Universidade do Minho
Escola de Engenharia

Ana Isabel Freitas Baptista

Bioprocessing of macroalgae for bioactive compounds production with food and feed applications

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University of Minho
School of Engineering
Department of Biological Engineering

Ana Isabel Freitas Baptista

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Work supervised by:

PhD José Manuel Salgado

PhD Professor Isabel Belo

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I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

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Bioprocessamento de macroalgas para a produção de compostos bioativos com aplicações alimentares

RESUMO

A indústria de macroalgas atual baseia-se na produção de um único composto, como alginatos, agar ou corantes, sendo que os subprodutos são tratados como resíduo. Assim, esta indústria enfrenta o desafio de desenvolver processos que permitam obter vários produtos com atividade biológica.

A fermentação em estado sólido (SSF) é um processo biotecnológico de baixo custo que pode produzir compostos bioativos, como enzimas e compostos antioxidantes.

As macroalgas verdes como a *Ulva rigida* têm potencial para ser usadas como ingrediente em aquacultura. No entanto, estas são difíceis de digerir para muitas espécies de peixe. Neste sentido, a SSF pode alterar a estrutura de polissacarídeos para facilitar a digestão das macroalgas e pode ainda produzir uma grande variedade de produtos com aplicações alimentares como proteínas, enzimas e compostos antioxidantes.

O principal objetivo deste projeto é o processamento sequencial da *U. rigida* por SSF e hidrólise enzimática (EH) para produzir produtos de valor acrescentado e aumentar o valor nutricional das macroalgas, promovendo assim uma economia circular. O passo da hidrólise enzimática foi otimizado pelo desenho experimental Box-Behnken.

Durante a SSF da *U. rigida* produziram-se celulases (40 ± 1 U/g) e xilanases (160 ± 4 U/g). Após a SSF, foi adicionado tampão para iniciar a EH que durou 72h. A variável que teve um maior efeito na libertação de compostos fenólicos, açúcares, atividade antioxidante e aumento da concentração de proteína foi a temperatura. A concentração máxima de compostos fenólicos e atividade antioxidante atingiu-se a uma temperatura intermédia (40°C), a conversão máxima de celulose a glucose e aumento da concentração de proteína atingiram-se à temperatura mais elevada (44°C). As condições ótimas da hidrólise enzimática para maximizar em conjunto as 4 variáveis foram 44°C, carga de sólido 30% w/v e pH 4,1. Nestas condições, atinge-se teoricamente 929 μM de equivalentes de Trolox/g, 1,56 mg de compostos fenólicos totais/g, 231,04 g de proteína/kg e 61% de conversão de celulose para glucose. Em todas as experiências, verificou-se uma diminuição da atividade da xilanase durante a hidrólise enzimática (72h), sendo que a redução foi menor nas experiências realizadas a menor temperatura (35°C).

O bioprocessamento da *U. rigida* por SSF e EH permitiu a obtenção de compostos antioxidantes, açúcares livres que podem ser fermentados noutros produtos de valor acrescentado ou energia e um sólido final enriquecido em proteína. No futuro, devem ser realizadas experiências de modo a aplicar estes produtos na aquacultura.

Palavras-chave: Bioprocessos; Fermentação em estado sólido; Hidrólise Enzimática; Macroalgas

Bioprocessing of macroalgae for bioactive compounds production with food and feed applications

ABSTRACT

The current seaweed industry is based on a single compound production, as alginates, carrageenan, agars, or colorants, being the remaining seaweed byproduct treated as waste. Thus, macroalgae industry faces the challenge of developing processes allowing to obtain multi-products with biological activities. Solid-state fermentation (SSF) is a low-cost biotechnology process that can produce bioactive compounds as enzymes and antioxidant compounds.

Green macroalgae as *Ulva rigida* have potential to be used as ingredient in aquaculture. However, they are difficult to digest by many species of fish. In this sense, SSF can alter the structure of polysaccharides to facilitate digestion of macroalgae, and it can also produce a wide variety of valuable products for feed applications, such as proteins, enzymes, and antioxidant compounds.

The main aim of this project is the sequential bioprocessing of *U. rigida* by SSF and enzymatic hydrolysis (EH) to produce value-added products and increase the nutritional value of macroalgae, promoting a circular economy. The EH stage was optimized by Box-Behnken experimental design.

During SSF were produced cellulases (40 ± 1 U/g) and xylanases (160 ± 4 U/g). After SSF, it was added the buffer to carry out EH during 72h. The variable that had a higher effect on release of phenolic compounds, sugars, antioxidant activity and increase the concentration of protein was the temperature. Maximum concentration of phenolic compounds and antioxidant activity was achieved with intermediate temperature (40°C), the maximum conversion of cellulose to glucose and increase of protein concentration were achieved with the higher temperature (44°C). The optimal conditions of EH to maximize jointly the 4 variables were 44°C, load of solid 30% w/v and pH 4,1. In these conditions, they were predicted an antioxidant activity of 929 μM of Trolox equivalents/g, 1,56 mg of total phenolic compounds U/g, 23,.04 g of crude protein/kg and 61% cellulose conversion to glucose. In all experiments it was observed a decrease of xylanase activity during EH, the reduction was lower in experiments performed with the lowest temperature (35°C).

The bioprocessing of *U. rigida* by SSF and EH allowed to obtain antioxidant compounds, free sugars that can be fermented to other value-added products or energy, and the final solid was enriched in protein. Future works should be performed to apply these products in aquaculture.

Key Words: Bioprocess; Enzymatic Hydrolysis; Macroalgae; Solid-state fermentation

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A α_w - water activity

C CGC_{max} – conversion of cellulose to glucose maximum

D DNS - dinitrosalicylic acid

DPPH - 2,2-Diphenyl-1-picrylhydrazyl

E EH – Enzymatic Hydrolysis

F FTIR - Fourier-Transform Infrared Spectroscopy

G GRAS – Generally Regarded As Safe

H HPLC - High Performance Liquid Chromatography

M MUM - Micoteca da Universidade do Minho

P PDA - Potato Dextrose Agar

PUFAs - long-chain polyunsaturated fatty acids

Q QAH - Quantitative Acid Hydrolysis

R rpm – rotations per minute

S SD - standard deviation

s:l - solid : liquid

SmF - Submerged Fermentation

SSF - Solid State Fermentation

T TPC - Total Phenolic Compounds

U USD - United States Dollar

W w/v - weight/volume

wt – weight

w/w – weight/weight

CONTEXT AND MOTIVATION

Macroalgae are photosynthetic organisms and they are more efficient in this process than terrestrial plants. Also, macroalgae grow at fast rates and can reach large sizes, which, along with the capacity to generate and store carbon resources causes that macroalgae are beginning to be regarded as an inexpensive and interesting biomass to be used as a substrate in biorefinery processes. Although there is yet a lot that can be studied with these plants, macroalgae have the potential to be refined into fractions, that can be used for a wide range of interesting bioactive compounds, for biofuel production and chemicals.

Solid-state fermentation (SSF) is an underexplored process, in the presence of macroalgae. This technology can process biomass into a spectrum of value-added products and allows to exploit all the raw material without producing wastes or producing less than is usually produced in submerged fermentation.

Thus, SSF with macroalgae as a substrate can be considered a biorefinery process, that will allow to produce and to extract value-added compounds and to use the remainder fermented macroalgae as fish feed, developing a circular economy.

AIMS

The global objective of this master's project is to bioprocess green macroalga (*U. rigida*) by sequential SSF and EH to produce value-added products as antioxidant phenolic compounds, release free sugars and increase the nutritional value of macroalgae to be used as fish feed in aquaculture, promoting a circular economy. It is intended to determine the optimal conditions of EH stage by Box-Behnken experimental design, evaluating different conditions to maximize the extraction of antioxidant compounds, free sugars and to increase protein content of macroalgae.

1. INTRODUCTION

1.1. Macroalgae

Macroalgae - also called seaweeds - have great potential, however, only recently have they been attracting more attention from researchers. This is mainly due to a number of favorable characteristics, such as their large biomass yields, fast growth rates and low needs of freshwater and terrestrial land for cultivation (Kostas, White and Cook, 2017; Fernandes *et al.*, 2019). These are chlorophyll containing organisms, which means that they can photosynthetically convert atmospheric carbon dioxide into a variety of metabolites and organic molecules (Sambusiti *et al.*, 2015). In contrast with terrestrial plants, macroalgae have, in average, a much higher photosynthetic efficiency (between 6 and 8 % for macroalgae, but only from 1.8 to 2.2% for terrestrial plants) (Chen *et al.*, 2015). Another advantage of macroalgae when compared to terrestrial plants is the low quantity of lignin they have (most of the times, lignin is absent), which dispenses the need for intensive pre-treatments prior to fermentation, meaning reduced costs and less energy spent (Trivedi *et al.*, 2015).

Furthermore, they can grow in a wide variety of environments, including fresh, salt, temperate and municipal wastewater (Masri *et al.*, 2018) and they constitute approximately 50% of biomass on Earth (Barbot, Al-Ghaili and Benz, 2016). As such, macroalgae potentially represent a significant source of renewable energy and a primary source of natural products (Ross *et al.*, 2008; de Almeida *et al.*, 2011), which makes this an interesting organism to exploit in biorefinery processes.

Their metabolism can differ accordingly to certain parameters, such as the water temperature, salinity, light, or available nutrients. This forces macroalgae to quick adaptions to new environmental conditions, which makes them produce a wide variety of secondary metabolites with biological activity (Rodrigues *et al.*, 2015). Some of the compounds that macroalgae might synthetize are carotenoids, terpenoids, vitamins, saturated and polyunsaturated fatty acids, antioxidants and polysaccharides, such as agar (de Almeida *et al.*, 2011). Their growth is influenced by the presence of dissolved nutrients in water, specifically, nitrogen, phosphorous and iron and their optimal growth temperature ranges from below 15°C (*Ascophyllum* spp. found in Northern hemisphere) to 25°C (*Ulva pertusa*, found in Japanese coastline) (Barbot, Al-Ghaili and Benz, 2016).

When it comes to structure, macroalgae are simple multicellular organisms, with simple reproductive structures, and they do not have advanced structures such as the ones present in most terrestrial plants like roots, stems, leaves or vascular tissue. Instead, they have a blade that is leaf-like, a stipe that is stem-like and a holdfast that matches roots in terrestrial plants (de Almeida *et al.*, 2011).

Some species reproduce asexually by means of vegetative growth, which means that new individuals will be genetically identical to their parent (Sudhakar *et al.*, 2018). However, the life cycles of macroalgae are diverse and they have combinations of sexual and asexual reproductive strategies (Roesijadi, Jones and Zhu, 2010).

According to the Food and Agriculture Organization of the United Nations (FAO), the annual production, in 2010, of marine macroalgae was over 16 million tons and over 96% of that production is from aquaculture. This production is valued in USD 7 billion, corresponding to a little over 6 billion Euros, however, to obtain the total value of the seaweed industry, it is necessary to consider the added value products obtained after processing of macroalgae (*aquaCase*, no date). Five years later, by 2015, total production almost doubled, achieving 30.4 million tons (Ferdouse *et al.*, 2018). By 2017, the commercial seaweeds market was valued at USD 13.07 billion - that is, 11.6 billion euros - and is expected to be above 18.5 billion euros by 2023 (*Markets and Markets*, no date). The *Laminaria*, *Undaria*, *Porphyra*, *Eucheuma/ Kappaphycus* and *Gracilaria* genera account for approximately 98% of world seaweed production (Pereira and Yarish, 2008). The East Asian countries are world's greatest contributors on algal biomass - they accounted for 95% of the world's supply in 2010 (Jung *et al.*, 2013). The more recent estimates suggest the existence of 72500 species worldwide (Guiry, 2012).

1.1.1. Types of macroalgae

There are three major groups of macroalgae, in a classification based on their photosynthetic pigmentation: *Chlorophyta* (green pigments), *Rhodophyta* (red pigments) and *Phaeophyta* (brown pigments) (Chen *et al.*, 2015). Their distribution depends on environmental factors, with emphasis on the quantity and quality of the sunlight (Sudhakar *et al.*, 2018).

