} glucose was the starting point of the comparative growth studies in shake flask. It should be noted that the glucose is the structural monomer of laminarin. Furthermore, the majority of the available data in literature was obtained in cultivations with glucose as C-source.

a) Strain 4

The results obtained for strain 4 are presented on **Figure 18**.

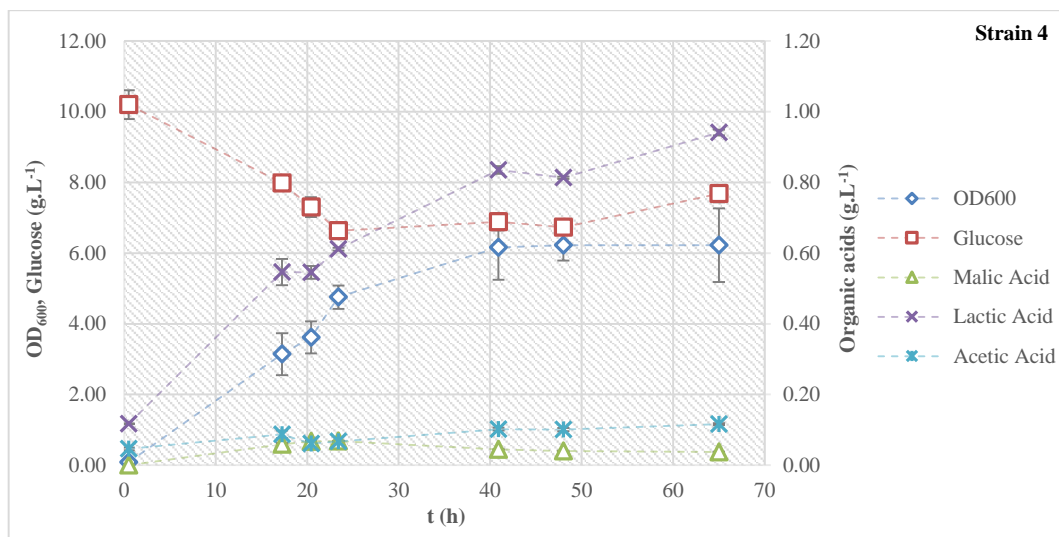


Figure 18 – Growth study in Medium 166 supplemented with 10 g.L^{-1} glucose (*R. marinus* strain 4).

A maximum OD₆₀₀ of 6.228 was observed at 48.00h. The strain did not consume all the glucose available in the medium. At 48.00h of fermentation, the glucose concentration of 6.63 g.L^{-1} was registered, representing an average volumetric substrate uptake rate (r_s) of $0.0723 \text{ g.L}^{-1}.\text{h}^{-1}$. Probably, after 40.92h of fermentation (OD₆₀₀ of 6.228) no growth occurred anymore, being the residual OD₆₀₀ increase of the same order of magnitude of concentration effect. During fermentation, negligible amounts of malic and acetic acids were produced (reaching 0.07 and 0.12 g.L^{-1} , respectively). On the other hand, lactic acid was a significant

by-product with a maximum concentration of 0.94 g.L^{-1} at the end of the fermentation. The production of the organic acids was responsible for the significant decrease of the average final pH to 4.47.

b) Strain 5

On **Figure 19**, the growth pattern obtained for strain 5 is presented.

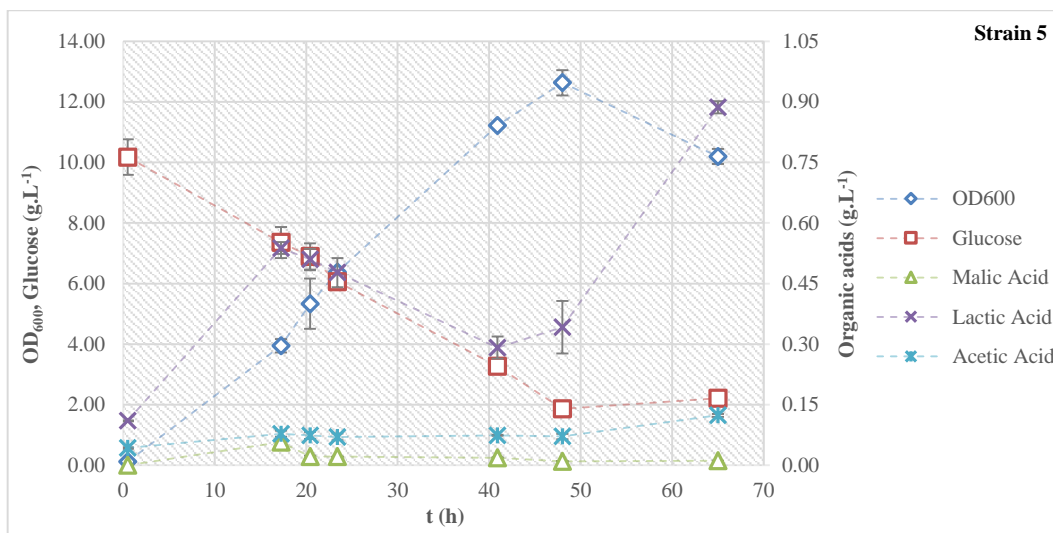


Figure 19 – Growth study in Medium 166 supplemented with 10 g.L^{-1} glucose (*R. marinus* strain 5).

Strain 5 consumed almost all the glucose available in the medium, attaining the minimum glucose concentration of 1.87 g.L^{-1} , which represents an average r_s of $0.173 \text{ g.L}^{-1} \cdot \text{h}^{-1}$. Accordingly, the maximum OD₆₀₀ of 12.63 was observed meaning that the biomass concentration was the double of that attained with strain 4. As observed for strain 4, the concentrations of malic and acetic acids were negligible (maximum of 0.06 and 0.12 g.L^{-1} , respectively) and large amounts of lactic acid were produced (0.89 g.L^{-1} at the end of the fermentation). The pH was also decreased to 4.69.

c) Strains 4 and 5

Strain 5 seemed to be more adapted to control conditions, since the maximum OD₆₀₀ was almost two-fold the maximum OD₆₀₀ observed for strain 4. None of the strains consumed all the glucose available in the media. For both, the glucose consumption was interrupted at 48.00h. Some hypothetical reasons are presented: the incubating temperature (60°C) was lower than the optimum described in literature (65°C) which may limit the C-sources consumption and the growth of the strains; a low 50% (v/v) head-space was used, leading to a low aeration rate, which directly affects the growth rate, since *R. marinus* is a strict aerobe; the pH at the end of the fermentations was below 5.00, which is out of the range described in literature as optimum for *R. marinus* growth, between 5.00 and 9.00

(Gomes et al., 2000). It should be noted that the initial Medium 166 pH had been adjusted to 8.00. The acidification of the medium was probably due to the organic acids production (mainly lactic acid).

Medium 166 was considered a suitable medium for *R. marinus* cultivation, especially for strain 5, but the relatively low N and P-sources (Nitrogen and Phosphorus sources) can be a limiting factor to the growth for experiments with higher concentrations of C-source. Given the high production of organic acids and the little *R. marinus* tolerance to low pH (**Chapter 3.1.2**), controlling the pH during the fermentation could also be a beneficial strategy to enhance the growth of the strains.

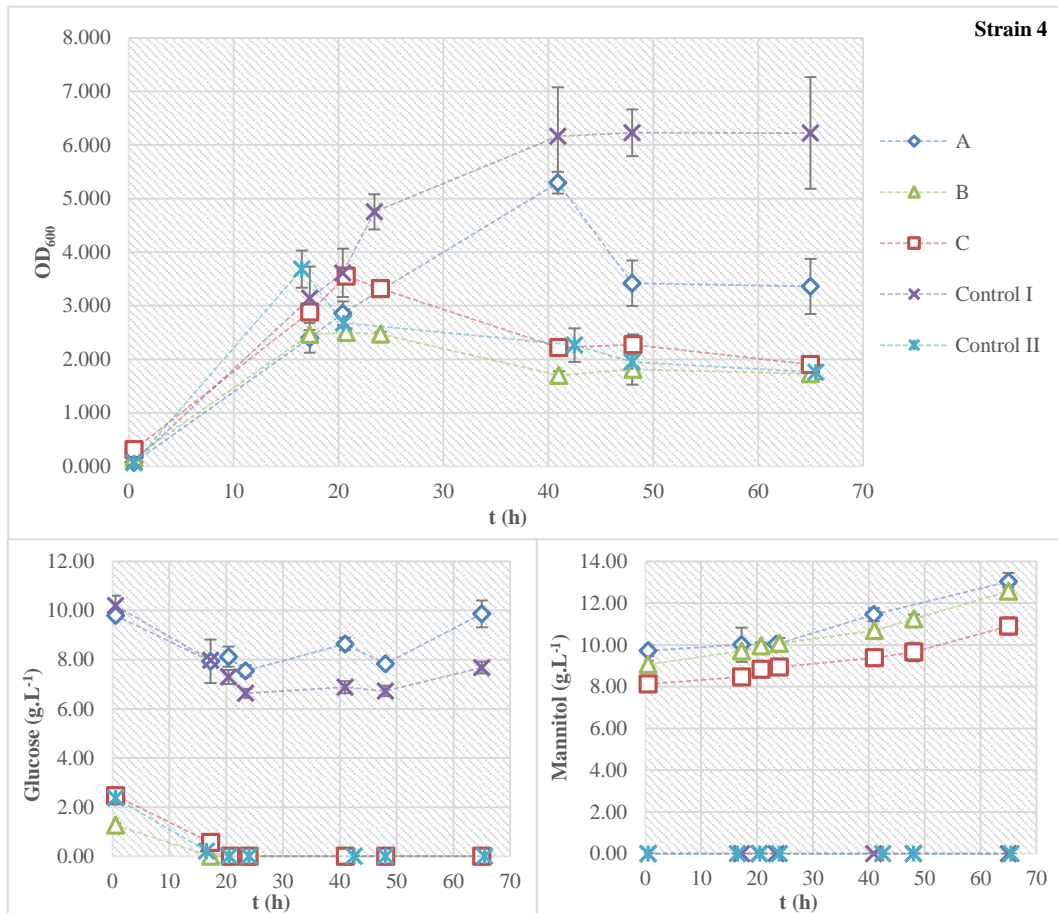
6.3.2.2. Mannitol as C-source

Studies performed by Matís revealed that *R. marinus* holds the enzymatic machinery to metabolize mannitol. This growth study aims to check how the strains behave in the presence of different concentrations of mannitol and glucose.

It should be noted that no mannitol consumption occurred during the assays. Consequently, the mannitol concentrations represented on the following graphs were crescent due to the aforementioned high evaporation rate occurring in shake flask cultivations.

a) Strain 4

On **Figure 20**, the time course of experiment is presented (OD_{600} , glucose and mannitol concentrations) for the assays with strain 4.



A – 10.0 g.L⁻¹ mannitol + 10.0 g.L⁻¹ glucose; B – 9.00 g.L⁻¹ mannitol + 1.00 g.L⁻¹ glucose; C – 2.00 g.L⁻¹ glucose + 8.00 g.L⁻¹ mannitol; Control I – 10.0 g.L⁻¹ glucose; Control II – 2.00 g.L⁻¹ glucose.

Figure 20 – Growth study in Medium 166 supplemented with different concentrations of mannitol (*R. marinus* strain 4).

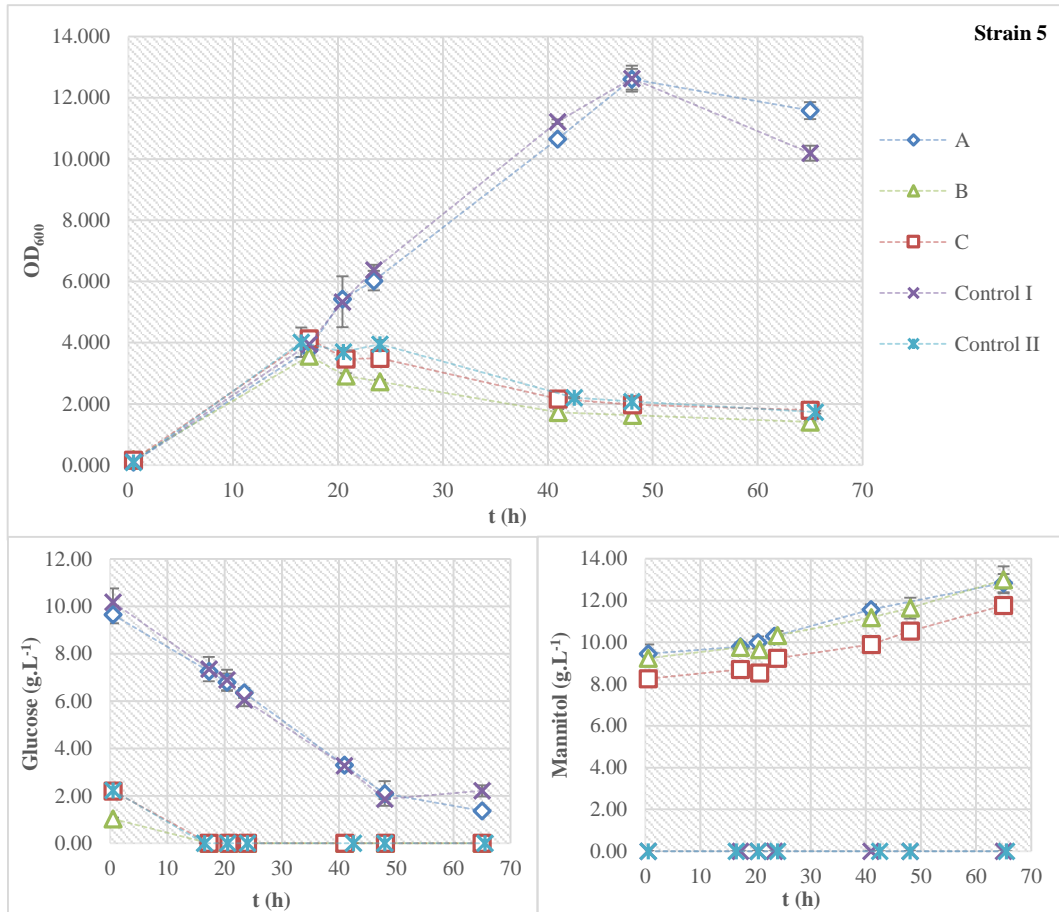
In Experiment A, no mannitol was consumed and the glucose consumption was lower than in Control I, which indicates that high amounts of mannitol in the medium affected the glucose uptake of strain 4. Interestingly, in the presence of mannitol, the strain produced higher amounts of by-products such as malic, lactic and acetic acids than in Control I. After 40.92h of incubation, the cells started to lyse.

Experiments B and C were performed with residual amounts of glucose to verify if the absence of mannitol consumption could be related to a hypothetical “glucose-effect”, since high amounts of glucose were available during the time course of Experiment A. However, in Experiments B and C, no mannitol consumption was observed as well. A maximum OD_{600} of 3.549 was observed at 20.75h for Experiment C comparing to 3.685 at 16.50h for Control

II, coinciding to the glucose depletion. Once again, the mannitol seemed to exert some inhibitory effect to glucose uptake, delaying the glucose depletion.

b) Strain 5

The growth patterns obtained in assays with strain 5 are presented on **Figure 21**.



A – 10.0 g.L⁻¹ mannitol + 10.0 g.L⁻¹ glucose; B – 9.00 g.L⁻¹ mannitol + 1.00 g.L⁻¹ glucose; C – 2.00 g.L⁻¹ glucose + 8.00 g.L⁻¹ mannitol; Control I – 10.0 g.L⁻¹ glucose; Control II – 2.00 g.L⁻¹ glucose.

Figure 21 – Growth study in Medium 166 supplemented with different concentrations of mannitol (*R. marinus* strain 5).

In Experiment A, no mannitol consumption was observed. The pattern of growth in the presence of mannitol was quite similar to Control I. In experiment A, the strain produced almost a half of the lactic acid produced in control. Maybe because of that, the OD₆₀₀ observed at the end of fermentation is slightly higher in the presence of mannitol.

In Experiments B and C, no mannitol was consumed as well. It was observed a maximum OD₆₀₀ of 4.128 at 17.25h for Experiment C compared to 4.017 at 16.50h for Control II. Once again, the pattern of growth in the presence of mannitol was similar to control.

c) Strains 4 and 5

The growth study revealed that none of the strains consumed mannitol under the imposed conditions. Ideally, the initial glucose would be metabolized to provide the energy for the enzymatic machinery production that would be required to mannitol metabolism. However, the cells did not establish the conditions for mannitol consumption. It could be related to the non-existence of a specific mannitol transporter or a regulation carrier on the cell. These results were reported to Matís.

Strain 4 presented a lower growth and a higher formation of by-products in the presence of mannitol. Contrary, strain 5 presented a similar pattern of growth and produced lower amounts of lactic acid. Although not metabolizing mannitol, strain 5 seemed to be more robust in its presence. The presence of mannitol in the growth medium can enable to reduce the NaCl concentration since it is one of the main compatible solutes reported in literature (Empadinhas and Da Costa, 2008).

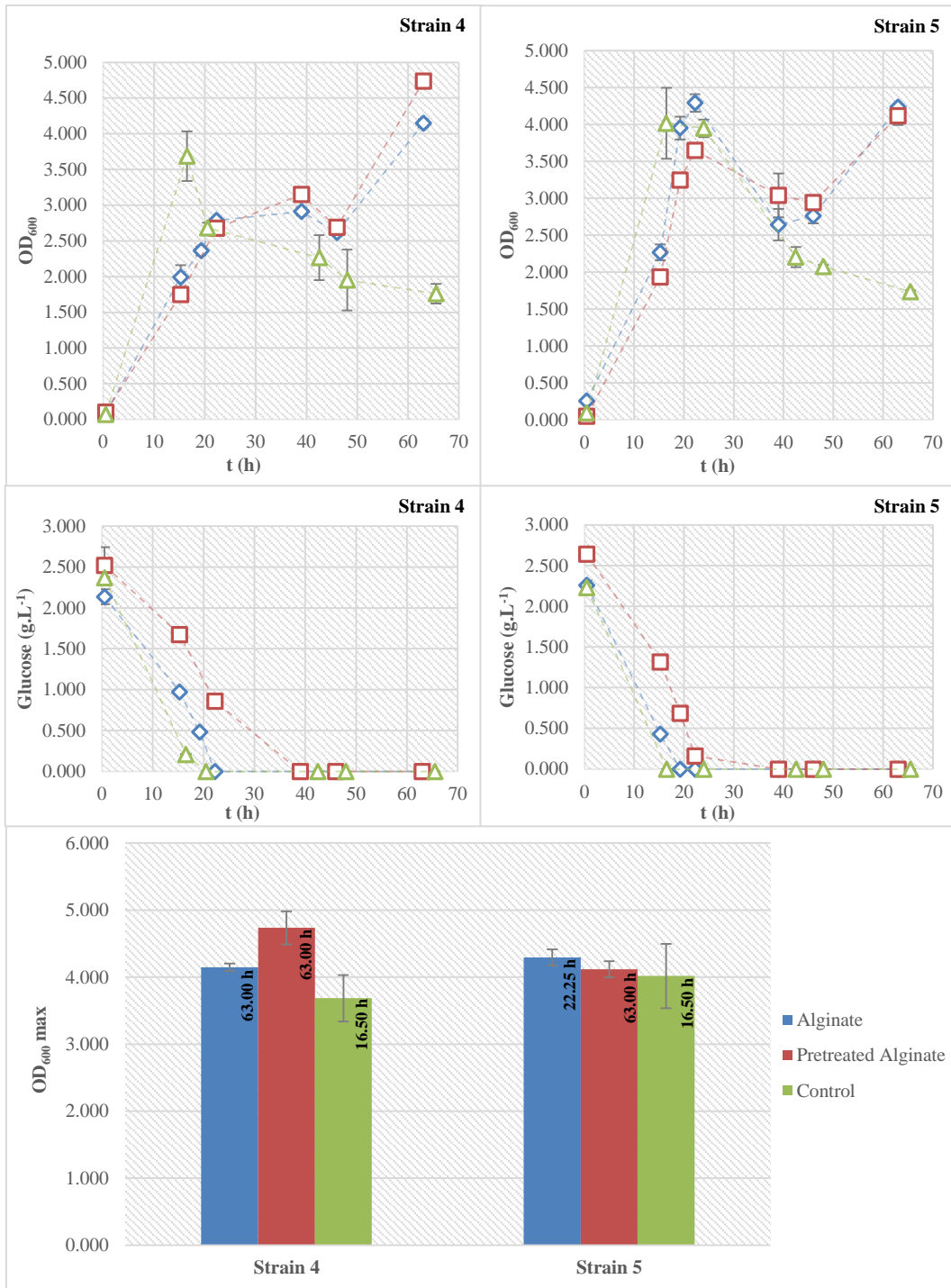
6.3.2.3. Alginate as C-source

Matís studies also revealed that *R. marinus* strains produce enzymes capable of hydrolyzing alginate and metabolizing the correspondent monomers. This growth study aimed to verify the strains behavior in the presence of alginate and pretreated alginate.

The growth studies were performed with dilute-acid hydrolyzed alginate, as the pure monomers are very expensive molecular biology products. Several attempts to establish an alginate HPLC calibration were performed but it was not possible to unequivocally identify the respective monomers in chromatograms using the HPLC settings implemented as routine at Biotrend. Two two major peaks representing M and G monomers from alginate hydrolysis were expected, but the chromatograms showed various peaks, including small peaks eluting at the same retention time as glucose and fructose. Alginate was analyzed against various standards and the sucrose peak overlapped, which could eventually be a contaminant in the commercial powder (ca 5.00% w/w) or another unidentified disaccharide.

a) Strains 4 and 5

The time course (OD₆₀₀ and glucose concentration) and the maximum OD₆₀₀ achieved during the growth studies with alginate and pretreated alginate as C-sources are presented on **Figure 22**.



Alginate – 8.00 g.L⁻¹ sodium alginate + 2.00 g.L⁻¹ glucose; Pretreated Alginate – 8.00 g.L⁻¹ dilute-acid hydrolyzed sodium alginate + 2.00 g.L⁻¹ glucose; Control – 2.00 g.L⁻¹ glucose.

Figure 22 – Time course (OD₆₀₀ and glucose concentration) and maximum OD₆₀₀ registered in cultivations in Medium 166 supplemented with alginate and pretreated alginate (*R. marinus* strains 4 and 5).

In general, by comparison with control assays, the presence of alginate in the media leads to a higher maximum OD₆₀₀, although the differences are not statistically significant.

In fermentations with strain 4, a lag phase was observed between the glucose depletion and the maximum OD₆₀₀ at 63.00 h, which can represent the necessary time that *R. marinus* needed to adapt its metabolism to alginate consumption. A slightly higher maximum OD₆₀₀ was observed with pretreated alginate as C-source. Concerning to strain 5, the aforementioned lag phase between the glucose depletion and the maximum OD₆₀₀ was only observed in fermentation with pretreated alginate as C-source. In the assay with alginate, the maximum OD₆₀₀ was achieved at 22.25 h. For both strains, the glucose depletion was achieved sooner in assays with non-pretreated alginate, which may indicate the presence of inhibitors that could be produced during the pretreatment process.

Taking into account the aforementioned effect of biomass concentration over time and the standard deviations, the OD₆₀₀ differences were not significant enough to infer if any alginate consumption occurred. Furthermore, the compound identified as residual fructose found in pretreated alginate was consumed during the correspondent assays, which directly influences the results, since more than one variable was varied at a time.