Starting with *Chlorophyta*, or green algae, there are around 700 to 7000 species and they occur mainly in bays, estuaries, and tide pools. *Chlorophyta* algae have simple thallus and are characterized by filamentous spongy fingers or paper-thin sheets. This group of macroalgae has, as major photosynthetic pigments, chlorophylls *a* and *b* – with the same ratio of chlorophyll *a* to *b* as land plants – and carotenoids, such as carotene and xanthophylls (Jung *et al.*, 2013; Sudhakar *et al.*, 2018; Leandro, Pereira and Gonçalves, 2020). Some of the most relevant species in this group are *Halimeda*, *Ulva* and *Codium* (Sudhakar *et al.*, 2018).

As for *Rhodophyta*, the group of red algae, they grow as filaments or sheets of cells. One interesting characteristic of *Rhodophyta* is that they can be parasites of other algae. This is the most abundant and most widespread group of algae, with records of 4000 species that can live either in deep

cold waters or warm shallow waters. The photosynthetic pigments that can be encountered in the *Rhodophyta* group are chlorophyll *a* and phycobilins (phycoerythrin and phycocyanin), which is responsible for their red color (Jung *et al.*, 2013; Sudhakar *et al.*, 2018; Leandro, Pereira and Gonçalves, 2020).

Lastly, the group of brown algae, *Phaeophyta*, comprises around 1500 species and they occur mainly in temperate and polar locations (North America, Europe, mid-Atlantic and Gulf of Mexico). Also, they can be found in rocky shores and this type of algae have preference for shallow and cold waters. These macroalgae species can grow up to 100 meters and are the ones that have the most complex thallus structure (Sudhakar *et al.*, 2018). The main photosynthetic pigments present in this type of algae are chlorophyll *a* and *c*, β-carotene and other xanthophylls (Jung *et al.*, 2013). Some of the most important species that belong to the *Phaeophyta* group are *Laminaria* and *Saccharina* (Sudhakar *et al.*, 2018).

The *Rhodophyta* and the *Phaeophyta* groups seem to be more present in Portuguese seas than *Chlorophyta* (*seaExpert*, no date; Rodrigues *et al.*, 2015).

1.1.2. Chemical composition of macroalgae

The chemical composition can vary between the different groups of macroalgae and it is influenced by seasonality and geographic locality (Wan *et al.*, 2019). Also, it can be influenced by the conditions in their habitat such as light, temperature, salinity, nutrients, and pollution. However, in every case, they contain high amounts of carbohydrates (up to 60%) and lower amounts of protein (around 10%) and even lower amounts of lipids (up to 3%).

The composition of the three groups of macroalgae regarding water content, carbohydrates, proteins, and lipids is presented in **Table 1**.

Table 1 - Composition of macroalgae (green, red and brown).

Compound	Green algae	Red algae	Brown algae	Reference
Water content (fresh mass)	70-85%	70-89%	79-90%	(Barbot, Al-Ghaili and Benz, 2016)
Carbohydrates (dry weight)	25-50%	30-60%	30-50%	(Jung *et al.*, 2013)
Protein (dry weight)	10-26%	35-47%	7-12%	(Miranda, Lopez-Alonso and Garcia-Vaquero, 2017)
Lipids (dry weight)	2-3%	0-3%	0-2%	(Jard *et al.*, 2013)

There is a large variety of carbohydrates present in macroalgae: alginate – that provides stability and flexibility - and cellulose are common in all types of macroalgae. Due to its abundance, easiness of processing and variety of applications, cellulose has been getting a lot of attention from researchers and macroalgae have been considered a potential source of cellulose (Siddhanta *et al.*, 2009). Also, in green algae we can encounter mannan, starch and ulvan; red algae contain agar, carrageenan and lignin and brown algae contain agar, laminarin, cellulose and others (Barbot, Al-Ghaili and Benz, 2016; Miranda, Lopez-Alonso and Garcia-Vaquero, 2017). The quantity of lignin is generally lower in macroalgae in comparison with terrestrial plants and its absence is important to microbial decomposition (Barbot, Al-Ghaili and Benz, 2016).

Regarding lipids, macroalgae contain a significant amount of PUFAs (long-chain polyunsaturated fatty acids). The content of unsaturated fatty acids is proportional to the potential antioxidant activity, as shown in a study with lipophilic extracts from 16 species of seaweeds (Huang and Wang, 2004). However, phenolic compounds are usually claimed to be the major active constituents responsible for the antioxidant activity of macroalgae. These phenolic compounds are highly present in green and brown macroalgae, which could be the reason of lower content in protein comparing to red algae (Kumar *et al.*, 2008).

Another major constituent of macroalgae are pigments, such as chlorophylls, carotenoids, phycobilins and xanthophylls and their abundance depends on the type of macroalgae as discussed in the above section.

As for micro-nutrients, macroalgae have high concentrations of minerals, such as calcium, magnesium and potassium, as well as glutamic acid (Barbot, Al-Ghaili and Benz, 2016). They also have high contents in iodine, being *Laminaria* spp. the best iodine accumulator among all living systems (Miranda, Lopez-Alonso and Garcia-Vaquero, 2017). Some heavy metals can also be found, such as arsenic and mercury, but the amount does not seem to pose any threat to the consumers' health (Garcia-Vaquero and Hayes, 2016).

Seasonal environmental changes can influence macroalgae's composition; during summer, they produce higher amounts of volatile solids – amount of organic substance in the solid fraction - and sugars whereas in spring they show higher content in proteins and minerals (Barbot, Al-Ghaili and Benz, 2016).

Ulva species, a green type of macroalgae, are listed in FAO as one of the main macroalgae for commercial use. This macroalgae is very common and can be found in marine and brackish waters, being widely distributed across the world. Also, *Ulva* species can be successfully product in an aquaculture environment (Lopes *et al.*, 2019).

In what concerns its composition, *Ulva* species are a major source of polysaccharides and oligosaccharides and, despite the lipidic profile not being intensively studied, they also represent an important nutritional role with major importance for PUFAs (Satpati and Pal, 2011; Lopes *et al.*, 2019). This species has also particular interest as a source of antioxidants and phenolic compounds and the amount of phenolic compounds positively correlates with the radical-scavenging activity, suggesting that phenolic compounds are the major contributor acting as free radical terminators (Mezghani *et al.*, 2016).

1.1.3. Biorefinery of macroalgae

Biorefineries transform renewable biomass into biofuels, food, chemicals, and other bio-based products. On simple terms, biorefineries are the operating units of bio-economies (Zollmann *et al.*, 2019). The main ideas in which a biorefinery process is based are the sustainable and renewable energy supply, saving foreign exchange reserves, reducing dependency on imported crude and other chemicals and the establishment of a circular economy. The final goal is to generate added value products, with benefits for the economy and the environment. While most researchers are focused on single-feedstock and single-product, one more advantageous approach is the co-production of multiple products from the same biomass, because this type of process leads to complete use of the raw material, close to zero-waste and maximum material valorization (Kazir *et al.*, 2019; Zahra *et al.*, 2019). The use of all content to produce high-value products makes the biorefinery process more profitable and sustainable, since it is increasing the biorefinery's global economic performance (Zahra *et al.*, 2019).

Due to limited crop yields and land availability, the future development of the biomass sector is largely uncertain (Zahra *et al.*, 2019). In this sense, macroalgae are excellent feedstocks [i.e., raw materials that are used in biorefineries (Cherubini, 2010)] since they have, not only high value components, but also compounds that are considered platform chemicals for the bio-based economy (Kostas, White and Cook, 2017). The fact that biorefineries based on terrestrial biomass are not sustainable at present due to environmental as well as economic issues enhances the potential of macroalgae (Jung *et al.*, 2013).

Several seaweed biorefinery processes have already been investigated. Sequential recovery of four fractions with economic interest – a liquid extract, containing nutrients suitable for use as food supplements, a lipid fraction, ulvan and finally a cellulose fraction – was reported by Trivedi *et al.* (Trivedi *et al.*, 2016). In a study using *Chaetomorpha linum*, the authors demonstrated the feasibility of the co-production of biogas and bioethanol, a process with low production of waste (Ben Yahmed *et al.*, 2016). Kostas, White and Cook reported the use of *Laminaria digitata*, a brown seaweed, to successfully produce bioethanol from the residues which remained after the extraction of two valuable polysaccharides (Kostas, White and Cook, 2017). Also, a lot of different products can be obtained from the residual algal biomass including products with application in food/feed, pharmaceutical, nutraceutical and cosmeceutical industries (Suganya *et al.*, 2016).

1.1.4. Applications of macroalgae

Seaweeds have a wide range of applications, being the more traditional ones the commercialization as food and soil fertilizer (van der Wal *et al.*, 2013). Nowadays, they have a wide range of applications and the components extracted from the macroalgae can potentially be applied in the food, medical and pharmaceutical industries, in the environmental fields, among others.

Macroalgae are valuable as a food resource as they are rich in vitamins, minerals, proteins, polysaccharides, and dietary fibers and are low in calories. Phycocolloids such as agar-agar, carrageenan and alginic acids that are present in brown and red algal cell walls are widely used in food industries (de Almeida *et al.*, 2011). Both agar-agar and carrageenan have gelling, thickening, and stabilizing properties, which allows them to be used as substitutes for gelatin and in dairies, respectively (Pangestuti and Kim, 2015). Also, algae can be applied in aquaculture, ruminant and swine feed industries (Miranda, Lopez-Alonso and Garcia-Vaquero, 2017).

In the medical and pharmaceutical field, macroalgae have also demonstrated to be interesting, as has been proved that various macroalgae have antibacterial, antifungal, and antiviral activities (Smit, 2004; de Souza Barros, Teixeira and Paixão, 2015). Besides that, some components present in macroalgae can be used for their antioxidant and anti-inflammatory potential, which is the case of some polysaccharides (Ananthi *et al.*, 2010). Some other examples of compounds with potential to be used in the pharmaceutical field are fucoxanthin that could have applications in cancer treatment, because it can induce cell cycle arrest and apoptosis, and also laminarin, because for its nutritional value it can play a role in prevention (Fleurence and Levine, 2016).

When it comes to the environmental field, besides being used as a fertilizer since ancient times, it can also be used to control pollution. It has even been developed a device, in 2011, called algal turf scrubber, which absorbs nutrients and is used to help filter aquaria and ponds (HydroMentia, no date).

Lastly, macroalgae can also be applied to produce biofuels (Chen *et al.*, 2015), in bioremediation processes (Sode *et al.*, 2013) and cosmetics – moisturizing care, photoprotection – and additives for cosmetics – preservatives, essential oils, antioxidants, dyes (Guillerme, Couteau and Coiffard, 2017).

1.1.5. Wastes from macroalgae processing

In the many processes, which use macroalgae for the applications mentioned above, there are, often, residues or wastes produced in the course.

For example, in the process of phycocolloid extraction there remains the cell wall as residue, in which can be found some impurities such as sand, salts and calcareous deposits, as well as sulfolipids, pigments, nucleic acids, other polysaccharides as cellulose, which can be used in chemical, pharmaceutical and fuel industries (López-Simeon *et al.*, 2012). Studies have also shown that after the extraction of some polysaccharides, such as agar and alginates, there remains a pulp containing high amounts of carbohydrates, proteins, lipids, and ash (Zahra *et al.*, 2019).

Currently seaweed wastes are used to produce fiber, glycerol, biofertilizers and organic acids but not exclusively. The residues from the alginate industry have also been used for the elimination of toxic heavy metals and for biomethane conversion. Also, make use of seaweed wastes to produce biomethane has already been tested and the results were promising (Barbot, Al-Ghaili and Benz, 2016).

In general, the quality of the wastes and, therefore, the application they can further have, depends on the initial composition of the macroalgal biomass and on processing done. For example, residues from phycobilin extraction have a high percentage of volatile solids and a lower percentage of ash (75% and 21%, respectively) while the remains from industrial biomass processing of *Laminaria japonica* have

almost the same percentage of volatile solids and ash (50,9% and 49,1%, respectively) (Barbot, Al-Ghaili and Benz, 2016).

However, it is possible to reduce the quantity of waste produced since one can co-extract additional valuable materials in the initial macroalgae processing, rather than treating them as waste (Kazir *et al.*, 2019; Zahra *et al.*, 2019).