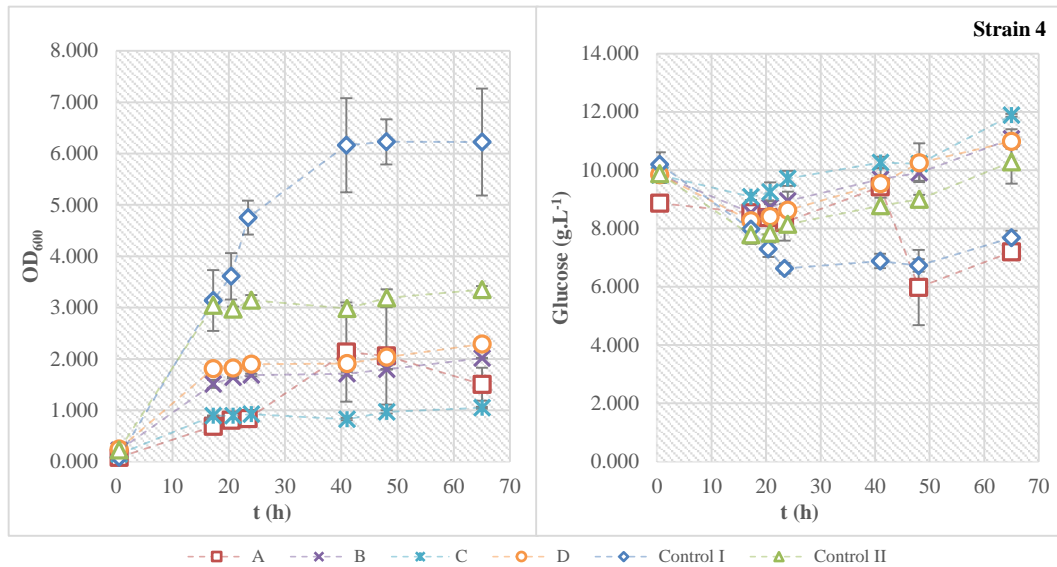
It should be noted that the high viscosity of alginate in aqueous solution restricted the maximum concentration to be applied in the growth medium, since the mass and oxygen transfer could be drastically reduced.

6.3.3. Na-source studies

The objective of this assay was to study the behavior of *R. marinus* when cultivated in media with low amounts of Cl⁻, a highly corrosive agent to stainless steel bioreactors. Sodium chloride (NaCl) is the major source of Cl⁻ in marine growth media. Standard Medium 166 contains 1.00% NaCl. The methodology was to partial or totally substitute the NaCl in the medium by a non-chloride sodium salt (Na₂SO₄) as described by Barclay (2002). The substitution was based on the molarity and the ionic strength of NaCl present in the control conditions.

a) Strain 4

The growth and glucose patterns obtained during the assays with strain 4 are presented on **Figure 23**.



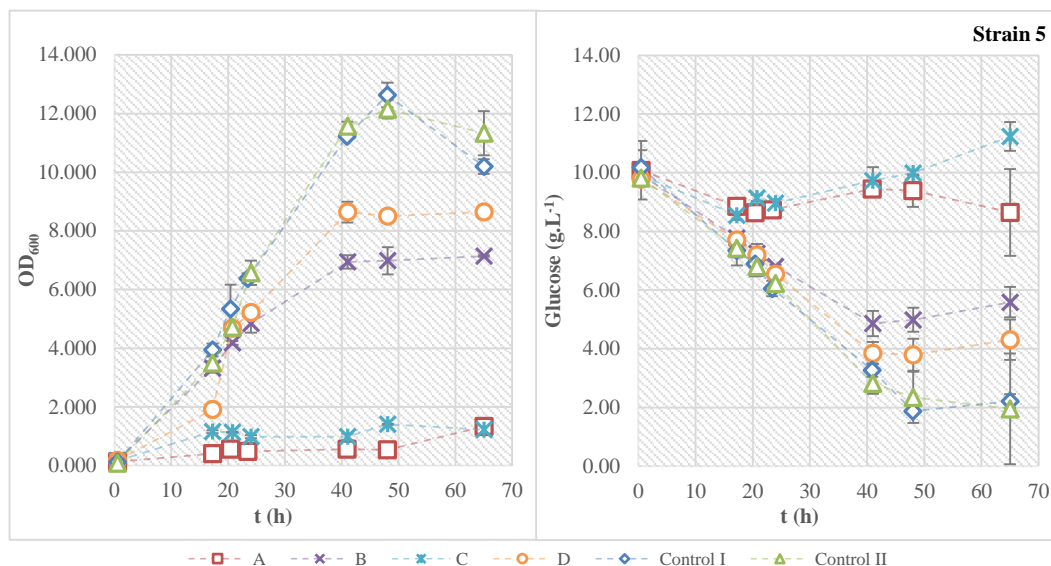
A – 1.22% Na₂SO₄; B – 0.608% Na₂SO₄ + 0.500% NaCl; C – 0.810% Na₂SO₄; D – 0.405% Na₂SO₄ + 0.500% NaCl; Control I – 1.00% NaCl; Control II – 0.500% NaCl.

Figure 23 – Growth study in Medium 166 with different concentrations of NaCl and Na₂SO₄ (*R. marinus* strain 4).

The strain 4 presented low growths and glucose consumptions in all the assays with partial and total substitution of NaCl. The highest OD₆₀₀ of 2.293 was observed at the end of the Experiment D (partial NaCl substitution according to ionic strength). A high concentration of acetic acid was observed in Experiments A and C (total NaCl substitution). Control II presented a maximum OD₆₀₀ of 3.189, approximately a half than OD₆₀₀ observed in Control I.

b) Strain 5

The growth and glucose patterns obtained for the assays with strain 5 are presented on **Figure 24**



A – 1.22% Na₂SO₄; B – 0.608% Na₂SO₄ + 0.500% NaCl; C – 0.810% Na₂SO₄; D – 0.405% Na₂SO₄ + 0.500% NaCl; Control I – 1.00% NaCl; Control II – 0.500% NaCl.

Figure 24 – Growth study in Medium 166 with different concentrations of NaCl and Na₂SO₄ (*R. marinus* strain 5).

The strain 5 presented very low growths in Experiments A and C and moderate growths in Experiments B and D. The highest OD₆₀₀ of 8.646 was observed at the end of the Experiment D (partial NaCl substitution according to ionic strength). High concentrations of acetic and lactic acids were observed in Experiments A and C, in which total NaCl substitution was carried out. Interestingly, Control II presented a very similar growth pattern relatively to Control I.

c) Strains 4 and 5

Both strains presented low to moderate growths and glucose consumption in the presence of Na₂SO₄. Furthermore, higher concentrations of by-products were observed, such as acetic and lactic acids, indicating that Na₂SO₄ could represent a stress factor to *R. marinus* growth and metabolism. Contrary to strain 4, strain 5 presented a very similar growth pattern when cultivated in 0.500% NaCl (Control II) relatively to 1.00% NaCl (Control I).

Barclay (2002) mentioned that 3.0 g.L⁻¹ Cl⁻ in the medium is an acceptable concentration to apply in stainless steel bioreactor in order to reduce the corrosion effect. Including all the chlorinated compounds present in Medium 166 with 0.500% NaCl, chloride totalizes 3.11 g.L⁻¹, which is only 3.8% above the stipulated concentration. Taking into account the high biomass production observed in the assay with strain 5 in Control II

(0.500% NaCl), the scale-up to bioreactor is feasible. Because of that, the results obtained in Control II are shown on **Figure 25** in detail.

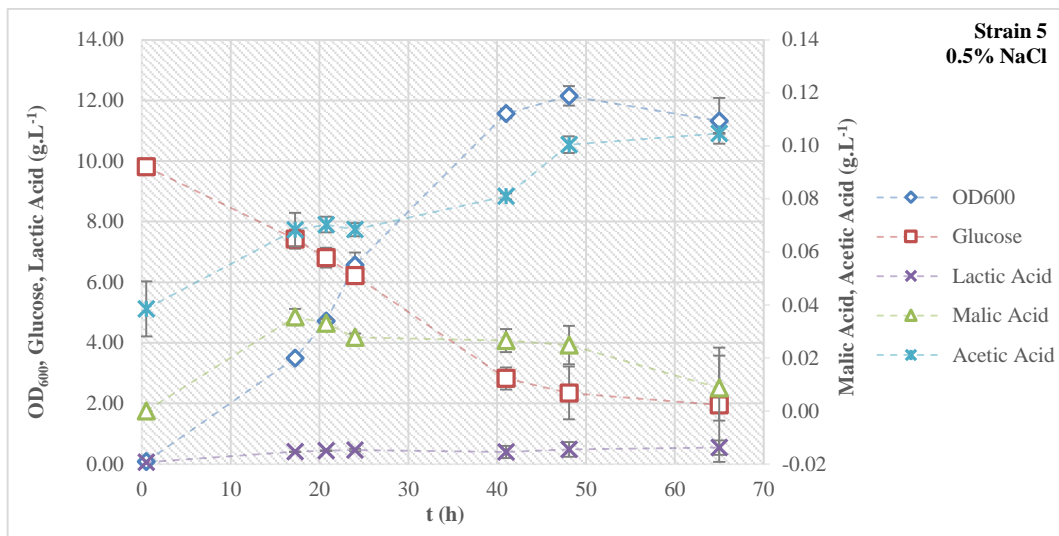


Figure 25 – Growth study in Medium 166 (0.500% NaCl) supplemented with 10 g.L⁻¹ glucose (*R. marinus* strain 5).

A maximum OD₆₀₀ of 12.15 was observed at 48.00h presenting an average r_s of 0.155 g.L⁻¹.h⁻¹. During the fermentation, the concentration of malic and acetic acids was negligible presenting a maximum of 0.04 and 0.10 g.L⁻¹, respectively. A 0.54 g.L⁻¹ lactic acid concentration was observed at the end of the fermentation. In addition to a similar maximum OD₆₀₀, the organic acids production was lower than that observed in cultivations with 1.00% NaCl (Control I).

6.3.4. Macroalgae as feedstock

The previous growth studies were carried out in order to a better understanding of the *R. marinus* behavior in the presence of the major brown macroalgal carbohydrates and different Na-sources. The purpose of this growth study was to test the behavior of *R. marinus* when cultivated in media with two different brown macroalgae, ASCO and LAM (raw and pretreated with dilute-acid). The biochemical profile of feedstock obtained by HPLC is presented on **Table 9**. It should be noted that the characterization obtained by HPLC refers to the liquid fraction with the soluble components of the milled brown macroalgae.

Table 9 – Biochemical profile of ASCO and LAM (raw and pretreated feedstock) by HPLC.

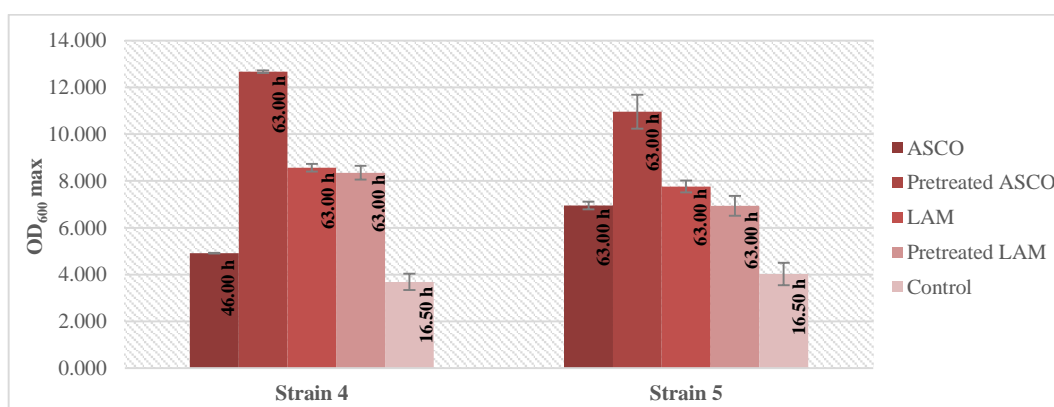
Components	Concentration (g.kg ⁻¹) ^a			
	ASCO		LAM	
	Raw	Pretreated	Raw	Pretreated
Lactose	0.00	0.00	2.15	0.00
Xylose	0.00	2.51	0.00	1.05
Mannitol	22.83	18.78	100.96	99.56
Citric acid	3.99	4.51	2.16	7.05
Tartaric acid	1.22	0.41	0.00	0.00
Pyruvic acid	0.00	0.15	0.52	0.76
Malic acid	0.00	0.00	0.00	0.33
Lactic acid	1.68	2.63	2.48	5.67
Acetic acid	0.00	1.89	0.00	0.00
Fumaric acid	0.01	0.01	0.03	0.03

^a gram of component per kg of feedstock

In general, a slight increment in the concentration of organic acids was observed after the pretreatment of macroalgae. *Ascophyllum nodosum* (ASCO) and *Laminaria digitata* (LAM) can contain up to 32% laminarin (w/w) (Kadam et al., 2015). LAM presents a higher content in mannitol than ASCO. An increment of glucose with the dilute-acid pretreatment was expected, but no glucose was observed in the chromatograms. It is described that the laminarin hydrolysis with HCl usually presents higher yields (Kadam et al., 2015). The fairly low total concentration of components in ASCO suggests that the pretreatment was not efficient or that the applied dilute-acid pretreatment could lead to a partial-hydrolysis of laminarin, releasing gluco-oligosaccharides that were not detected in the HPLC system used.

a) Strains 4 and 5

The maximum OD₆₀₀ achieved during the growth studies with alginate and hydrolyzed alginate as C-sources are presented on **Figure 26**.



ASCO – 8.00 g.L⁻¹ ASCO + 2.00 g.L⁻¹ glucose; Pretreated ASCO – 8.00 g.L⁻¹ dilute-acid hydrolyzed ASCO + 2.00 g.L⁻¹ glucose; LAM – 8.00 g.L⁻¹ ASCO + 2.00 g.L⁻¹ glucose; Pretreated LAM – 8.00 g.L⁻¹ dilute-acid hydrolyzed LAM + 2.00 g.L⁻¹ glucose; Control – 2.00 g.L⁻¹ glucose.

Figure 26 – Maximum OD₆₀₀ registered in cultivations in Medium 166 supplemented with raw and pretreated ASCO and LAM (*R. marinus* strains 4 and 5).

In general, the cultivations with the two species of brown macroalgae presented a higher maximum OD₆₀₀ than the controls. It should be noted that the fermentations were interrupted at 63h of incubation (see the General Considerations), but the stationary phase had not been reached, as presented on **Figure 27**.

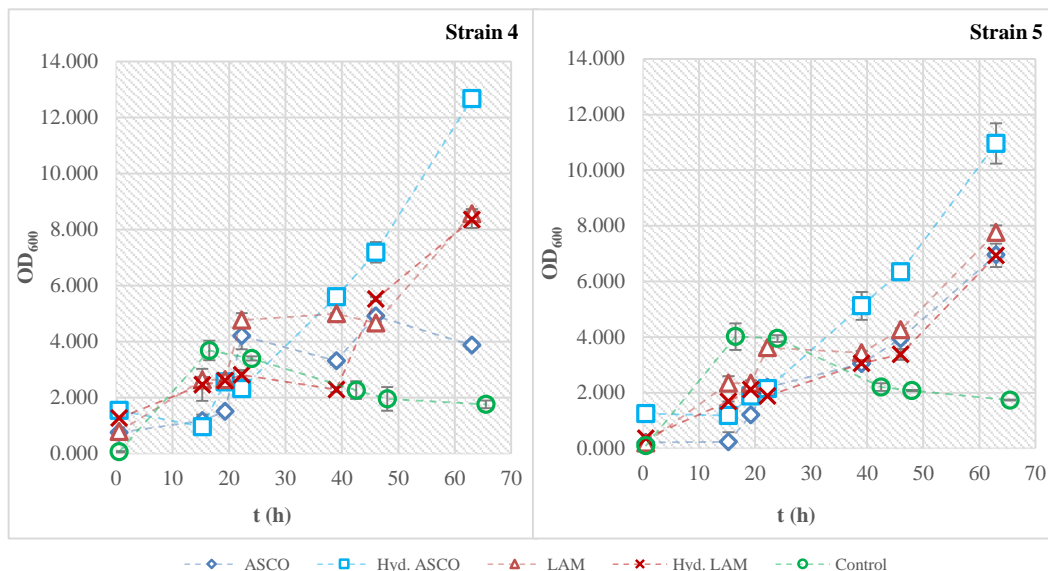


Figure 27 – Growth study in Medium 166 supplemented with raw and pretreated ASCO and LAM (*R. marinus* strains 4 and 5).

In all the assays, the initial glucose in the media was depleted in the first 24h of incubation but, as expected (see growth studies results), no mannitol consumption was observed. During fermentation, no increase of glucose concentration was observed, which means that if laminarin saccharification occurred, the releasing glucose was readily consumed by *R. marinus*. The assays with pretreated ASCO presented the highest maximum OD₆₀₀ by both strains (particularly strain 4 with 12.676), representing two to three-fold the maximum OD₆₀₀ observed in growths including non-pretreated ASCO. Contrary, the growths with pretreated LAM presented a slightly lower maximum OD₆₀₀ in comparison with non-pretreated LAM.

The data is still inconclusive, since laminarin and alginate monomers consumption could not be monitored. The main conclusion drawn from this study is that both strains of *R. marinus* are able to grow in media enriched with ASCO and LAM (with or without pretreatment). Furthermore, the OD₆₀₀ results could be to imprecise, since those feedstocks present to much insoluble compounds (up to 41% of dry weight) that directly affect the measurements. For the same reasons, the DCW procedure cannot be applied, since the insoluble particles remained in the pellet along with the cells. Although being more time-consuming, other procedures for measuring the biomass concentration, such as cell counting in Neubauer chamber and standard agar plate count, must be adopted in future studies.

It should be noted that macroalgal biomass present high amounts of salts. Considering that $3.00 \text{ g.L}^{-1} \text{ Cl}^{-}$ in the medium is the maximum concentration to apply in stainless steel bioreactor (Barclay, 2002) and the Cl^{-} contained in the two tested macroalgae (see **Chapter 5.7.5**), the maximum concentrations of 53.7 g.L^{-1} and 33.0 g.L^{-1} (ASCO and LAM, respectively), could be applied as feedstock for fermentation. Those concentrations are quite low, since only 60% of the dry weight (maximum) are carbohydrates. For using those products as feedstock fermentations in large scale, nutrient enrichment or salt removal should be applied, which obviously increase the process costs.

6.4. Fermentation in Bioreactor

6.4.1. General considerations

The main objective of the fermentation in bioreactor was to validate the results obtained in shake flask cultivation of *R. marinus* strain 5 in Medium 166 with 0.500% NaCl and 10.0 g.L^{-1} glucose. In a second stage of the fermentation, feeding strategies were tested aiming to enhance the final biomass concentration.

The feasibility of the scale-up of the process to bioreactor with low chloride content is very important to the BlueGenics project, since the bioproducts screened by Mafis as being marketable compounds could be mass-produced. As described before, the application of macroalgae as feedstock for fermentation could be an asset to the bioprocess, but the usage of defined media supplemented with glucose can also be economically viable, since the compounds of interest are HVLV products.

Gomes et al. (2000) showed that no growth of *R. marinus* occurs at pH 5.00 or below. The results in shake flask revealed that an acidification of the medium occurs during the fermentation, so for the bioreactor cultivation the pH was controlled to 7.00, which is described in literature as optimum for growth (Alfredsson et al., 1988). The purpose was to avoid growth inhibition due to the low pH.

As already studied, the evaporation rate is much less in bioreactor than that in shake flasks, withdrawing the considerations about the concentration effect during the fermentation.

This fermentation was performed with initial agitation of 200 rpm at $60 \text{ }^{\circ}\text{C}$, pH of 7.00 and a dissolved oxygen cascade. At 29.25 h of batch fermentation, a fed-batch stage was performed with four different feeds.

6.4.2. Batch and fed-batch stages

The time course of experiment is presented (OD₆₀₀ and DCW) is presented on **Figure 28**.

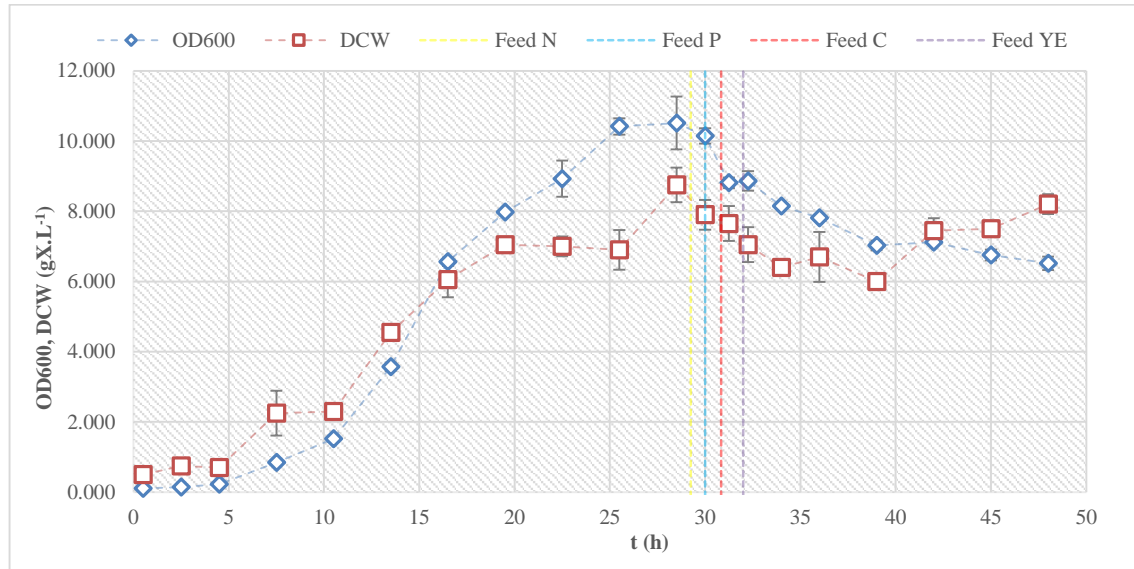


Figure 28 – Fermentation in bioreactor (*R. marinus* strain 5) in Medium 166 (0.500% NaCl) – OD₆₀₀ and DCW during the time.

The first deduction to be drawn from the graphic was that the ratio between OD₆₀₀ and the DCW varies during the fermentation (ranging from 0.193 to 1.51 L.g⁻¹), validating the preliminary studies in shake flasks. The OD₆₀₀ was a useful growth indicator mainly because it provides almost in-time results. However, the calculations made directly with the DCW, which was also carried out point to point, are more relevant for practical applications.

The exponential growth phase was observed between 4.50 and 19.50h. The maximum growth was achieved after 28.50h, with 8.75 gX.L⁻¹ (OD₆₀₀ of 10.515), representing an average volumetric growth rate (r_x) of 0.295 gX.L⁻¹.h⁻¹.

The natural logarithm of OD₆₀₀ and DCW concentration during the fermentation time is presented on **Figure 29**.

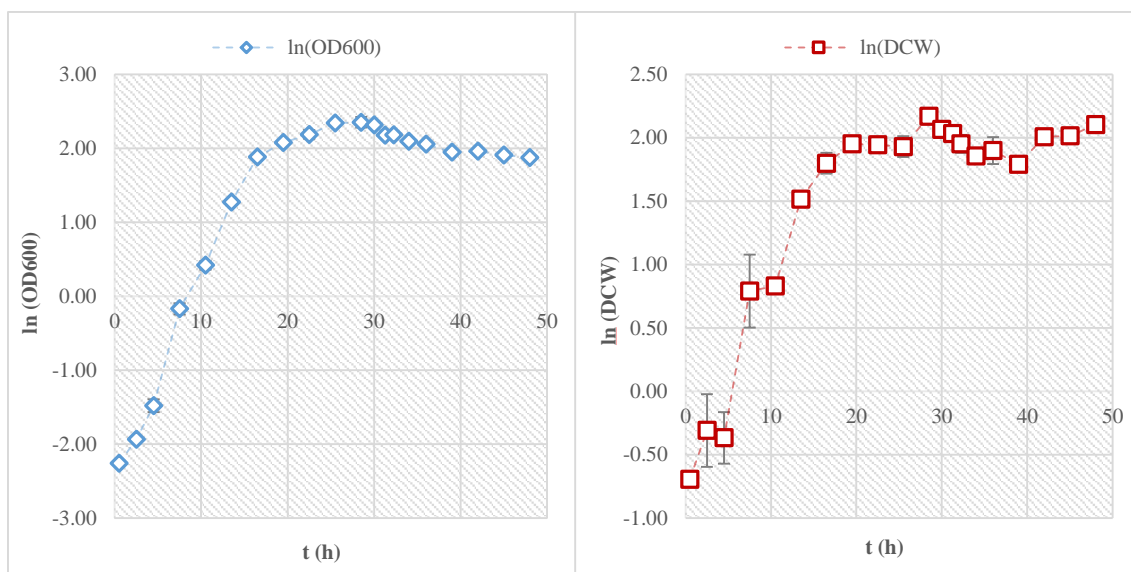


Figure 29 – Fermentation in bioreactor (*R. marinus* strain 5) in Medium 166 (0.500% NaCl) – ln(OD₆₀₀) and ln(DCW) during the time.