1.2. Solid-state fermentation

Solid-state fermentation (SSF) is a cost-effective bioprocess technology, with potential applications in a variety of areas, such as the feed, food, fuel and chemical industries, but also the production of pharmaceutical products and biologically active secondary metabolites – for example, pigments and antibiotics (Thomas, Larroche and Pandey, 2013; Singhania *et al.*, 2015). SSF systems seem to be promising to produce value-added products, such as biopharmaceuticals. Also, this technology has been used for the development of bioprocesses for instance bioremediation and biodegradation of hazardous compounds and biological detoxification of agro-industrial residues (Pandey, 2003).

SSF is a three-phase heterogeneous process, composed by solid, liquid and gaseous phases (Costa *et al.*, 2018). It is a fermentation process that occurs in the absence, or near absence, of free water; however, the solid substrate must contain enough moisture to support the microorganism's growth and metabolic activity (Hölker, Höfer and Lenz, 2004; Thomas, Larroche and Pandey, 2013; Cerda *et al.*, 2019). The solid matrix where the process occurs can either be the source of carbon or an inert material to support the microorganisms' growth (Salgado *et al.*, 2014a; Oliveira *et al.*, 2016; Costa *et al.*, 2018).

SSF offers many advantages when compared with classic submerged fermentation (SmF), in which the microorganisms grow in liquid medium, with high contents of free water (Soccol *et al.*, 2017; Wang *et al.*, 2019). SSF has lower energy requirements and higher productivities, produces lesser wastewater, the products have extended stability, the production costs are lower, and it is less prone to problems with substrate inhibition, hence it allows higher final concentration of product (Pandey, 2003; Hölker, Höfer and Lenz, 2004; Hölker and Lenz, 2005; Barrios-González, 2012; Soccol *et al.*, 2017; El-Mansi *et al.*, 2019; Wang *et al.*, 2019). Also, when it comes to environmental related issues, the fact that SSF is conducted in near absence of free water results in minimum water consumption and a low production of effluent water in the process. The fact that SSF is performed at low water activities, reduces

the growth of contaminating bacteria and yeasts, thus, in certain cases, semi-sterile conditions may be applied, reducing the energy needed for sterilization (Hölker and Lenz, 2005; Soccol *et al.*, 2017). Yet another benefit regarding the sustainability of the process is the utilization of low-cost agro-industrial residues as carbon and energy sources (Hölker and Lenz, 2005; Thomas, Larroche and Pandey, 2013). The advantages cited can bring direct economic advantages, therefore the economic efficiency is higher for SSF than SmF (Hölker and Lenz, 2005; Soccol *et al.*, 2017).

Several studies have been conducted comparing both types of fermentation. In a study conducted by Díaz-Godínez *et al.*, 2001, the production of exopectinases by *Aspergillus niger* in SSF and SmF was reviewed, and the research concluded that the production of biomass in SSF was higher, independently of the variables tested. Also, the exopectinase production was enhanced by using SSF over SmF. The SSF process may offer advantages in terms of enzyme activity because of reduced proteolysis (Díaz-Godínez *et al.*, 2001).

One other interesting factor of SSF is that it provides the cultivated microorganisms an environment as close as possible to their natural habitat, from where they were isolated (Hölker, Höfer and Lenz, 2004; Thomas, Larroche and Pandey, 2013; Oliveira *et al.*, 2017). This seems to be the main factor behind the higher productivity yields in SSF when compared with SmF, even if optimal conditions for growth are used (Thomas, Larroche and Pandey, 2013).

Nevertheless, the SSF process has also some disadvantages, such as problems with heat build-up, difficulties in controlling process parameters (like pH, temperature, moisture), difficulties on scale-up and higher impurity of the product (Hölker, Höfer and Lenz, 2004; Couto and Sanromán, 2006).

1.2.1. Macroalgae as solid substrate in SSF

In the beginning of this sector, it was mentioned that "the substrate for SSF must contain enough moisture to support the microorganism's growth and metabolic activity". As we have seen in section 1.1.2, macroalgae's water content is over 70%, almost in every type of algae, meaning that macroalgae are a suitable substrate for SSF (Barbot, Al-Ghaili and Benz, 2016). Besides that, the substrates commonly contain some macromolecular structure, such as cellulose, starch, lignocellulose, or fibers, so the algae's composition is adequate as well (El-Mansi *et al.*, 2019). Also, many other characteristics cited in this report for macroalgae make this an appropriate substrate, for example, fast growth rates, high photosynthetic efficiency and large quantity of carbohydrates, minerals, and amino acids. Macroalgae are a valuable feedstock as they serve as both physical support and nutritional source, allowing for biofuel, biochemical and biometabolites production (General *et al.*, 2014; Fernandes *et al.*, 2019). The fact that

algal biomass is scarcely used as food source and that it is rich in carbohydrates, proteins and lipids makes it a valuable source for the growth of fungal cultures (General *et al.*, 2014).

The microorganisms that have been used together with macroalgae in SSF, as well as products that have already been produced when using macroalgae as a substrate will be reported further ahead.

1.2.2. Microorganisms used in SSF

It was already mentioned before that not all microorganisms are suitable for use in SSF. This is because this process is performed in near absence of free water, which was reported as an advantage for this process because it diminishes contamination, however it limits the organisms that can be used. In this sense, fungi and yeast are usually considered suitable organisms for SSF while bacteria have not been so commonly used in this process. This is because fungi and yeast have lower water activity (α_w) requirements, typically around 0,5-0,6 α_w, whilst bacteria have water activity requirements around 0,8-0,9 α_w (Costa *et al.*, 2018). However, several studies have proved that bacterial cells can be manipulated and managed for SSF processes (Gupta *et al.*, 2008; Mukherjee, Adhikari and Rai, 2008). Besides that, in general, filamentous fungi adapt better to a solid substrate, because the hyphal growth allows the fungi to better penetrate the substrates (Graminha *et al.*, 2008; El-Mansi *et al.*, 2019). The choice of the microorganism seems to be apparently linked with the selection of the substrate and the product that is intended (Costa *et al.*, 2018).

Some of the bacterial genera that have been studied in SSF processes are *Bacillus* sp., *Pseudomonas* sp. and *Streptococcus* sp. For example, different strains of *Burkholderia* have been used to produce biodiesel, while *Pseudomonas aeruginosa* has already been used, having as final applications, flavor, and fragrance esters (Liu *et al.*, 2014; Aguieiras *et al.*, 2018). In the case of the fungi, many more species can be nominated, namely species of *Rhizopus*, *Aspergillus*, *Penicillium*, *Yarrowia* and *Candida* (Couto and Sanromán, 2006; Aguieiras *et al.*, 2018).

After choosing the microorganism, is important to consider the identification of the physiology of the microorganism and the physico-chemical factors it needs to grow, including temperature, pH, aeration, water, activity, moisture and so on. When it comes to moisture, for the reasons presented before, fungi have lower requirements and, generally, approximately 40 to 60 % is enough moisture to cultivate fungi (Costa *et al.*, 2018).

The microorganisms that have already been used together with macroalgae in this fermentation process are mainly fungi, for instance *Aspergillus niger*, *Cladosporium sphaerospermum*, *Clostridium*

acetobutylicum and *Clostridium beijerinckii* (van der Wal *et al.*, 2013; Trivedi *et al.*, 2015; Fernandes *et al.*, 2019).

Aspergillus ibericus

Aspergillus ibericus is a species form the black aspergilli group that was isolated from wine grapes from Portugal and Spain. It does not produce mycotoxins and has GRAS (generally regarded as safe) status (Serra *et al.*, 2006; Oliveira *et al.*, 2016; Fernandes *et al.*, 2019). Its hyphal growth allows the fungi to effectively penetrate the substrate, thus allowing better growth conditions under SSF (Salgado *et al.*, 2014b; Fernandes *et al.*, 2019). The *Aspergillus* species are among the fungi species that have been reported as very efficient for different enzymes production (Serra *et al.*, 2006; Oliveira *et al.*, 2016).

Aspergillus ibericus is a good producer of enzymes, such as cellulase and xylanase, under SSF processing and also oxidative phenolic compounds (Leite *et al.*, 2016; Sousa *et al.*, 2020; Treichel *et al.*, 2020). It has also been used to produce lipase and to improve the protein content in fermented solids (Salgado *et al.*, 2014b; Oliveira *et al.*, 2016; Sadh, Duhan and Duhan, 2018; Sousa *et al.*, 2018).

1.2.3. Products obtained by SSF

Some applications of SSF have already been referred, such as in feed, food, fuel and chemicals industries, the production of biologically active secondary metabolites or pharmaceutical products and the development of bioprocesses. Numerous studies have already been published reporting a significant number of products that can be produced by SSF. Amongst them, we can encounter a large number of enzymes such as cellulase, phytase, amylase, protease, lipase and many more (Trivedi *et al.*, 2015; Cerda *et al.*, 2016; Leite *et al.*, 2016; Novelli, Barros and Fleuri, 2016; Costa *et al.*, 2018) and secondary metabolites and bioactive compounds, such as organic acids such as lactic acid, citric acid or bio-ethanol; antibiotics and other metabolites (Kumar *et al.*, 2003; Barrios-González, 2012; Singhania *et al.*, 2015; Costa *et al.*, 2018). Also, the use of SSF to improve the nutritional value of agricultural residues, specifically protein enrichment, has been gaining attention, because it represents a potential solution to produce cheaper animal feed (Graminha *et al.*, 2008). The enrichment of protein content by SSF, along with the increase of antioxidant activity, was achieved by Kupski *et al.* in rice bran (Kupski *et al.*, 2012). There are many other studies that attained an increase of antioxidant activity by SSF and this may happen because of the release of phenolic compounds from macromolecules such as polysaccharides (Magro *et al.*, 2019).

There are also several processes reported which use macroalgae as a substrate for SSF. General *et al.*, used *Laminaria japonica* as a substrate for a fungus that can be isolated from indoor house dust (Villanueva-Lozano *et al.*, 2017) in SSF and they were able to obtain natural red and yellow pigments, which can be used in the industries of food, cosmetics and pharmaceuticals with various applications (General *et al.*, 2014). In a different sector, Trivedi *et al.* used a marine fungus in the process of SSF to obtain cellulases, which were then used for its saccharification potential with *Ulva fasciata* to finally produce bioethanol (Trivedi *et al.*, 2015). Also using *Ulva*, but a different species – *rigida* – the process of SSF was used to produce lignocellulolytic enzymes, having as final goal the application has aquaculture feed (Fernandes *et al.*, 2019). The use of one substrate in SSF process can lead to a wide variety of value-added products, because it can be combined with different operational conditions and different strains of microorganisms (Marín, Sánchez and Artola, 2019).

The studies mentioned show the large spectrum of applications that macroalgae and SSF can have, contributing, in most cases, to the development of a circular economy.

Enzymes

The SSF process is cost-effective and it requires low-cost substrates, and, because of that, it is becoming a more common choice for enzymes production for its several benefits such as enzyme titer, low labor cost and lower capital input (Singhania *et al.*, 2015). Besides, the amount of microbial enzymes produced during SSF with filamentous fungi commonly exceeds that produced by SmF (Barrios-González, 2012).

One type of enzymes being, most commonly, produced by SSF are cellulases (Yoon *et al.*, 2014; Singhania *et al.*, 2015; Fernandes *et al.*, 2019). This enzyme is involved in the hydrolysis reaction of the β-1,4-glycosidic linkage in cellulose, which is a dominant component of the plant cell wall and is commonly present together with hemicellulose and lignin. Cellulases are on top three of the most profitable enzymes, mainly due to a wide variety of applications in the industries of pulp and paper, textile, food and animal feed, beverages and detergents and can also be important in biofuels production (Yoon *et al.*, 2014; Cerda *et al.*, 2019; Marín, Sánchez and Artola, 2019). It was already reported that filamentous fungi are better producers of cellulase on SSF than on SmF and, additionally, a study conducted by Tewalt and Schilling that the cellulases produced on SSF by a specific species of fungi had better performance in hydrolysis efficiency, compared to those produced by SmF (Shrestha *et al.*, 2010;

Tewalt and Schilling, 2010). Among the fungal strains that produce cellulases, *Aspergillus*, *Trichoderma*, *Penicillium* and *Fusarium* genera stand out (Brijwani, Oberoi and Vadlani, 2010).