The μ_{\max} calculated with OD₆₀₀ data was 0.359h^{-1} , which differs from μ_{\max} calculated with DCW data, 0.208h^{-1} . This difference might be explained by the relatively high error associated with the DCW measurements at the initial stages of the culture, including at the onset of the exponential growth phase, due to the relatively low biomass concentration. In the literature, little information about *R. marinus* kinetics of growth was published. On **Table 10**, the available μ_{\max} at different NaCl concentrations at 60 and 65°C are presented. The growth rates from published works have been obtained by extracting the approximate biomass time-points from the available graphs

Table 10 – Comparison of approximate μ_{\max} of *R. marinus* for cultivations at 60 and 65°C with different NaCl concentrations.

	T (°C)	% NaCl (w/v)					Reference	
		0.500	1.00	2.00	3.00	4.00		5.00
μ_{\max} (h ⁻¹)	60	0.208 ^a	-	-	-	-	-	Present work
		0.359	-	-	-	-	-	
	65	-	0.328	-	0.288	-	0.217	Silva et al. (1999)
		0.391	0.419	0.441	0.388	0.329	0.247	Alfredsson et al. (1988)
65	-	0.610	-	0.477	-	0.278	Silva et al. (1999)	

^acalculated with DCW data. All the other rates was calculated with OD data.

All the available data in literature was calculated from optical density measurements. For comparison, the μ_{\max} of 0.359h^{-1} was taken into account. The μ_{\max} in this work was higher than the observed by Silva et al. (1999) for higher NaCl concentrations at the same temperature. Strain 5 seemed to be more robust to lower salinities. Even at the optimum temperature of growth (65°C), Alfredsson et al. (1988) described a slightly higher μ_{\max} (0.391h^{-1}) for the growth with 0.500% NaCl. In general, the performance at the exponential

phase of *R. marinus* strain 5 was quite acceptable, since the cultivation was carried out at a lower temperature and NaCl concentration than the optimum conditions.

At the end of the experiment (48.00h), the DCW in bioreactor was 8.20 gX.L⁻¹. It should be noticed that 300 mL of broth was withdrawn from bioreactor for sampling and all the feeds totalized 130 mL of extra volume to the bioreactor, which imparts a dilution effect of biomass and other medium components.

The main HPLC, ammonia and phosphate results are presented on **Figure 30**.

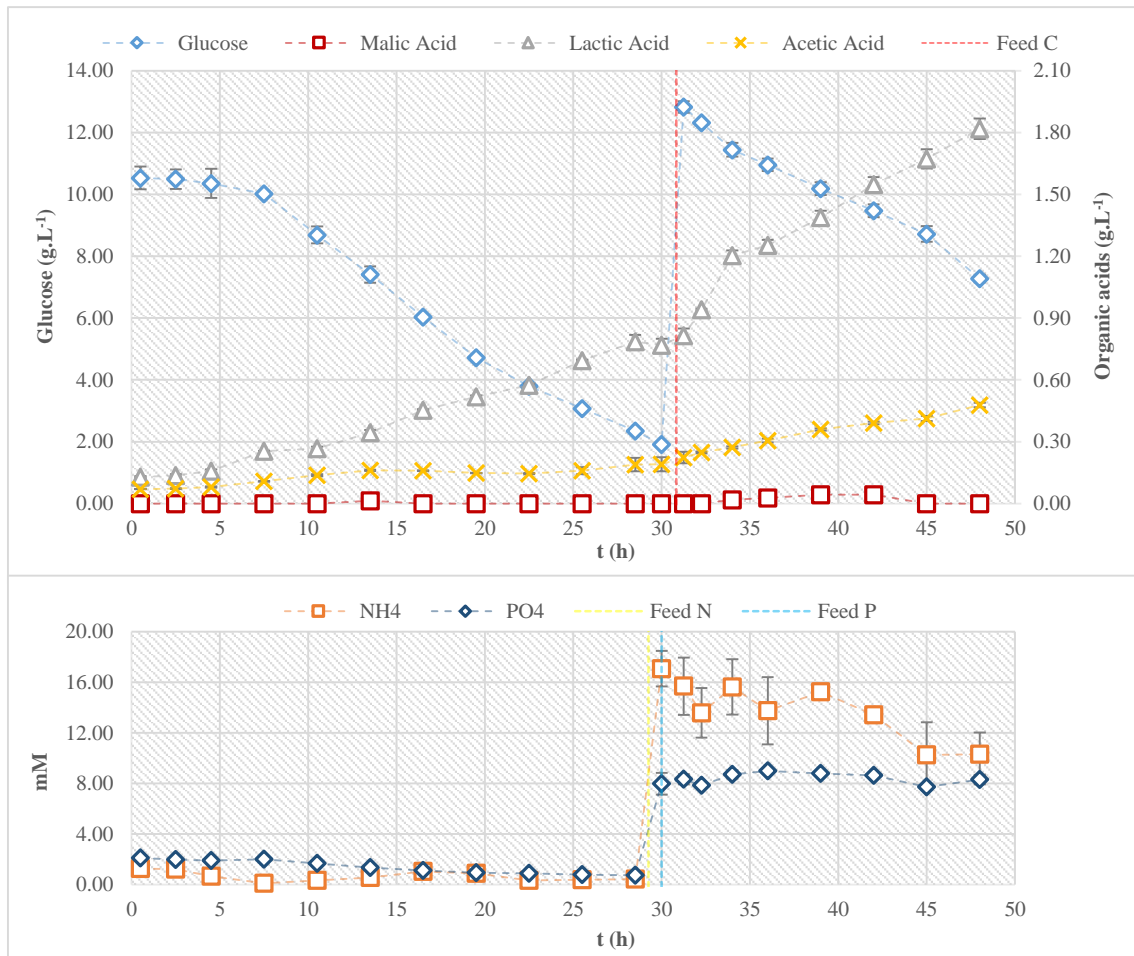


Figure 30 – Fermentation in bioreactor (*R. marinus* strain 5) in Medium 166 (0.500% NaCl) – glucose, organic acids, ammonia and phosphate during the time.

During the fermentation, the main by-product detected by HPLC was eluted at the typical retention time of lactic acid, as occurred in the shake flask cultivations. The production of compounds detected as malic and acetic acids production remained low. The HPLC results validated that until 4.50 h of fermentation, the strain remained in a lag phase, since no glucose consumption was observed. Thereafter, during the exponential phase, the glucose consumption accompanied the bacterial growth. Between 25.50 and 28.50h, an OD₆₀₀ stabilization was observed but the glucose consumption still occurred. At 28.50h (the

maximum DCW), an average r_s of $0.293 \text{ g.S.L}^{-1}.\text{h}^{-1}$ was observed. For this calculation, only glucose was considered although other residual carbon sources such as lactose and pyruvic acid were present since the beginning of the fermentation.

In general, when bacteria interrupts the growth by the lack of a specific nutrient, a timely feed often results in an almost instantaneous change in oxygen consumption due to the acceleration of the bacterial metabolism. Because of that, the on-line results from bioreactor presented on **Figure 31** were essential for the in-time bioreactor operation.

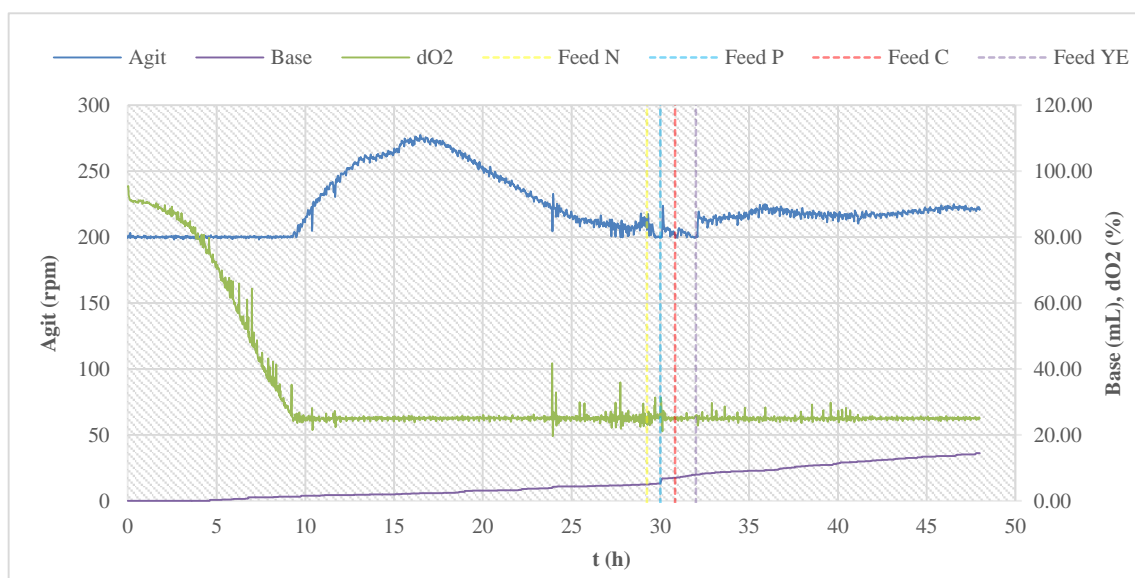


Figure 31 – Fermentation in bioreactor (*R. marinus* strain 5) in Medium 166 (0.500% NaCl) – on-line results from BioCommand®.

The oxygen consumption is directly related to the agitation increase once the dissolved oxygen concentration reaches the defined set-point. The agitation increased during exponential growth phase and decreased during the transitional growth phase, remaining next to the minimum at 29.00h. The NaOH addition corresponds to data called “Base”.

It was hypothesized that the nitrogen initially present in Medium 166 was depleted, so the bioreactor was fed with a N-source ($(\text{NH}_4)_2\text{SO}_4$) at 29.25h. The results indicated that between 0.50 and 28.50h, the ammonia concentration declined from 1.27mM to 0.44mM. After feeding, the ammonia concentration rose to 17.06mM. A further rise in agitation was expected if N was the sole limiting nutrient, but in practice, this was not the case.

The phosphate initially available in the medium was also quite low, so the bioreactor was fed with a P-source (KH_2PO_4) at 30.00h. Between 0.50 and 28.50h, the phosphate concentration declined from 2.09mM to 0.73mM. After feeding, the phosphate concentration rose to 7.98mM. A slight increase in agitation after the feed P was observed.

With no immediate response by the strain, it was decided to feed the bioreactor with glucose (30.83h) and yeast extract (32.00h) to final concentrations of 10.0 and 1.00 g.L^{-1} , respectively, restoring the initial concentrations in Medium 166. After feeding the bioreactor with yeast extract, a noticeable increase of agitation was registered, which probably means that some micronutrient present in yeast extract was missing in the medium.

After all the feed strategies, glucose and ammonia were gradually consumed and organic acids continued to be produced. At 39.00h, the strain seemed to slowly restart to grow (DCW curve), but that was not confirmed by the OD measurements. The experiment was interrupted at 48.00h.

The feeds were added in the beginning of the stationary growth phase, causing a long lag phase to the reactivation of the culture. At this stage, the metabolism changes are often too slow, making it impossible to conclude which of the feeds contained the limiting substrate. Probably, the strain response could be faster if the feeds were applied at the time of inflection of agitation curve (17.00h), enhancing the exponential growth phase. It is likely that most of the nutrients were being consumed for maintenance purposes and that a richer medium should be used from the beginning of the fermentation if higher biomass concentrations are planned to be obtained.

For comparison between the results obtained in bioreactor and shake flask, the OD₆₀₀ and glucose profiles are presented on **Figure 32**.

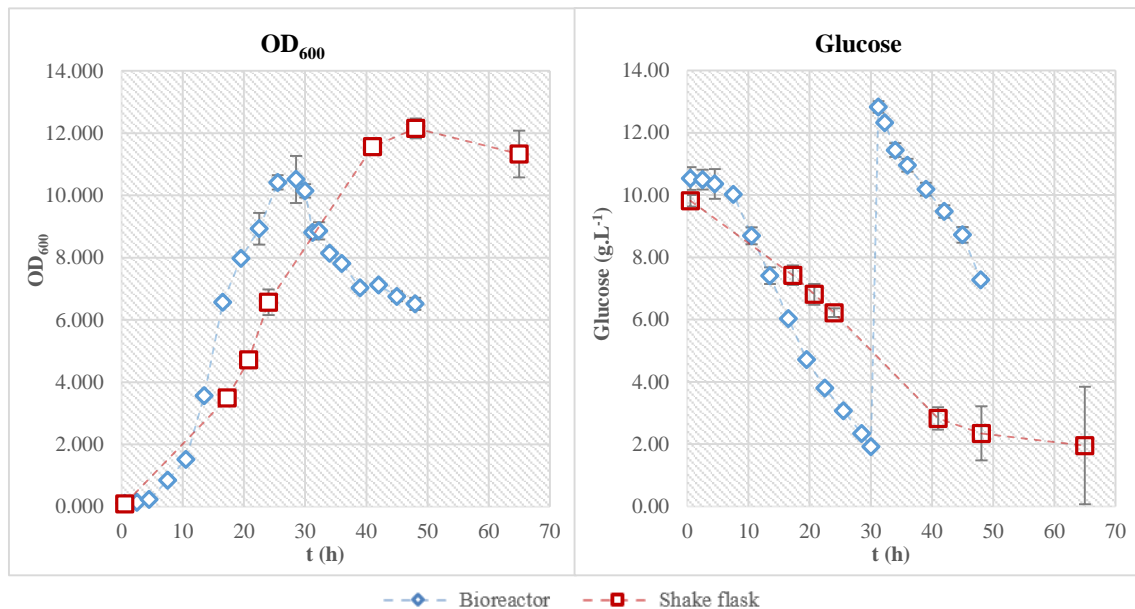


Figure 32 – Fermentation in bioreactor and shake flask (*R. marinus* strain 5) in Medium 166 (0.500% NaCl) – comparison of OD₆₀₀ and glucose data.

Figure 32 seems to show that the maximum OD₆₀₀ was achieved more rapidly in bioreactor, since the oxygen was not a limiting factor. Taking into account the concentration effect in shake flask cultivations due to evaporation, the maximum OD₆₀₀ was numerically quite similar between the systems (10.52 and 12.15 in bioreactor and shake flask cultivations, respectively). The average r_s observed in bioreactor was about 2-fold that observed in shake flask. The comparison between the batch stage of bioreactor assay and the cultivations in shake flask permitted to validate the bioprocess as scalable, revealing very similar, although faster, profiles. From the bioreactor assay, it was possible to recover about 58.0 g of wet biomass per liter of fermentation broth to send to BlueGenics partners in order to perform further screening tests.

7. Conclusions

From the five strains of *R. marinus* supplied by Matís in solid Marine Broth, three of them were successfully acclimatized to cultivation in liquid Medium 166 and cryopreserved in glycerol. Only strains 4 and 5 were effectively recultivated in liquid media and subject of study in the following assays.

During the evaporation study, it was shown that, at 60°C, the medium water loss by evaporation from shake flask could be a key abiotic factor to be taken into account, since a half of the working volume can evaporate after only 72 h of incubation. The growth studies were performed in 100mL shake flasks with 50mL working volume, in which 25% of evaporation was observed after 65h of incubation.

The growth studies in Medium 166 supplemented with 10.0 g.L⁻¹ glucose showed that strain 5 presented higher C-source consumption and growth, although none of the strains consumed all the glucose available in the media. During cultivations in Medium 166 supplemented with mannitol, none of strains consumed such C-source. Strain 5 presented a similar pattern of growth to the presented by controls with glucose, seeming to be more robust than strain 4 with the presence of mannitol in the medium than strain 4. During the growth studies in the presence of alginate and dilute-acid hydrolyzed alginate, the growth differences between the controls and the tests were not significant enough to infer if any alginate consumption occurred.

In growth studies with varying Na-sources, both strains presented low/moderate growths in the presence of Na₂SO₄, in addition to higher concentrations of by-products. The partial and total substitution of NaCl was not successful, since Na₂SO₄ seem to represent a stress factor to *R. marinus* growth. Interestingly, strain 5 presented a very similar growth pattern when cultivated in 0.500% NaCl relatively to the standard 1.00% NaCl. The Medium 166 with 0.500% NaCl totalizes 3.11 g.L⁻¹ of chloride. Accordingly to Barclay (2002), the scale-up to bioreactor is feasible.

The cultivation of *R. marinus* strains in brown macroalgae (ASCO and LAM) as feedstock for growth was successful. Although, in the operational conditions imposed in shake flask assays, mannitol was not consumed and it was not possible to clearly monitor the alginate and laminarin saccharification and metabolization.

From all the results, the more interesting to validate in controlled bioreactor was the cultivation of *R. marinus* strain 5 in Medium 166 with 0.500% NaCl supplemented with 10.0g.L⁻¹ glucose. The feasibility of the scale-up to bioreactor was proved, since the process presented a μ_{\max} of 0.208h⁻¹ based on DCW data and 0.369h⁻¹ based on OD₆₀₀ data, a maximum DCW of 8.75 gX.L⁻¹ (at 28.50h), an average r_X of 0.295 gX.L⁻¹.h⁻¹ and an average r_S of 0.293 gS.L⁻¹.h⁻¹ in the batch stage. In this way, the results obtained in shake flask were successfully validated as scalable to larger volumes. In a second stage of the fermentation, feeding strategies with N, C and P-sources were performed in the beginning of stationary growth phase. To enhance the final biomass concentration, the bioreactor should be fed somewhen in exponential growth phase, at the inflection point of agitation, which represents the maximum oxygen consumption.

In this project, the results showed that it is possible to enhance the *R. marinus* biomass production using glucose as sole C-source. However, the process of saccharification and/or fermentation of mannitol, alginate and laminarin by *R. marinus* strains was not completely understood and characterized. Within the framework of this master's thesis, they were shown the first insights about the biorefinery perspective of cultivating *R. marinus* using brown macroalgae as feedstock, but the project will continue in the BlueGenics context.

As mentioned through all this work, the implementation of a bioprocess with a cheap feedstock, requiring a minimal pretreatment and using a wild strain as sole biocatalyst could be very advantageous in a SSF system under the biorefinery concept. More research has to be performed in this sense.

8. Future Prospects

The experiments that should follow this work include the study of other operational conditions in controlled bioreactor such as C-sources concentration and respective ratios, carbon to nitrogen and phosphate ratios, dissolved oxygen concentrations and eventually assays with higher temperatures. For that, it is imperative to establish reliable procedures to effectively determine the alginate and laminarin levels of saccharification over the fermentation time.

The role of the salts present in the media as well as the effect of low temperatures in enzymatic activity of alginases and laminarinases should also be investigated.

Regarding to the effectiveness of the process, more information about the pretended bioactive compound(s) should be provided by BlueGenics partners in order to optimize the product yield. During this work, it was stipulated that the obtained biomass concentration is directly proportional to the targeted compound, but often intracellular product concentration varies with the bacterial growth stage and the environmental conditions such as nutrient depletion.

9. References

- Alderkamp AC, Van Rijssel M, Bolhuis H. Characterization of marine bacteria and the activity of their enzyme systems involved in degradation of the algal storage glucan laminarin. *FEMS Microbiology Ecology*. 2007;59:108-17.
- Alfredsson GA, Kristjansson JK, Hjörleifsdóttir S, Stetter KO. *Rhodothermus marinus*, gen. nov., sp. nov., a Thermophilic, Halophilic Bacterium from Submarine Hot Springs in Iceland. *Journal of General Microbiology*. 1988;134:299-306.
- Andrade PB, Barbosa M, Matos RP, Lopes G, Vinholes J, Mouga T, et al. Valuable compounds in macroalgae extracts. *Food Chemistry*. 2013;138:1819-28.
- Anon. Corrosion resistance of the austenitic chromium-nickel stainless steels in chemical environments. INCO (Int Nickel Co, Inc) Corros Man. 1963.
- Atabani AE, Silitonga AS, Badruddin IA, Mahlia TMI, Masjuki HH, Mekhilef S. A comprehensive review on biodiesel as an alternative energy resource and its characteristics. *Renewable and Sustainable Energy Reviews*. 2012;16:2070-93.
- Azmat R, Hayat A, Khanum T, Talat R, Uddin F. The inhibition of bean plant metabolism by Cd metal and atrazine III: Effect of seaweed *Codium iyengarii* on metal, herbicide toxicity and rhizosphere of the soil. *Biotechnology*. 2006;5:85-9.
- Banin E, Israely T, Fine M, Loya Y, Rosenberg E. Role of endosymbiotic zooxanthellae and coral mucus in the adhesion of the coral-bleaching pathogen *Vibrio shiloi* to its host. *FEMS Microbiology Letters*. 2001;199:33-7.
- Barclay WR. Reducing corrosion in a fermentor by providing sodium with a non-chloride sodium salt. Google Patents; 2002.
- Barreto MdC, Seca AM, Costa AC, Neto AI, Simões N. Potencial biotecnológico do mar dos Açores. *Boletim de Biotecnologia*. 2014;1645-5878.
- Becker EW. *Microalgae: Biotechnology and Microbiology*. Cambridge University Press ed1994.
- Bilan MI, Kusaykin MI, Grachev AA, Tsvetkova EA, Zvyagintseva TN, Nifantiev NE, et al. Effect of Enzyme Preparation from the Marine Mollusk *Littorina kurila* on Fucoidan from the Brown Alga *Fucus distichus*. *Biochemistry (00062979)*. 2005;70:1321-6.
- Bixler HJ, Porse H. A decade of change in the seaweed hydrocolloids industry. *Journal of Applied Phycology*. 2011;23:321-35.
- Björnsdóttir SH, Blondal T, Hreggvidsson GO, Eggertsson G, Petursdóttir S, Hjörleifsdóttir S, et al. *Rhodothermus marinus*: Physiology and molecular biology. *Extremophiles*. 2006;10:1-16.
- Bruton T, Lyons H, Lerat Y, Stanley M, Rasmussen MB. A Review of the Potential of Marine Algae As A Source of Biofuel in Ireland. 2009.
- Buerger S, Spoering A, Gavriš E, Leslin C, Ling L, Epstein SS. Microbial scout hypothesis and microbial discovery. *Applied and Environmental Microbiology*. 2012;78:3229-33.
- Cardoso SM, Carvalho LG, Silva PJ, Rodrigues MS, Pereira OR, Pereira L. Bioproducts from seaweeds: A review with special focus on the Iberian Peninsula. *Current Organic Chemistry*. 2014;18:896-917.
- Chandia NP, Matsuhira B, Vasquez AE. Alginic acids in *Lessonia trabeculata*: Characterization by formic acid hydrolysis and FT-IR spectroscopy. *Carbohydrate Polymers*. 2001;46:81-7.
- Cheng JJ, Timilsina GR. Status and barriers of advanced biofuel technologies: A review. *Renewable Energy*. 2011;36:3541-9.