Another type of interesting enzymes are xylanases, which are glycosidases and catalyze the hydrolysis of 1,4-β-D-xylosidic linkages in xylan (Salgado *et al.*, 2014b). These enzymes can have applications in food and beverage industries, feedstock improvement by improving animal feed digestibility and improvement of quality of lignocellulosic residues (Pal and Khanum, 2010). Xylanase production in SSF processes is mainly reported using *Aspergillus* species (Salgado *et al.*, 2014b; Cerda *et al.*, 2019). As reported for cellulase, the strains that are better producers of this enzyme, have better performances under SSF (Ang *et al.*, 2013).

Enzymatic preparations containing both these enzymes, with the main function being the use in pulp, paper, fuel, and other chemical industries, have also been used as food additives in ruminant feed to aid in feed digestion (Graminha *et al.*, 2008).

1.3. Macroalgae in fish diets in aquaculture

In aquaculture, nutrition is one of the most important parameters, because it usually represents about 50% of the production cost. Furthermore, protein is the most expensive component in fish feed (Craig, Kuhn and Schwarz, 2017).

The inset of macroalgae as an ingredient for aquaculture derives from the current pursuit for new economically competitive ingredients (Tacon, Hasan and Metian, 2011). Because macroalgae have the capacity to produce a wide variety of secondary metabolites, which have a wide variety of biological properties - such as antioxidant, anti-bacterial or anti-tumoral and many more – they have great potential to be used in animal feed or to have their biologically active compounds extracted to be turn macroalgae species into potential novel feed ingredients in aquaculture. Some studies already reported the results for the incorporation of macroalgae in fish's diet. Some of the benefits reported were improved growth rates, metabolic rates enhanced and was verified an increase in certain beneficial compounds, such as pigments or iodine (Miranda, Lopez-Alonso and Garcia-Vaquero, 2017).

1.3.1. Nutritional components of macroalgae for aquafeeds

As we have seen before, macroalgae's composition varies accordingly to the type of macroalgae, the season of harvest, geographic location, and environmental conditions. The sampling methodology could also affect the results when determining macroalgae's chemical composition (Patarra *et al.*, 2011).

However, in every case, a macroalgae based diet has as main constituents' carbohydrates, followed by proteins and lipids (Amaro *et al.*, 2019).

There are two possible types of diets that fish farmers use, however most of them use complete diets. These are typically made up of: 18-50% protein; 10-25% lipids; 15-20% carbohydrates; <8.5% ash; <1.5% phosphorus; <10 % water; and trace amounts of vitamins and minerals (Craig, Kuhn and Schwarz, 2017), which is similar to the composition of macroalgae, in general.

Macroalgae's protein content is very high – red macroalgae have the highest amount, followed by green algae and lastly brown algae – being comparable to foods rich in protein such as soybean, eggs or fish (Miranda, Lopez-Alonso and Garcia-Vaquero, 2017). Also, in most cases, they are considered a rich source of essential amino acids and acidic amino acids, having higher content than terrestrial plants (Fleurence, 1999, 2004). Since proteins are one of the most expensive nutrients in animal feed, the high levels reported for macroalgae could suggest its incorporation into animal feed as a high-quality protein source (Miranda, Lopez-Alonso and Garcia-Vaquero, 2017). A seasonal study showed that there is an inverse relationship between protein content, which is higher in winter, and polysaccharide content, which is higher in summer (Wells *et al.*, 2017). These polysaccharides are used in marine algae as energy storage and structural elements and the most complex polysaccharides, known as dietary fiber, cannot be digested easily as we will see later in this report (Wells *et al.*, 2017). However, polysaccharides can influence the overall quality of the fish's diet since they act as emulsifiers. The feed stability, viscosity and texture would vary accordingly to the amount of polysaccharides in the diet (Wan *et al.*, 2019).

Regarding lipids, their levels are low in every algae group, however the ones present can have an important role in fish's diet. Seaweeds are mainly constituted by phospholipids and glycolipids, being long-chain polyunsaturated fatty acids (PUFAs) – omega 6 and omega 3 fatty acids –, alongside with carotenoids, the worthiest as elements in functional foods (Holdt and Kraan, 2011). For example, *Ulva* spp. has high levels of octadecatetraenoic acid, as well as essential dietary eicosapentaenoic and docosahexaenoic acids, which makes it unique since these lipids are generally absent in plants (Miranda, Lopez-Alonso and Garcia-Vaquero, 2017). As for carotenoids, they are also important components because they can possess a range of properties, such as antioxidant, antitumor and anti-inflammatory activity (Wan *et al.*, 2019).

Macroalgae are also a rich source of minerals, such as iodine, copper, iron, selenium and zinc and vitamins, such as vitamin C (Miranda, Lopez-Alonso and Garcia-Vaquero, 2017; Wells *et al.*, 2017). Vitamins are essential micronutrients because they serve as precursors for enzyme cofactors, are needed in essential metabolic functions and animals have lost the capacity to produce them, so they must obtain

it from external sources (Wells *et al.*, 2017). Also, they can play important roles in promoting immunological responses (Wan *et al.*, 2019). Seaweeds are also a good source of other vitamins – B-group, A and E – and the concentrations of some of them, such as β-carotene (pro-vitamin A) exceed the values found in conventional foods considered to be rich sources of these compounds, in this case, carrots (Wells *et al.*, 2017). In aquaculture feed, vitamin E can be particularly important as it can serve as an antioxidant, preventing the macroalgal PUFAs from oxidation (Wan *et al.*, 2019).

Phenolic compounds, as known as polyphenols, are also interesting incorporations in the diet since they can have therapeutic functions, such as antioxidant, anticancer and antibacterial activities (Wan *et al.*, 2019).

1.3.2. Digestibility of macroalgae

While simple carbohydrates are easily digested and absorbed by the gut wall, this may not be necessarily true for more complex carbohydrates as cellulose, xylan and phycocolloids. This may happen due to a lack or low presence of enzymes that degrade carbohydrates and this will make it more difficult for fish to fully consume all the nutrients within the algae (Wan *et al.*, 2019).

Also, proteins in most algae are digested less completely than reference proteins – such as casein, form milk - and it is probably due to inhibitory fibers (Wells *et al.*, 2017). It is probable that the mild digestibility of proteins is due to the existence of network-like structure composed of proteins and polysaccharides (Jard *et al.*, 2013). It is also important to consider the presence of anti-nutritional factors, as is the case with phlorotannins, that are present in brown macroalgae and can interfere with the bioavailability and digestibility of proteins (Garcia-Vaquero and Hayes, 2016).

The digestibility of macroalgae can be increased by using a pretreatment method (Jard *et al.*, 2013). For example, SSF with fungi can increase the digestibility, since the enzymes produced can break the bonds between hemicellulose and cellulose with lignin (Sousa *et al.*, 2018).

2. MATERIALS AND METHODS

2.1. Raw material

During this work, the green algae *Ulva rigida* was used. The macroalgae was provided dry and powder by Algaplus in 2020, a Portuguese company based in Aveiro. They were stored in a dry place, avoiding exposure to light.

2.2. Reagents

The reagents used during this work are described on **Table 2**, such as the companies that provided those reagents.

Table 2 - List of reagents used during this study.

Reagents	Company
3,5-dinitrosalicylic acid	Acros Organics
Agar	Labkem
Xylan (Beechwood)	Megazyme
CMC	Sigma-Aldrich
Folin-Ciocalteu reagent	Panreac
Gallic Acid	Acros Organics
Glacial acetic acid	Fisher Scientific
Glucose	VWR
Methanol	Fisher Scientific
Peptone	Acros Organics
Sulfuric acid	Fluka
Tween-80	Fisher Scientific

2.3. Microorganisms

The filamentous fungi *Aspergillus ibericus* MUM 03.49 was used during this study and it was obtained from *Micoteca da Universidade do Minho* (MUM) culture collection (Braga, Portugal). It was revived in slants with potato dextrose agar (PDA) medium (4 g/L potato extract, 20 g/L dextrose and 15

g/L agar). In order to use the fungus in SSF, it was incubated in PDA medium at 25°C for 7 days. During the experiment period, the species was preserved at 4°C.

2.4. *Ulva rigida* characterization

The macroalgae *Ulva rigida* used in this study was initially characterized and different analysis were performed, namely, phenolic compounds, protein, lipids, humidity, ashes, salt and cellulase. The methods used in the determination of each parameter are described in sectors further ahead.

2.4.1. Phenolic compounds determination

Phenolic compounds were determined by the Folin-Ciocalteu method (Benzie and Devaki, 2017). In order to analyze them in the macroalgae, a previous extraction was performed with distilled water using a ratio of 1:10 w/v.

In tubes, 100 μL of sample was added (for the blank, 0,1 mL of distilled water), as well as 2 mL of Na_2CO_3 at 15%, 500 μL of Folin-Ciocalteu reagent and 7,4 mL of distilled water in duplicate. The tubes were placed in a bath at 50°C for 5 minutes and, after cooling at room temperature, the tubes were vortexed. Absorbance was read at 740 nm.

The calibration curve was constructed with gallic acid standard solutions between 0 g/L and 2 g/L.

2.4.2. Protein quantification

The Kjeldahl method was used to determine the total nitrogen in the solid sample. This method is used in the measurement of protein content of biological materials, as so in the determination of nitrogen in inorganic materials, solids, or liquids.

Kjeldahl method can be divided in two steps - the first one a digestion and the second one a titration. In the first step, a digestion of the sample is performed by heating with concentrated sulfuric acid in the presence of a catalyzer, in this case, selenium (or red Hg, the second one more efficient than the first but also with more environmental implications). This step is responsible for the reduction of organic nitrogen to ammonia, which is recovered in solution in the form of ammonium sulfate:

$$Norganic + H_2SO_4 + cataliser \rightarrow CO_2 + H_2O + (NH_4)_2SO_4$$

After digestion, the ammonium is displaced by a strong base in excess, being used aqueous NaOH at 400 g/L. This is, stoichiometrically:

$$(NH_4)_2SO_4 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$

The resultant solution with NH_3 is distillated with vapor that drags with it the NH_3, being this recovered in a solution of boric acid:

$$NH_3 + H_3BO_3 \rightarrow NH_4^+ + H_2BO_3^-$$

The borate of the acidic solution is titrated (second step) with sulfuric acid to quantify the quantity of ammonium according to the reaction (*Cole-Parmer Scientific Experts*, no date; ExpotechUSA, no date; PanReac AppliChem, no date):

$$2H^+ + {SO_4}^{2-} + 2{H_2BO_3}^- + 2NH_4^+ \rightarrow 2H_3BO_3 + (NH_3)_2SO_3 + H_2O$$

The Kjeldahl method protocol consists in turning on the thermoblock (Tecator system 1007/6) which should achieve the 420°C. In each digestion tube, 0,5 g of the sample are placed and then it is added 10 mL of H_2SO_4 concentrated and a tablet of catalyzer selenium (Tecator S/3.5). The solution is carefully mixed and placed on the thermoblock previously heated to 420°C. After digestion, the titration is performed with the addition of an alkali solution.

2.4.3. Cellulose, hemicellulose, and lignin quantification

To determine the amounts of cellulose, hemicellulose, and lignin present on the fermented solid, a quantitative acid hydrolysis (QAH) was performed, based on Hoebler *et al.*, 1989, with some modifications. This includes a first stage incubation with 72% wt H_2SO_4 at 30°C for 1 h and a second stage after dilution to 4% wt H_2SO_4 at 121°C for 1 h.