- Cherubini F. The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. *Energy Conversion and Management*. 2010;51:1412-21.
- Cherubini F, Jungmeier G, Wellisch M, Willke T, Skiadas I, Van Ree R, et al. Toward a common classification approach for biorefinery systems. *Biofuels, Bioproducts and Biorefining*. 2009;3:534-46.
- Chhatbar M, Meena R, Prasad K, Siddhanta AK. Microwave assisted rapid method for hydrolysis of sodium alginate for M/G ratio determination. *Carbohydrate Polymers*. 2009;76:650-6.
- Choi WY, Han JG, Lee CG, Song CH, Kim JS, Seo YC, et al. Bioethanol production from *Ulva pertusa* Kjellman by high-temperature liquefaction. *Chemical and Biochemical Engineering Quarterly*. 2012;26:15-21.
- Colle PN, Sassi JF, Rogniaux H, Marfaing H, Helbert W. Ulvan lyases isolated from the flavobacteria *Persicivirga ulvanivorans* are the first members of a new polysaccharide lyase family. *Journal of Biological Chemistry*. 2011;286:42063-71.
- Csonka LN. Physiological and genetic responses of bacteria to osmotic stress. *Microbiological Reviews*. 1989;53:121-47.
- Dahlberg L, Holst O, Kristjansson JK. Thermostable xylanolytic enzymes from *Rhodothermus marinus* grown on xylan. *Applied Microbiology and Biotechnology*. 1993;40:63-8.
- Dahlberg LAL, Holst OP, Anker L. Thermostable xylanase from a strain of *Rhodothermus marinus*. Google Patents; 1995.
- Daniel R, Berteau O, Jozefonvicz J, Goasdoue N. Degradation of algal (*Ascophyllum nodosum*) fucoidan by an enzymatic activity contained in digestive glands of the marine mollusc *Pecten maximus*. *Carbohydrate Research*. 1999;322:291-7.
- Dash HR, Mangwani N, Chakraborty J, Kumari S, Das S. Marine bacteria: Potential candidates for enhanced bioremediation. *Applied Microbiology and Biotechnology*. 2013;97:561-71.
- De Silva SS. *Tropical Mariculture*. 1992.
- Ducatti DRB, Massi A, Nosedá MD, Duarte MER, Dondoni A. Production of carbohydrate building blocks from red seaweed polysaccharides. Efficient conversion of galactans into C-glycosyl aldehydes. *Organic and Biomolecular Chemistry*. 2009;7:576-88.
- Elnashar MMM, Mostafa H, Morsy NA, Awad GEA. Biocatalysts: Isolation, Identification, and Immobilization of Thermally Stable Lipase onto Three Novel Biopolymeric Supports. *Industrial & Engineering Chemistry Research*. 2013;52:14760-7.
- Empadinhas N, Da Costa MS. Osmoadaptation mechanisms in prokaryotes: Distribution of compatible solutes. *International Microbiology*. 2008;11:151-61.
- Eom SH, Kim YM, Kim SK. Marine bacteria: Potential sources for compounds to overcome antibiotic resistance. *Applied Microbiology and Biotechnology*. 2013;97:4763-73.
- Epstein W. Osmoregulation by potassium transport in *Escherichia coli*. *FEMS Microbiology Letters*. 1986;39:73-8.
- FAO. *FAO Statistical Yearbook 2012: World Food and Agriculture*. 2012.
- Fleurence J. Seaweed proteins: Biochemical, nutritional aspects and potential uses. *Trends in Food Science and Technology*. 1999;10:25-8.
- Galinski EA. Osmoadaptation in bacteria. *Advances in Microbial Physiology*. 1995;37:273-328.
- Gao K, McKinley KR. Use of macroalgae for marine biomass production and CO₂ remediation: a review. *Journal of Applied Phycology*. 1994;6:45-60.

Garrity GM, Bell JA, Lilburn TG. Taxonomic outline of the prokaryotes. *Bergey's Manual of Systematic Bacteriology*. 2004.

Ge L, Wang P, Mou H. Study on saccharification techniques of seaweed wastes for the transformation of ethanol. *Renewable Energy*. 2011;36:84-9.

Gomes I, Gomes J, Steiner W. Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium *Rhodothermus marinus*: Production and partial characterization. *Bioresource Technology*. 2003;90:207-14.

Gomes J, Gomes I, Terler K, Gubala N, Ditzelmüller G, Steiner W. Optimisation of culture medium and conditions for α -l-Arabinofuranosidase production by the extreme thermophilic eubacterium *Rhodothermus marinus*. *Enzyme and Microbial Technology*. 2000;27:414-22.

Gomes J, Steiner W. Production of a high activity of an extremely thermostable β -mannanase by the thermophilic eubacterium *Rhodothermus marinus*, grown on locust bean gum. *Biotechnology Letters*. 1998;20:729-33.

Grasdalen H, Larsen B, Smisrod O. ^{13}C -n.m.r. studies of monomeric composition and sequence in alginate. *Carbohydrate Research*. 1981;89:179-91.

Guibet M, Colin S, Barbeyron T, Genicot S, Kloareg B, Michel G, et al. Degradation of λ -carrageenan by *Pseudoalteromonas carrageenovora* λ -carrageenase: A new family of glycoside hydrolases unrelated to κ - and ι -carrageenases. *Biochemical Journal*. 2007;404:105-14.

Gunaseelan VN. Anaerobic digestion of biomass for methane production: A review. *Biomass and Bioenergy*. 1997;13:83-114.

Gupta S, Abu-Ghannam N, Scannell AGM. Growth and kinetics of *Lactobacillus plantarum* in the fermentation of edible Irish brown seaweeds. *Food and Bioproducts Processing*. 2011;89:346-55.

Hartmann EM, Durighello E, Pible O, Nogales B, Beltrametti F, Bosch R, et al. Proteomics meets blue biotechnology: A wealth of novelties and opportunities. *Marine Genomics*. 2014;17:35-42.

Hjörleifsdóttir S, Pétursdóttir SK, Korpela J, Torsti AM, Mattila P, Kristjánsson JK. Screening for restriction endonucleases in aerobic, thermophilic eubacteria. *Biotechnology Techniques*. 1996;10:13-8.

Hjörleifsdóttir S, Ritterbusch W, Petursdottir SK, Kristjansson JK. Thermostabilities of DNA ligases and DNA polymerases from four genera of thermophilic eubacteria. *Biotechnology Letters*. 1997;19:147-50.

Ho DP, Ngo HH, Guo W. A mini review on renewable sources for biofuel. *Bioresource Technology*. 2014;169:742-9.

Hobel CFV, Hreggvidsson GÖ, Marteinsonn VT, Bahrani-Mougeot F, Einarsson JM, Kristjánsson JK. Cloning, expression, and characterization of a highly thermostable family 18 chitinase from *Rhodothermus marinus*. *Extremophiles*. 2005;9:53-64.

Holtkamp AD, Kelly S, Ulber R, Lang S. Fucoidans and fucoidanases-focus on techniques for molecular structure elucidation and modification of marine polysaccharides. *Applied Microbiology and Biotechnology*. 2009;82:1-11.

Horn SJ, Aasen IM, Emptyvstgaard K. Ethanol production from seaweed extract. *Journal of Industrial Microbiology and Biotechnology*. 2000;25:249-54.

Hreggviðsson GÓ, Matís, 2015. Personal Communication

Hreggvidsson GO, Kaiste E, Holst O, Eggertsson G, Palsdottir A, Kristjansson JK. An extremely thermostable cellulase from the thermophilic eubacterium *Rhodothermus marinus*. *Applied and Environmental Microbiology*. 1996;62:3047-9.

- Huber R, Wilharm T, Huber D, Trincone A, Burggraf S, König H, et al. Aquifex pyrophilus gen.nov. sp.nov., represents a novel group of marine hyperthermophilic hydrogen-oxidizing bacteria. Systematic and Applied Microbiology. 1992;15:340-51.
- Hurd CL, Harrison PJ, Bischof K, Lobban CS. Seaweed Ecology and Physiology: Cambridge University Press; 2014.
- IEA, 2007. World Energy Outlook 2007. Available at http://www.worldenergyoutlook.org/media/weowebsite/2008-1994/WEO_2007.pdf (accessed 10 Oct. 2014).
- Jang JS, Cho YK, Jeong GT, Kim SK. Optimization of saccharification and ethanol production by simultaneous saccharification and fermentation (SSF) from seaweed, *Saccharina japonica*. Bioprocess and Biosystems Engineering. 2012a;35:11-8.
- Jang SS, Shirai Y, Uchida M, Wakisaka M. Production of mono sugar from acid hydrolysis of seaweed. African Journal of Biotechnology. 2012b;11:1953-63.
- Jensen A. Present and future needs for algae and algal products. Hydrobiologia. 1993;260-261:15-23.
- Jiang P, Li J, Han F, Duan G, Lu X, Gu Y, et al. Antibiofilm activity of an exopolysaccharide from marine bacterium *Vibrio* sp. QY101. PLoS ONE. 2011;6.
- John RP, Anisha GS, Nampoothiri KM, Pandey A. Micro and macroalgal biomass: A renewable source for bioethanol. Bioresource Technology. 2011;102:186-93.
- Jorge CD, Sampaio MM, Hreggvidsson GÓ, Kristjánson JK, Santos H. A highly thermostable trehalase from the thermophilic bacterium *Rhodothermus marinus*. Extremophiles. 2007;11:115-22.
- Joux F, Jeffrey WH, Lebaron P, Mitchell DL. Marine bacterial isolates display diverse responses to UV-B radiation. Applied and environmental microbiology. 1999;65:3820-7.
- Jung KA, Lim SR, Kim Y, Park JM. Potentials of macroalgae as feedstocks for biorefinery. Bioresource Technology. 2013;135:182-90.
- Kadam SU, Tiwari BK, O'Donnell CP. Extraction, structure and biofunctional activities of laminarin from brown algae. International Journal of Food Science & Technology. 2015;50:24-31.
- Kamm B, Kamm M. Biorefineries - Multi product processes. Advances in Biochemical Engineering/Biotechnology 2006. p. 175-204.
- Khairy HM, El-Shafay SM. Seasonal variations in the biochemical composition of some common seaweed species from the coast of Abu Qir Bay, Alexandria, Egypt. Oceanologia. 2013;55:435-52.
- Klinke HB, Thomsen AB, Ahring BK. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Applied Microbiology and Biotechnology. 2004;66:10-26.
- Krah M, Misselwitz R, Politz O, Thomsen KK, Welfle H, Borriss R. The laminarinase from thermophilic eubacterium *Rhodothermus marinus* conformation, stability, and identification of active site carboxylic residues by site-directed mutagenesis. European Journal of Biochemistry. 1998;257:101-11.
- Kusaykin MI, Chizhov AO, Grachev AA, Alekseeva SA, Bakunina IY, Nedashkovskaya OI, et al. A comparative study of specificity of fucoidanases from marine microorganisms and invertebrates. In: Anderson R, Brodie J, Onsøyen E, Critchley A, editors. Eighteenth International Seaweed Symposium: Springer Netherlands; 2007. p. 143-7.
- Kushner DJ. The Halobacteriaceae. The Bacteria. 1985;8:171-214.
- Lahaye M. Marine algae as sources of fibres: Determination of soluble and insoluble dietary fibre contents in some 'sea vegetables'. Journal of the Science of Food and Agriculture. 1991;54:587-94.

- Lahaye M, Robic A. Structure and function properties of Ulvan, a polysaccharide from green seaweeds. *Biomacromolecules*. 2007;8:1765-74.
- Lammens TM, Franssen MCR, Scott EL, Sanders JPM. Availability of protein-derived amino acids as feedstock for the production of bio-based chemicals. *Biomass and Bioenergy*. 2012;44:168-81.
- Lanyi JK. Salt dependent properties of proteins from extremely halophilic bacteria. *Bacteriological Reviews*. 1974;38:272-90.
- Lauro FM, McDougald D, Thomas T, Williams TJ, Egan S, Rice S, et al. The genomic basis of trophic strategy in marine bacteria. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106:15527-33.
- Leon O, Quintana L, Peruzzo G, Slebe JC. Purification and properties of an extracellular agarase from *Alteromonas* sp. strain C-1. *Applied and Environmental Microbiology*. 1992;58:4060-3.
- Li K, Liu S, Liu X. An overview of algae bioethanol production. *International Journal of Energy Research*. 2014;38:965-77.
- Lin H, Qin S. Tipping points in seaweed genetic engineering: Scaling up opportunities in the next decade. *Marine Drugs*. 2014;12:3025-45.
- Lippert K, Galinski EA. Enzyme stabilization by ectoine-type compatible solutes: Protection against heating, freezing and drying. *Applied Microbiology and Biotechnology*. 1992;37:61-5.
- Lobban CS, Harrison PJ. *Seaweed Ecology and Physiology*: Cambridge University Press; 1994.
- Lobban CS, Wynne MJ. *The Biology of Seaweeds*. 1981.
- Lü J, Sheahan C, Fu P. Metabolic engineering of algae for fourth generation biofuels production. *Energy & Environmental Science*. 2011;4:2451-66.
- Lutnaes BF, Strand Å, Pétursdóttir SK, Liaaen-Jensen S. Carotenoids of thermophilic bacteria—*Rhodothermus marinus* from submarine Icelandic hot springs. *Biochemical Systematics and Ecology*. 2004;32:455-68.
- Ma LY, Chi ZM, Li J, Wu LF. Overexpression of alginate lyase of *Pseudoalteromonas elyakovii* in *Escherichia coli*, purification, and characterization of the recombinant alginate lyase. *World J Microbiol Biotechnol*. 2008;24:89-96.
- Macleod RA, Onofrey E. Nutrition and metabolism of marine bacteria. III. The relation of sodium and potassium to growth. *Journal of cellular physiology*. 1957;50:389-401.
- Margesin R, Schinner F. Biodegradation and bioremediation of hydrocarbons in extreme environments. *Applied Microbiology and Biotechnology*. 2001;56:650-63.
- McHugh DJ. *A guide to the seaweed industry*: Food and Agriculture Organization of the United Nations Rome; 2003.
- Meinita MDN, Hong YK, Jeong GT. Detoxification of acidic catalyzed hydrolysate of *Kappaphycus alvarezii* (cottonii). *Bioprocess and Biosystems Engineering*. 2012a;35:93-8.
- Meinita MDN, Kang JY, Jeong GT, Koo HM, Park SM, Hong YK. Bioethanol production from the acid hydrolysate of the carrageenophyte *Kappaphycus alvarezii* (cottonii). *Journal of Applied Phycology*. 2012b;24:857-62.
- Michel G, Chantalat L, Fanchon E, Henrissat B, Kloareg B, Dideberg O. The α -carrageenase of *Alteromonas fortis*: A β -helix fold-containing enzyme for the degradation of a highly polyanionic polysaccharide. *Journal of Biological Chemistry*. 2001;276:40202-9.