A sample of about 0,5 g was weighted into a glass cup, 5 mL of 72 % H_2SO_4 were added to the cup and then they were placed in a water bath at 30°C during 1 hour with periodic agitation (every 10 minutes) - first stage. After this period, the reaction was stopped with the addition of distilled water and

the glass cup's content was transferred for flasks. The waste that stayed attached to the walls was dragged with distilled water, which was added to dilute the solution up at 4% (w/w) H_2SO_4. The flasks were closed and introduced in the autoclave during 1 hour at 121°C - second stage. Posteriorly, the flasks were cooled and was determined the losses originated during the second stage by weighting the flasks. The entire content of each flask was filtered through a Gooch crucible with known weight. The Gooch crucibles with insoluble fraction were placed on an oven at 105°C. After 24 h the crucibles were cooled on a desiccator containing silica gel and then weighted. This determination was performed in duplicate. The filtrate was analyzed by High Performance Liquid Chromatography (HPLC) system for measure of sugars (glucose, xylose, and arabinose) and acetic acid. Using a Jasco830-IR intelligent refractive-index detector and a Varian MetaCarb 87H column. The column was eluted with 0.005 M H_2SO_4 and the flux was 0,5 mL/min at 60°C. Calibration curves were constructed with glucose, xylose, arabinose, and acetic acid standard solutions between 0,1 g/L e 10 g/L. With the data of sugars concentrations (glucose, xylose, arabinose, and acetic acid) was calculated the content in polymers (CP). The CP, glucan (CGn), xylan (CXn), arabinan (CArn), and acetyl groups (CGA) were calculated according to **Equation A** and expressed as grams of polymer per 100 grams of dry waste.

$$CP\ (\%) = F * SCF * \frac{[S]}{\rho} * \frac{W + WHS * H}{WHS * (1 - H)} * 100$$

Equation A

where F is a factor which corrects degradation of sugars (1,04 for CGn, 1,088 for CXn/CArn and 1,00 for CGA), SCF is a stoichiometric correction factor to take in account the increase in molecular weight during hydrolysis (162/180 for CGn, 132/150 for CXn/CArn and 43/60 for CGA), S is the monomer concentration in g/L, ρ is the density of the analyzed dissolution in g/L (as the samples were diluted in water for HPLC analysis the value is about 1000 g/L), W is the weight of added water in grams and corrected to take account the losses during second stage of QHA, WHS is the total weight in grams of humid waste and H is the humidity in grams of water/grams of humid waste. Cellulose (grams of cellulose per 100 grams of dry waste) and hemicellulose (grams of hemicellulose per 100 grams of dry waste) content were determined according to **Equation B** and **Equation C**, respectively.

$$Cellulose\ (\%) = CG_n$$

Equation B

$$Hemicellulose\ (\%) = CX_n + CAr_n + CG_A$$

Equation C

The increase weight of the Gooch container matches to Klason lignin, thus the content of lignin (grams of lignin per 100 grams of dry waste) was calculated according to **Equation D**.

$$Lignin\ (\%) = \frac{(WCDS - WC) * (1 - CE)}{WCHS * (1 - H)} * 100$$

Equation D

wherein WCDS is the weight of Gooch container with dry sample in grams, WC is the weight of Gooch container in grams, WCHS is the weight of Gooch container with humid initial sample who underwent to the QHA in grams and H is the humidity in grams of water/grams of humid waste. The parameter CE is added to remove the value of ashes and is calculated by **Equation E**.

$$CE = (WCDS - WC\ ) - \left((WCDS - WC) * \frac{Ash}{100}\right)$$

Equation E

2.4.4. Ashes determination

After drying the Gooch crucible at 105°C during 24 h, its content was added to a porcelain container (previously dried at 105°C for 24 hours). The container was weighted before and after the content of the Gooch crucible was added. The porcelain container with the solid was placed in the muffle furnace at 550°C for 2 hours, until constant weight. After cooling in the desiccator containing silica gel for about 15 minutes, it was weighed. Ash percentage (grams of ash per 100 grams of dry solid) is given by **Equation F**.

$$Ash\ (\%) = \frac{WCA - WC}{(WCHS - WC) \times (1 - H)} \times 100$$

Equation F

where WCA is the weight of porcelain container with ash in grams, WC is the weight of porcelain container in grams, WCHS is the weight of porcelain container with humid waste in grams and H is the humidity in grams of water/grams of humid waste.

2.4.5. Antioxidant activity determination

Directly on an Elisa plate, 200 μL of sample were pipetted and 100 μL of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were added to the same well (Benzie and Devaki, 2017). For the blank, 100 μL of water were added, instead of DPPH. For the control, 200 μL of water were added, instead of the sample.

The calibration curve was constructed with Trolox standard solutions with, concentrations between 3,125 and 100 microM.

The final result is expressed in millimoles of Trolox equivalent per gram of dry solid substrate. To achieve this result, firstly the scavenging activity (%) is calculated with **Equation G**.

$$Scavenging\ activity\ (\%) = \frac{1 - Absc}{Mean\ of\ absorbances} \times 100$$

Equation G

where Absc represents absorbance corrected, or, in other words, the absorbance read minus the blank. Finally, with this result and using the calibration curve constructed, the final result is reached.

2.4.6. Salt determination

Salt content was determined by adding 100 mL of water to 5 g of algae and it was stirred for 24h. After that time, the mixture was filtered, and the liquid retrieved. The liquid was added to a previously weighed cup and left to dry in an oven at 55°C for 48h.

2.4.7. Lipids quantification

Total lipids were determined by Soxhlet extraction, using petroleum ether as a solvent, at 70°C using a FOSS Soxtec 8000 apparatus.

2.5. Sequential SSF and enzymatic hydrolysis (EH)

SSF and EH were performed sequentially and it was only added a citrate buffer on the beginning of EH, following the methodology described by Fernandes *et al.* (Fernandes *et al.*, 2019). SSF by *Aspergillus ibericus* was conducted in Erlenmeyer flasks of 500 mL, where 10 g of *U. rigida* were weighted and water was added to reach a humidity of 75% (wet basis). The flasks were sterilized at 121°C during 15 min. After sterilization, in the laminar flow hood, the Erlenmeyer flasks were inoculated with 2 mL of a spore solution prepared by adding peptone (0,1% w/v) and Tween 80 (0,001% w/v), the concentration was adjusted to $1*10^6$ cells/mL. The fermentation process was conducted for 5 days at 25°C.

EH with enzymes produced by fungus were performed. Three parameters of EH were optimized using a Box-Behnken experimental design (temperature, load of solid and pH). After SSF, sequentially different quantities of citrate buffer were added to the fermented solid, to adjust the load of solid and pH, according to **Table 3**. The EH was carried out in orbital shaker at 150 rpm and the temperatures defined for each experiment (Table 3). These values were obtained using experimental Box-Behnken design, which is an incomplete factorial design, combined in blocks, and its main advantage is the reduction of the number of experiments, when comparing to other experimental designs (Czyrski and Sznura, 2019). It was also added thymol, which works as an antifungal to prevent the consumption of released sugars during EH (Salehi *et al.*, 2018).

Table 3 – Matrix of experiments obtained by Box-Behnken experimental design.

Runs	Temperature (ºC)	Load of solid (% w/v)	pH buffer
1	44	20	4
2	39,5	20	4,6
3	39,5	20	4,6
4	35	20	4
5	44	10	4,6
6	39,5	10	4
7	44	20	5,2
8	35	20	5,2
9	39,5	10	5,2
10	35	10	4,6
11	39,5	20	4,6
12	35	30	4,6
13	39,5	30	5,2
14	44	30	4,6
15	39,5	30	4

Samples were collected at time 0, 4h, 8h, 24h, 32h, 48h and 72h. The samples collected were centrifuged at 8000 rpm for 5 minutes and stored at -20°C until analysis. By the end of the EH time, the resulting mixture was centrifuged at 9000 rpm for 10 minutes and the solid was dried at 60°C during the weekend. Afterwards, the resulting solid and the samples collected were used in different analysis described in the next sectors and the sectors before (phenolic compounds, protein, hemicellulose, cellulose, lignin, ashes, and antioxidant activity).

2.6. Reducing sugars determination

Free reducing sugars were measured by the DNS method (Miller, 1959).

To each tube 0,1 mL of the sample was added and 0,1 mL of DNS reagent in duplicate (for the blank measurement 0,1 mL of distilled water was used). The tubes were placed in a bath at 100°C for 5 minutes. After cooling, 1 mL of water was added to the mixture and the absorbance was read at 540 nm.

The calibration curve for this method was constructed with glucose standard solutions, with concentrations between 0 g/L and 4 g/L.

The maximum conversion of cellulose to glucose (CGC_{max}) during EH was calculated following the equation described in Romani et al. (Romaní *et al.*, 2011) using the values of glucose analyzed by HPLC:

$$CGC_t = CGC_{max} \frac{t}{t + t_{1/2}}$$

Equation H

where CGC_t is the cellulose-to-glucose conversion achieved at time t, calculated as:

$$CGC_t = 100 \frac{G_t - G_{t0}}{G_{pot}}$$

Equation I

whereas CGC_{max} is the cellulose-to-glucose conversion predicted for an infinite reaction time, t is the EH time (h), t1/2 (h) is the time needed to achieve CGC= $CGC_{max}/2$, Gt is the glucose concentration (g/L) achieved at time t, G_{t0} is the glucose concentration at the beginning of the experiments, and G_{pot} represents the potential glucose concentration (calculated assuming total cellulose conversion into glucose).

2.7. Cellulase and xylanase quantification

The quantification of cellulase and xylanase was performed according to the method described by Sousa *et al.*, 2020.The procedure for determination of cellulases activity was to add 250 µL of cellulase substrate (CMC 1% in 0.1 M sodium acetate buffer, pH 4.6) to test tubes and then, 250 µL of diluted sample in buffer. The test tubes were placed on a bath at 50°C for 30 minutes. After 30 minutes it was added 500 µL of DNS and then the test tubes were placed on a bath at 100°C for 5 minutes. Finally, 5 mL of distilled water was added to each tube and the absorbance was read at 540 nm. The blank was performed with sodium acetate buffer and the addition of the sample after the 30 minutes incubation. A calibration curve was constructed with glucose standard solutions in buffer between 0 g/L and 2 g/L.

The procedure to determine the xylanases activity was the same as for the determination of cellulases activity but the duration of the reaction was only 15 minutes instead of 30 minutes and the substrate solution was beechwood xylan (2%).

2.8. Statistical analysis

Results are presented as the mean ± standard deviation (SD) of at least two replicates. The analyses were performed using Microsoft Office Excel software. Statistically significant differences of the several assays were evaluated by a one-way ANOVA. A significant difference was considered if $p < 0.05$ applying the Tukey multiple-comparisons test. Statistical analyses were performed using GraphPad Prism 9 software. Statistical analysis of Box-Behnken experimental design was performed by Statistica 10 software.

3. RESULTS AND DISCUSSION

During the research work was studied a sequential biological treatment of a green macroalgae (*U. rigida*). A sequential solid-state fermentation (SSF) and enzymatic hydrolysis (EH) were performed to upgrade their nutritional value as aquaculture feed and produce value added products, following biorefinery concept. For that, the macroalgae was fermented by *A. ibericus* for 5 days, followed by EH using the enzymes produced by fungus. The EH stage was optimized using a Box-Behnken experimental design. Three variables (temperature, load of solid and pH of the buffer) were tested to determine the optimal conditions to extract phenolic and antioxidant compounds, release of sugars and to increase the protein content of *U. rigida*. In addition, the stability of enzymes during EH and reduction of non-starch polysaccharides were studied.

3.1. Characterization of *Ulva rigida*

The *Ulva rigida* was firstly characterized to have a control of the composition of the macroalgae and so that further on it could allow us to know how the compound variated in each condition after fermentation and EH. It was determined the amount of phenolic compounds, crude protein, lipids, ashes, salt and lignocellulosic composition. The results are shown in **Table 4**.

Table 4 – Characterization of *Ulva rigida*. Results expressed in g per kg of dry matter.

Analysis	Result (g/kg)
Phenolic compounds	0.72 ± 0.06
Crude protein	169.1 ± 0.7
Lipids	10.7 ± 0.2
Ashes	379 ± 5
Salt	415 ± 9
Cellulose	95 ± 14
Hemicellulose	117 ± 8
Lignin	2.2 ± 0.1

It was observed that *U. rigida* is have a higher protein content than lipids. These results agree with previous studies, such as the one conducted by Taboada, Millán and Míguez, that also used *U. rigida*

and where the amount of protein was 178 g/kg and fat content was approximately 9 g/kg. Furthermore, the work performed by Taboada, Millán and Míguez determined that most of the fat content was unsaturated, with a larger percentage of polyunsaturated fats. In what concerns protein, the study determined that the content in essential amino acids was generally high, which indicated proteins of good quality. The amino acids present in higher quantity in *U. rigida* were glutamic acid, arginine, and aspartic acid (Taboada, Millán and Míguez, 2010). As it was already stated in Chapter 1, the amount of protein is usually higher in red algae, while the amount of fats is low in all types of algae.

The amounts of cellulose and hemicellulose are also high, with a total of both above 200 g/kg. As for lignin, its content is low, as expected.

It was also determined that the amount of ashes and salt was much higher than the rest of the parameters. This is because the macroalgae were not washed after harvesting. In other studies, the content of ashes in *Ulva* species were between 170 to 310 g/kg (Plaza, Cifuentes and Ibáñez, 2008; Taboada, Millán and Míguez, 2010). These differences can be linked to washing stages of the macroalgae.

3.2. Sequential SSF and EH

3.2.1. Production of biocompounds during SSF

The first stage was the fermentation of the macroalgae by *A. ibericus* over 5 days. Then, to carry out the second stage (EH), a buffer was added to fermented solid. The production of enzymes by the filamentous fungi was analyzed after 5 days of SSF. **Figure 1** shows the production of xylanase and cellulase during the five-day fermentation with *Ulva rigida* and *Aspergillus ibericus*.

The production of cellulase by SSF of green macroalgae (*Ulva fasciata*) was also performed by (Trivedi *et al.*, 2015). They achieved a production of 10 U/g using a marine fungus *Cladosporium sphaerospermum*. The production of xylanase and cellulase by SSF of *U. rigida* using *A. ibericus* was already studied achieving 350 U xylanase/g and 50 U cellulase/g after 7 days of SSF. They observed a reduction of enzyme production (200 and 10 U/g, respectively) when seaweed was washed (Fernandes *et al.*, 2019). Thus, this process related to harvesting of seaweeds have a high influence in enzyme production.

In this case, it was verified a higher production of xylanase than cellulase, which was also verified in the study referred above. In this work, the production of xylanase achieved (160 ± 4) U/g, as for cellulase it was achieved (40 ± 1) U/g. These values are below those achieved by Fernandes *et al.*, however the fermentation was only performed during 5 days in this work.

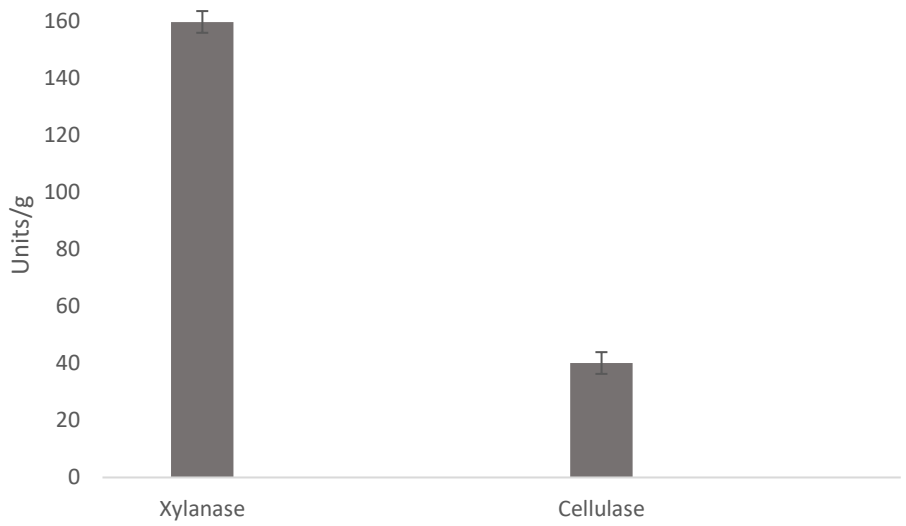

Figure 1 - Production of cellulase and xylanase by SSF. The results represent the average of two independent experiments and error bars represent SD.

It was also determined the amount of phenolic compounds and antioxidant activity after fermentation and these values were determined for the extraction with 50 mL of buffer. Phenolic compounds increased after fermentation (1,711±0,005 mg/g), when compared to the amount present in *Ulva rigida* [(0,72±0,06) mg/g]. SSF has already been used in different studies to improve the content in phenolic compounds, for example in the study performed by Leite *et al.* (Martins *et al.*, 2011; Leite *et al.*, 2019). As for antioxidant activity, it was determined to be (4,294±0,042) µmol of Trolox equivalents/g of dry solid.

3.3. Optimization of EH stage by Box-Behnken Experimental Design

3.3.1. Release of phenolic and antioxidant compounds

Phenolic compounds increased during EH stage, being that, in most rounds performed the higher quantity was achieved in the last time quantified, at 72h. **Table 5** shows the difference between final and initial amounts of phenolic compounds antioxidant compounds detected in each round and compares the values observed with the values predicted by the model. It can be observed that there is a good adjustment between the values obtained experimentally and the values predicted, being that the biggest differences in antioxidant activity were in rounds 1 and 8 and in phenolic compounds were verified very low differences.

Table 5 - Results of antioxidant activity and total phenolic compounds studied in Box-Behnken experimental design.

Runs	Antioxidant activity (μmol TE/g)		Phenolic compounds (mg/mL)	
	Observed	Predicted	Observed	Predicted
1	269 ± 1	284	0.32 ± 0.03	0.34
2	288 ± 44	284	0.47 ± 0.06	0.48
3	286 ± 8	284	0.48 ± 0.03	0.48
4	124 ± 23	123	0.25 ± 0.01	0.25
5	185 ± 42	176	0.39 ± 0.20	0.34
6	308.07 ± 6.31	301.60	0.36 ± 0.02	0.38
7	21.04 ± 18.82	22.97	0.32 ± 0.04	0.31
8	115.05 ± 41.28	100.13	0.28 ± 0.04	0.26
9	-64 ± 8	-58	0.39 ± 0.04	0.44
10	139 ± 19	148	0.14 ± 0.02	0.12
11	279 ± 10	284	0.48 ± 0.04	0.48
12	247 ± 20	256	0.38 ± 0.20	0.42
13	275.07 ± 19.43	281.54	0.53 ± 0.03	0.52
14	320 ± 22	311	0.32 ± 0.03	0.33
15	212 ± 14	206	0.64 ± 0.01	0.59

In Table 5, the values of antioxidant activity and phenolic compounds are the variation between the initial and the maximum value achieved. The higher variation was verified in round 15 (conditions: 39,5°C; load of solid 30% w/v; pH 4), where it was also achieved the highest content in phenolic compounds (0,825±0,004 mg/mL). The increase that was verified in all rounds performed may be related with the presence of carbohydrate hydrolyzing enzymes produced by the fungi in SSF, because it causes the hydrolysis of the phenolic conjugates (Sousa *et al.*, 2020).

As for antioxidants, the differences verified were higher in rounds 6 and 14, however the higher content in antioxidants was achieved in round 4 (conditions: 35°C; load of solid 20% w/v; pH 4), being that value 398,18±6,07 μM of Trolox equivalents. On the other hand, round 7 showed a lower variation of antioxidant activity, however it can be explained by a high initial concentration. In round 9 a negative value is obtained, however it can be explained by a higher initial value for activity than on the other rounds. Furthermore, this round was performed with pH 5,2 and load of solid of 10% and, as will be further

shown, these conditions negatively affect the antioxidant activity. The liberation of phenolic compounds and increase in antioxidant activity after EH was also observed by Fernandes *et al.*, 2019.

Figures 2 and **3** are three-dimensional response surface curve plots that allow to determine the interaction of independent variables and the optimum levels that have the most significant effect on the dependent variable. It can be observed that the amount of phenolics extracted increases with the increase of temperature, until it reaches an intermediate temperature and then it slightly decreases. As for the load of solid, an increase in this parameter translates into an increase on total phenols. As for antioxidant activity, it increases with the decrease of pH and it increases with an increment on the load of solid. However, a slight decrease is noted in the antioxidant activity after a certain value of pH.

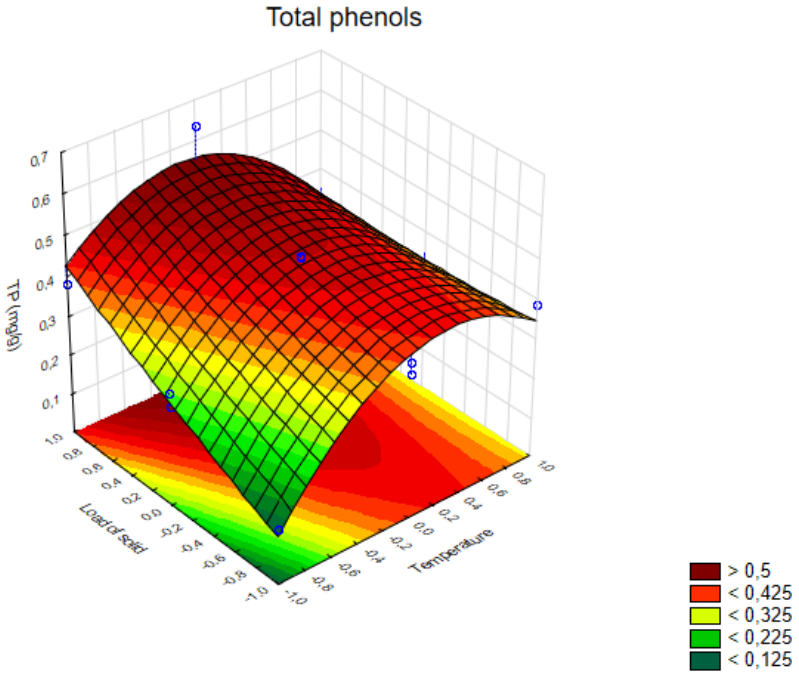

Figure 2 - Response surface for total phenolic compounds as a function of temperature and load of solid.

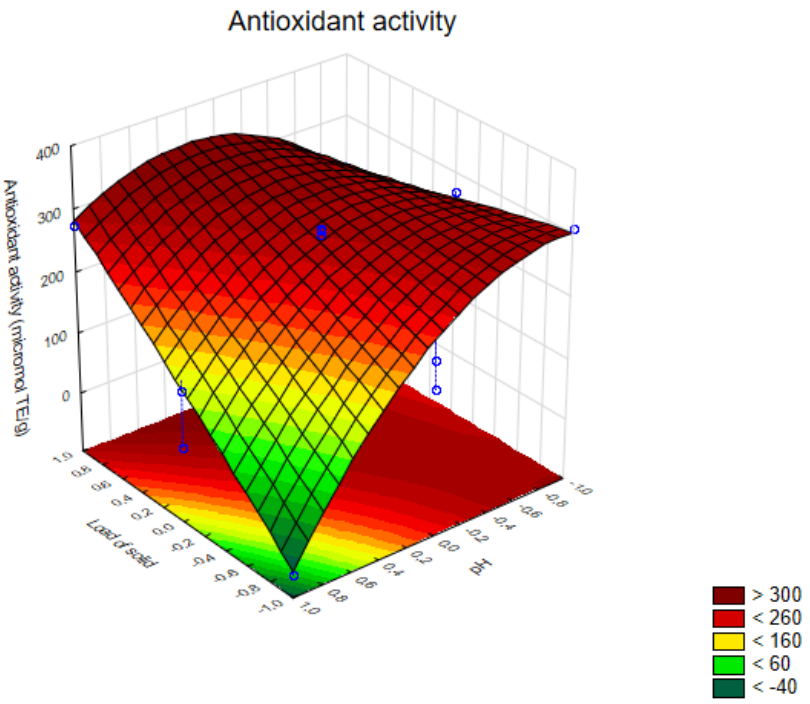

Figure 3 – Response surface for antioxidant activity as a function of pH and load of solid.

Table 6 lists regression coefficients and their statistical significance, as well as the statistical parameters that measure the suitability of the model. The determination coefficient (R^2) was 0.995 for antioxidant activity and 0.953 for total phenolic compounds, which demonstrates a satisfactory adjustment of the model.

Table 6 - Regression coefficients of model parameters antioxidant activity and phenolic compounds.

Coefficients	Antioxidant activity	TPC
Constant	284.13***	0.475***
X_1	21.10***	0.034***
$X_1 \cdot X_1$	-55.94***	-0.180***
X_2	60.82***	0.074***
$X_2 \cdot X_2$	-5.44	0.010**
X_3	-70.84***	-0.004**
$X_3 \cdot X_3$	-95.79***	-0.005**
$X_1 \cdot X_2$	6.77	-0.079***
$X_1 \cdot X_3$	-59.68***	-0.007**
$X_2 \cdot X_3$	108.68***	-0.033***
Coefficients of determination		
R^2	0.995	0.953
$R^{2\ adj}$	0.985	0.868
X_1: Temperature; X_2: Load of solid; X_3: pH; ***significant at 99%; **significant at 95%; *significant at 90%; TPC: total phenolic compounds; CGC_{max}: conversion cellulose to glucose maximum		

3.3.2. Release of sugars during EH

The sugar release during EH was evaluated on samples collected during EH. The results are presented in terms of maximum conversion of glucose to cellulose (**Table 7**).