Moreira L, Nobre MF, Sá-Correia I, Da Costa MS. Genomic typing and fatty acid composition of *Rhodothermus marinus*. Systematic and Applied Microbiology. 1996;19:83-90.

Morrice LM, McLean MW, Williamson FB, Long WF. β -Agarases I and II from *Pseudomonas atlantica*: Purifications and some properties. European Journal of Biochemistry. 1983;135:553-8.

Netalgae.eu, 2012. Portuguese Seaweed Industry. Available at <http://www.netalgae.eu/uploadedfiles/Portugal.pdf> (accessed 15 Nov. 2014).

Nolan M, Tindall BJ, Pomrenke H, Lapidus A, Copeland A, del Rio TG, et al. Complete genome sequence of *Rhodothermus marinus* type strain (R-10 T). Standards in Genomic Sciences. 2009;1:283-91.

Nunes OC, Donato MM, Da Costa MS. Isolation and characterization of *Rhodothermus* strains from S. Miguel, Azores. Systematic and Applied Microbiology. 1992;15:92-7.

Nunes OC, Manaia CM, Da Costa MS, Santos H. Compatible solutes in the thermophilic bacteria *Rhodothermus marinus* and *Thermus thermophilus*. Applied and Environmental Microbiology. 1995;61:2351-7.

Nyvall Collén P, Sassi J-F, Rogniaux H, Marfaing H, Helbert W. Ulvan lyases isolated from the flavobacterium *Persicivirga ulvanivorans* are the first members of a new polysaccharide lyase family. Journal of Biological Chemistry. 2011.

Okami Y. Marine microorganisms as a source of bioactive agents. Microbial Ecology. 1986;12:65-78.

Omar HH, Abdullatif BM, El-Kazan MM, El-Gendy AM. Red sea water and biochemical composition of seaweeds at southern coast of Jeddah, Saudi Arabia. Life Science Journal. 2013;10:1073-80.

Pan Y-Y, Wang S-T, Chuang L-T, Chang Y-W, Chen C-NN. Isolation of thermo-tolerant and high lipid content green microalgae: Oil accumulation is predominantly controlled by photosystem efficiency during stress treatments in *Desmodesmus*. Bioresource Technology. 2011;102:10510-7.

Paolucci M, Fabbrocini A, Volpe MG, Varricchio E, Coccia E. Development of biopolymers as binders for feed for farmed aquatic organisms. Aquaculture. 2011;1.

Patarra RF, Buschmann AH, Abreu MH, Neto AI. Cultivo de macroalgas nos Açores... Oportunidades e desafios. 2014.

Pereira L, Amado AM, Ribeiro-Claro PJA, van de Velde F. Vibrational spectroscopy (FTIR-ATR and FT-RAMAN), - A Rapid and Useful Tool for Phycocolloid Analysis. 2nd International Conference On Biomedical Electronics and Devices. 2009.

Petursdottir SK, Hreggvidsson GO, Da Costa MS, Kristjansson JK. Genetic diversity analysis of *Rhodothermus* reflects geographical origin of the isolates. Extremophiles. 2000;4:267-74.

Phillips GO, Williams PA. Handbook of Hydrocolloids: CRC Press; 2000.

Politz O, Krah M, Thomsen KK, Borriss R. A highly thermostable endo-(1,4)- β -mannanase from the marine bacterium *Rhodothermus marinus*. Applied Microbiology and Biotechnology. 2000;53:715-21.

Potin P, Sanseau A, Le Gall Y, Rochas C, Kloareg B. Purification and characterization of a new k-carrageenase from a marine Cytophaga-like bacterium. European Journal of Biochemistry. 1991;201:241-7.

Prokazyme, 2014. Thermostable alginate lyases. Available at <http://prokazyme.com/index.php/enzyme-dev-amylomics.html> (accessed 7 Oct. 2014).

Qin S, Jiang P, Tseng CK. Molecular biotechnology of marine algae in China. Hydrobiologia. 2004;512:21-6.

Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, et al. The path forward for biofuels and biomaterials. Science. 2006;311:484-9.

Rainbow PS. Biomonitoring of heavy metal availability in the marine environment. *Marine Pollution Bulletin*. 1995;31:183-92.

Reddy CRK, Gupta MK, Mantri VA, Jha B. Seaweed protoplasts: Status, biotechnological perspectives and needs. *Journal of Applied Phycology*. 2008;20:619-32.

Reith JH. Bio-offshore: grootschalige teelt van zeeieren in combinatie met offshore windparken in de Noordzee: ECN, Energy research Centre of the Netherlands; 2005.

Roesijadi G, Jones S, Snowden-Swan L, Zhu Y. Macroalgae as a biomass feedstock: a preliminary analysis, PNNL 19944. 2010.

Rohani-Ghadikolaei K, Abdulalian E, Ng WK. Evaluation of the proximate, fatty acid and mineral composition of representative green, brown and red seaweeds from the Persian Gulf of Iran as potential food and feed resources. *Journal of Food Science and Technology*. 2012;49:774-80.

Ruby EG, Jannasch HW. Physiological characteristics of *Thiomicrospira* sp. strain L-12 isolated from deep-sea hydrothermal vents. *Journal of Bacteriology*. 1982;149:161-5.

Sauer T, Galinski EA. Bacterial milking: A novel bioprocess for production of compatible solutes. *Biotechnology and Bioengineering*. 1998;57:306-13.

Sawabe T, Ohtsuka M, Ezura Y. Novel alginate lyases from marine bacterium *Alteromonas* sp. strain H-4. *Carbohydrate Research*. 1997;304:69-76.

Schaumann K, Weide G. Enzymatic degradation of alginate by marine fungi. In: Lindstrom S, Gabrielson P, editors. Thirteenth International Seaweed Symposium: Springer Netherlands; 1990. p. 589-96.

Schiraldi C, De Rosa M. The production of biocatalysts and biomolecules from extremophiles. *Trends in Biotechnology*. 2002;20:515-21.

Seaaroundus.org, 2014.Exclusive Economic Zones (EEZ). Available at <http://www.seaaroundus.org/eez/> (accessed 28 Jan. 2015).

Silva Z, Borges N, Martins LO, Wait R, Da Costa MS, Santos H. Combined effect of the growth temperature and salinity of the medium on the accumulation of compatible solutes by *Rhodothermus marinus* and *Rhodothermus obamensis*. *Extremophiles*. 1999;3:163-72.

Silva Z, Horta C, Da Costa MS, Chung AP, Rainey FA. Polyphasic evidence for the reclassification of *Rhodothermus obamensis* (Sako et al. 1996) as a member of the species *Rhodothermus marinus* (Alfredsson et al. 1988). *International Journal of Systematic and Evolutionary Microbiology*. 2000;50:1457-61.

Sleator RD, Hill C. Bacterial osmoadaptation: The role of osmolytes in bacterial stress and virulence. *FEMS Microbiology Reviews*. 2002;26:49-71.

Smetacek V, Zingone A. Green and golden seaweed tides on the rise. *Nature*. 2013;504:84-8.

Spilliaert R, Hreggvidsson GO, Kristjansson JK, Eggertsson G, Palsdottir A. Cloning and Sequencing of a *Rhodothermus marinus* Gene, *bglA*, Coding for a Thermostable β -Glucanase and its Expression in *Escherichia coli*. *European Journal of Biochemistry*. 1994;224:923-30.

Sydney EB, da Silva TE, Tokarski A, Novak AC, de Carvalho JC, Woiciechowski AL, et al. Screening of microalgae with potential for biodiesel production and nutrient removal from treated domestic sewage. *Applied Energy*. 2011;88:3291-4.

Sze P. *A Biology of the Algae*. WCB/McGraw-Hill ed1998.

Takeuchi K, Fujioka Y, Kawasaki Y, Shirayama Y. Impacts of high concentration of CO₂ on marine organisms; a modification of CO₂ ocean sequestration. *Energy Conversion and Management*. 1997;38:S337-S41.

Van De Velde F, De Ruiter GA. Carrageenan. Biopolymers Vol 6: Polysaccharides II: Polysaccharides from Eukaryotes. 2002;6:245-74.

Van Zyl WH, Lynd LR, Den Haan R, McBride JE. Consolidated bioprocessing for bioethanol production using *Saccharomyces cerevisiae*. Advances in Biochemical Engineering/Biotechnology 2007. p. 205-35.

Viola E. Pre-treatment of *Zostera marina* and *Ulva armoricana* for Production of Bioethanol and Biogas: ENEA; 2009.

Wang H, Ji B, Wang J, Guo F, Zhou W, Gao L, et al. Growth and biochemical composition of filamentous microalgae *Tribonema* sp. as potential biofuel feedstock. Bioprocess and Biosystems Engineering. 2014.

Wargacki AJ, Leonard E, Win MN, Regitsky DD, Santos CNS, Kim PB, et al. An engineered microbial platform for direct biofuel production from brown macroalgae. Science. 2012;335:308-13.

Wei N, Quarterman J, Jin YS. Marine macroalgae: An untapped resource for producing fuels and chemicals. Trends in Biotechnology. 2013;31:70-7.

Wong HC, Liu SH. Characterization of the low-salinity stress in *Vibrio vulnificus*. Journal of Food Protection. 2008;71:416-9.

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN. Living with water stress: Evolution of osmolyte systems. Science. 1982;217:1214-22.

Yoon H-J, Hashimoto W, Miyake O, Okamoto M, Mikami B, Murata K. Overexpression in *Escherichia coli*, Purification, and Characterization of *Sphingomonas* sp. A1 Alginate Lyases. Protein Expression and Purification. 2000;19:84-90.

Yoon M, Choi JI, Lee JW, Park DH. Improvement of saccharification process for bioethanol production from *Undaria* sp. by gamma irradiation. Radiation Physics and Chemistry. 2012;81:999-1002.

Yu S, Blennow A, Bojko M, Madsen F, Olsen CE, Engelsen SB. Physico-chemical Characterization of Floridean Starch of Red Algae. Starch - Stärke. 2002;54:66-74.

Zaccari G, Wachtel E, Eisenberg H. Solution structure of halophilic malate dehydrogenase from small-angle neutron and X-ray scattering and ultracentrifugation. Journal of Molecular Biology. 1986;190:97-106.

Zhou C, Ma H. Ultrasonic degradation of polysaccharide from a red algae (*Porphyra yezoensis*). Journal of Agricultural and Food Chemistry. 2006;54:2223-8.

Zobell CE, Morita RY. Barophilic bacteria in some deep sea sediments. Journal of bacteriology. 1957;73:563-8.