Table 7 - Results observed and predicted of sugar release during EH.

Runs	CGC_{max} (%)	
	Observed	Predicted
1	71	74
2	22	19
3	15	19
4	14	16
5	62	61
6	15	14
7	61	59
8	38	35
9	12	16
10	23	22
11	21	19
12	22	23
13	18	20
14	66	67
15	22	18

The values observed were, in all rounds, similar to the values predicted, being that the biggest difference was verified in round 3 (it was observed 14,85% when it was predicted 19%). According to **Table 7**, the highest value was predicted to appear in round 1 (conditions: 44°C; load of solid 20% w/v; pH 4,6) and the observed value confirmed this tendency. On the other hand, the lowest rate of conversion from cellulose to glucose was expected in round 6 (conditions: 39,5°C; load of solid 10% w/v; pH 4), however it was verified in round 9 (conditions: 39,5°C; load of solid 10% w/v; pH 5,2), that has the same temperature and load of solid as round 6 but was performed with buffer with a different pH. It can be also verified that the rounds performed at 44°C allowed a higher conversion of cellulose to glucose that

others performed at lower temperatures. A study performed by Liu *et al.* also achieved a greater degree of conversion with an increase of the temperature (Liu *et al.*, 2012). In another study, conducted by Harun and Danquah, the optimal temperature for the conversion of cellulose to glucose on EH was determined to be 40°C so, although in this study the conversion increases with the increase of temperature until 44°C, it could be expected that the conversion would soon decrease if higher temperatures were tested (Harun and Danquah, 2011).

Figure 4 shows the effect of temperature and load of solid in the conversion of cellulose to glucose. The load of solid used in EH step did not interfere with the conversion of cellulose to glucose, however, an increase in the temperature at which the EH was performed correlates to an increase on the conversion of cellulose to glucose. Regression coefficients and their statistical significance, as well as the statistical parameters that measure the suitability of the model for the conversion of cellulose to glucose are presented in **Annex 1**.

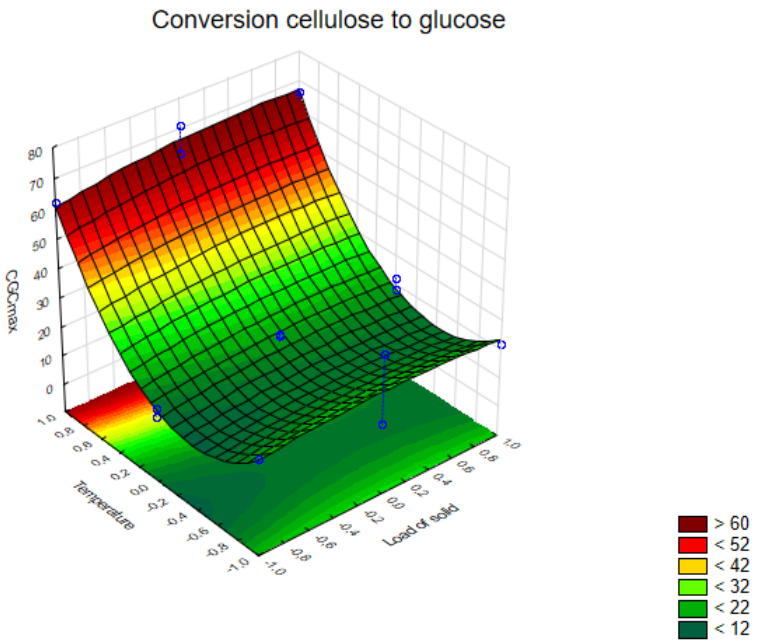

Figure 4 - Response surface for conversion of cellulose to glucose as a function of temperature and load of solid.

3.4. Stability of lignocellulolytic enzymes during EH

In all the runs tested, the stability of xylanase decreased during the 72 hours of EH. The results are presented in **Figure 5.**

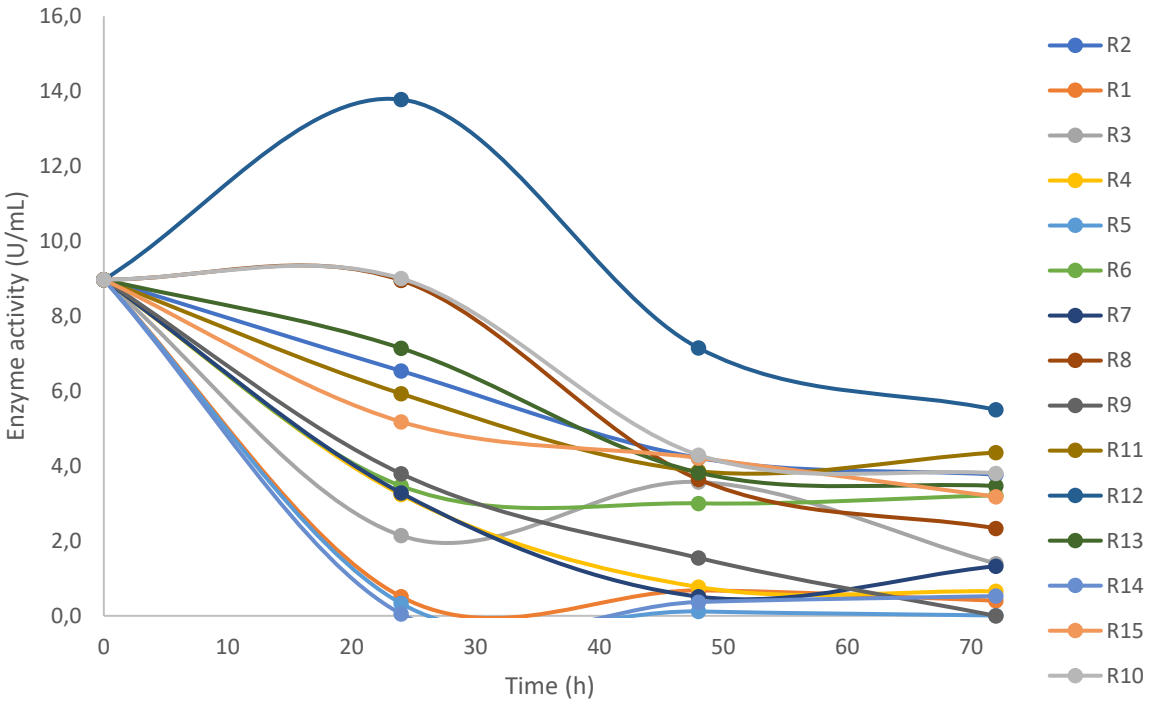

Figure 5 - Kinetics of xylanase activity during 72 hours of EH.

Although all rounds present a reduction in xylanase activity, it is clear that the decrease is less significative in some rounds than others. For instance, round 11 (conditions: 39,5°C; load of solid 20% w/v; pH 4,6) has the lower decay of activity, while round 5 (conditions: 44°C; load of solid 10% w/v; pH 4,6) has the higher decay of activity. Furthermore, all the five rounds that had a higher decay rate (Rounds 1, 4, 5, 9 and 14) were performed under at least one of these conditions: 44°C and pH 4. On the contrary, in the five rounds that had a slower decay of activity (2, 8, 11, 12 and 13) none of these conditions was implemented. In what concerns the load of solid, it was not possible to correlate with the enzyme activity, which means that the enzyme stability was mainly affected by temperature and pH.

In what concerns cellulase, its activity did not have a clear behavior, which hinders the analysis of the results, presented in **Figure 6**.

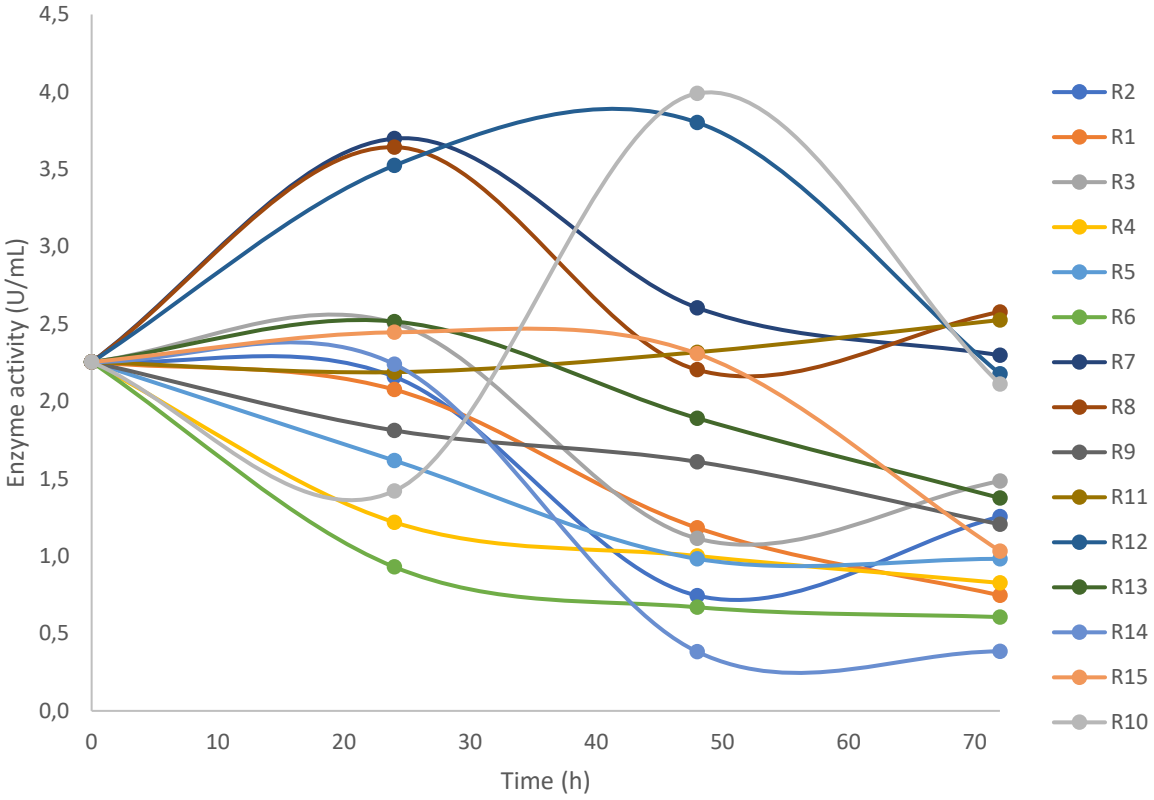

Figure 6 - Kinetics of cellulase activity between 0h and 72h of EH.

In some rounds, the enzyme activity increases from time 0 to time 24 and then decreases. On the other hand, in round 11 (conditions: 39,5°C; load of solid 20% w/v; pH 4,6) the cellulase activity continuously increased from 0 to 72h. Additionally, in round 10 (conditions: 35°C; load of solid 10% w/v; pH 4,6) the final value is also higher than the value at 24h, however, there is an increase between 24h and 48h and a decrease between 48h and 72h.

When comparing the rounds with higher and lower decay of activity during time, the rounds with a bigger decay of activity (1, 7, 12, 14 and 15) were mainly performed at higher temperatures except for round 12. These rounds also did not have a pH in common, but all of them were performed with a load of solid of 20% or 30%. By contrast, in rounds 2,4,5,6 and 9, the decline of activity was slower than in the other rounds. Similarly, these rounds have neither temperature or pH in common, being that these rounds were performed at all three temperatures and pH. In what concerns load of solid, all five rounds that had a slower decline of cellulase activity were performed either with 10 or 20% of load of solid. The main conclusion that can be withdrawn from these results is than cellulase is more stable than xylanase.

3.5. Characterization of solid after sequential SSF and EH

The resulting solids were analyzed to determine the amount of lignin, ashes, and protein. The results are presented in **Table 8** and **Figures 7** and **8**, respectively.

Table 8 - Results observed and predicted by the model of the content in protein in the solid after SSF+HE.

Runs	Protein (mg/g)	
	Observed	Predicted
1	206.07 ± 0.51	215.4
2	234 ± 11	235
3	230 ± 4	235
4	242 ± 4	274
5	226 ± 15	208
6	249 ± 18	225
7	203 ± 0	218
8	236 ± 3	271
9	267 ± 24	253
10	256 ± 4	288
11	231 ± 0	235
12	235 ± 7	227
13	229 ± 1	188
14	230 ± 1	195
15	239 ± 12	216

The lowest value of protein was in round 7 (conditions: 44°C; load of solid 20% w/v; pH 5,2) and the maximum value was achieved in round 9 (conditions: 39,5°C; load of solid 10% w/v; pH 5,2). The mean of the values is 234 and it can be observed that all the rounds performed at 35°C are above the mean, as well as two of the rounds performed at 39,5°C, with pH 4.

In what concerns ashes quantification, bigger differences are presented between rounds performed. The lowest value (142,08±57,19 g/kg) was obtained in round 6 (conditions: 39,5°C; load of solid 10% w/v; pH 4), while the highest values (464,07±146,30 and 491,70±77,13 g/kg) were achieved

in rounds 3 (conditions: 39,5°C; load of solid 20% w/v; pH 4,6) and 14 (conditions: 44°C; load of solid 30% w/v; pH 4,6), respectively. Regression coefficients and their statistical significance, as well as the statistical parameters that measure the suitability of the model for protein quantification are presented in **Annex 1**.

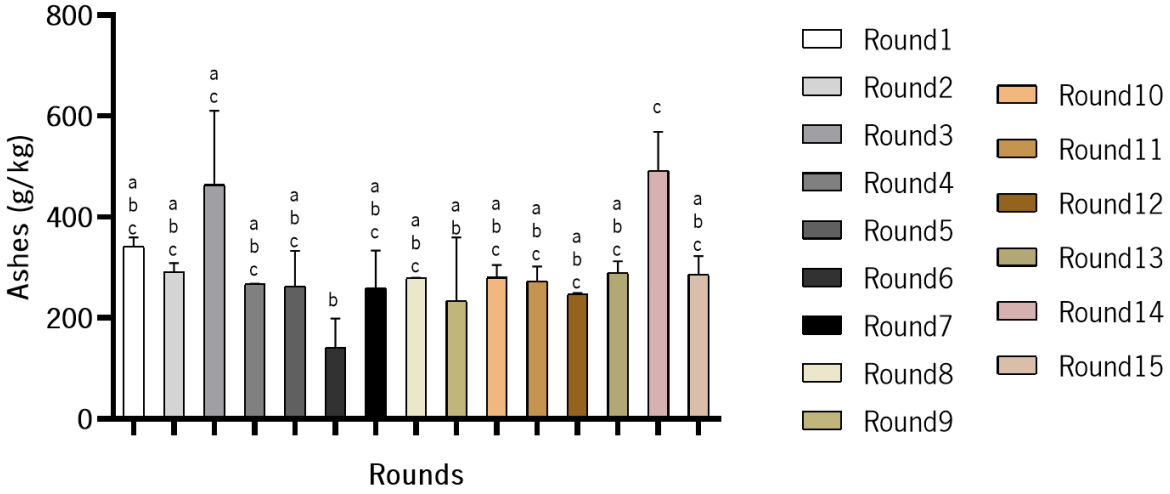

Figure 7 - Ashes quantification on the resulting solid after sequential SSF and EH. The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

The quantification of lignin had maximum values (335,98±29,32 and 305,87±33,82 g/kg) in rounds 9 (conditions: 39,5°C; load of solid 10% w/v; pH 5,2) and 10 (conditions: 35°C; load of solid 10% w/v; pH 4,6), respectively. The lowest amount of lignin (205,58±18,18) was determined in round 7 (conditions: 44°C; load of solid 20% w/v; pH 5,2).

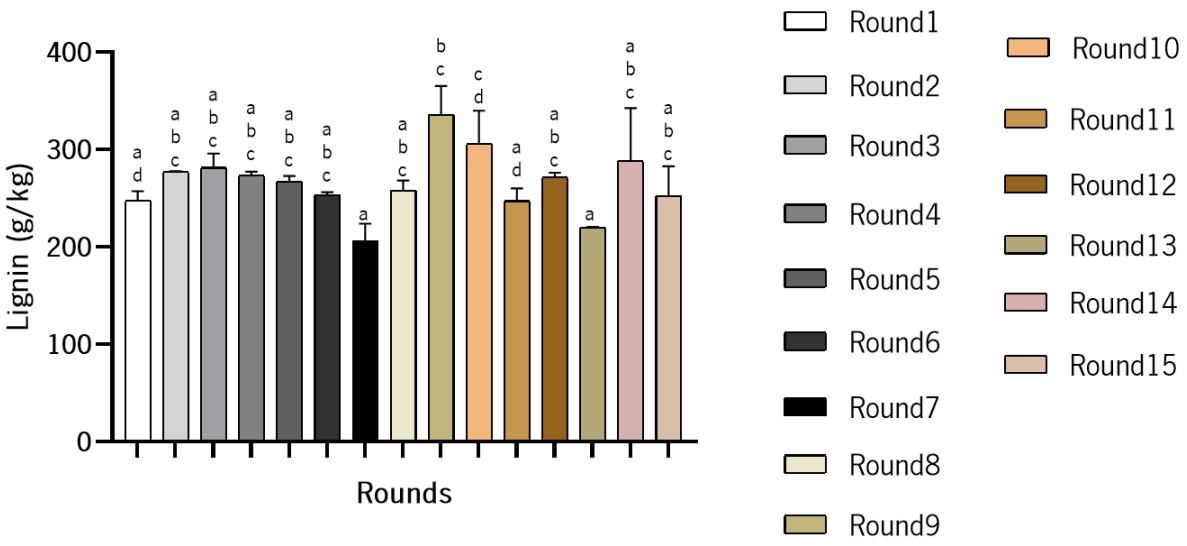

Figure 8 - Lignin quantification on the resulting solid after sequential SSF and EH. The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

Figure 9 shows the comparison between the maximum value obtained in each determination and the initial amount present in *Ulva rigida*. In comparison with the results obtained for *U. rigida* before fermentation (169,08±0,66 g/kg), protein always increased, with a maximum increment of 57,8%, which is a significant difference. The difference between lignin concentration after SSF+HE and initial amount in algae is also significative and it was more than 10 times higher than the initial amount present in *Ulva rigida*. On the other hand, the quantification of ashes shows that the difference between the amount already present in *Ulva rigida* is not significantly different from the values presented after SSF+HE.

Protein in the final solid increases due to the presence of protein from the fungal biomass. Also, the increase of ashes and lignin, as well as proteins, may be due to an effect of concentration in the final solid after EH.

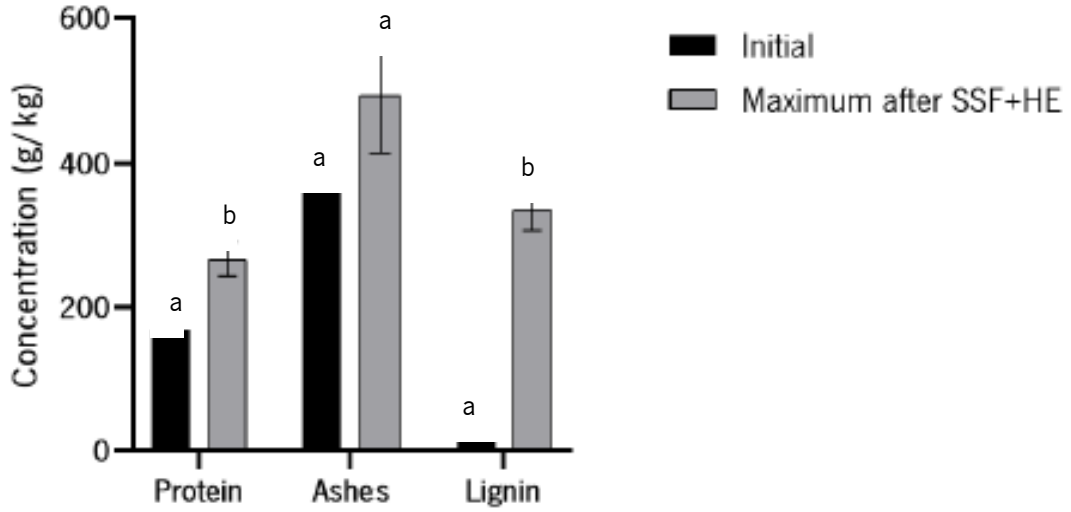

Figure 9 - Comparison between the initial values present in *Ulva rigida* and the maximum values obtained after SSF+HE. The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

3.6. Optimal conditions of sequential SSF and EH

The optimal conditions are presented in **Table 9** for each variable separately in specific and also a multiple optimization for all variables at once.

Table 9 - Optimum conditions for each variable predicted by the model.

				Dependent variables			
	Temperature (°C)	**Load of solid (% w/v)**	**pH**	**Antioxidant activity (μmol TE/g)**	**TPC (mg/mL)**	**Protein (mg/g)**	**CGC_{max} (%)**
Single optimization	41	30	4.7	345			
	39	30	4.0		0.59		
	35	10	5.2			301	
	45	27	4.0				75
Multiple optimization	44	30	4.1	279	0.47	231	61

As such, a new round of sequential SSF and EH must be performed, with the optimal conditions described in **Table 9**: 44°C; load of solid 30% w/v; pH 4,1. These conditions allow an increase in protein content in the final solid of around 37%, when comparing to the initial concentration present in *Ulva rigida.*

The future work must comprise the analysis of antioxidant activity, phenolic compounds, conversion of cellulose to glucose, protein, and the analysis of enzyme stability from EH in the optimal conditions determined in this work.

Additionally, the fermented solid obtained must be added to fish feed to determine the response of fish to the new form of food.

CONCLUSION AND FUTURE PERSPECTIVES

The initial part of this study allowed to characterize the *Ulva rigida* used during all experiments performed in this work. It was verified that *Ulva rigida* contained high amounts of protein, salt, and organic matter and, on the contrary, a small quantity of lipids and phenolic compounds.

Sequential SSF and EH of *U. rigida* allowed to produce antioxidant compounds, releases fermentable sugars, and increase the protein content of macroalgae. Different parameters of enzymatic hydrolysis were optimized by Box-Behnken experimental design.

The phenolic compounds showed an increase from 0 to 72h in almost every round and the highest content in phenolic compounds was verified at 39,5°C, with a load of solid of 30% and pH 4. As for antioxidant activity, only in seven of the rounds performed the highest value was obtained at 72h. Despite the higher variations were verified in rounds 6 and 14, the highest antioxidant activity was verified in round 4, where the conditions were 35°C, load of solid of 20% and pH 4. During EH was also quantified the release of sugars. The values obtained were similar to the values predicted by the model and it was verified that the rounds performed at a higher temperature, 44°C, allowed a higher conversion of cellulose to glucose, when compared to the other temperatures tested. During the EH is also clear a decrease in xylanase activity and it was concluded that its stability was mainly affected by high temperature and lower pH. As for cellulase, the results did not allow a clear interpretation, however from 24h of EH until 72h, the most common evolution was a decrease of activity. The conditions that maximized all variables dependents jointly were 44°C, 30% load of solid and pH 4,1.

After the EH, the remaining solid showed an increase in protein and ashes when compared to initial unfermented *Ulva rigida* but not significative, however lignin concentration increased significatively.

Further investigation is necessary, including the use of the fermented solid as fish feed and the response of fish to that kind of food and also determine the use of other biocompounds produced in the process.

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ANNEXES

Annex 1 - Regression coefficients of model parameters of protein and conversion of cellulose to glucose.

Coefficients	Protein	CGC_{max}
Constant	234.65***	19.00***
X_1	-27.96***	20.53***
$X_1 \cdot X_1$	9.66**	26.60***
X_2	-18.59***	1.86
$X_2 \cdot X_2$	-14.53***	-2.37
X_3	-0.09	0.96
$X_3 \cdot X_3$	0.40	0.38
$X_1 \cdot X_2$	12.00	1.32
$X_1 \cdot X_3$	1.43	-8.38**
$X_2 \cdot X_3$	-13.75*	-0.24
R^2	0.954	0.985
$R^{2\ adj}$	0.872	0.959
X_1: Temperature; X_2: Load of solid; X_3: pH; ***significant at 99%; **significant at 95%; *significant at 90%; TPC: total phenolic compounds; CGC_{max}: conversion cellulose to glucose maximum		